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PLANT DERIVED ANTICANCER AGENTS: A GREEN APPROACH TO CANCER PROGRESSION

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Index

Introduction

Cancer is one of the leading causes of death in modern society and nowadays, it is among the top study priorities for researchers to develop new effective cures and preventive strategies (Pucci et al., 2019; Debela et al., 2021).

The mechanism underlying the multistep process of carcinogenesis results from unspecific behaviour of cells overcoming the signals that regulate normal cell proliferation and growth. This leads to an accumulation of a series of mutations and progressive invasion, metastasis and malignancy (Copper, 2000).

Current treatments in cancer therapy include chemotherapy, radiation therapy, natural antioxidants, targeted therapy, nanoparticles and extra-vesicle based-therapy (Debela et al., 2021; Cecchin et al., 2023). Unfortunately, conventional approaches such as chemotherapy can put a strain on patients increasing a further damage of healthy cells and tissues (Moses et al., 2003). For this reason, researchers focus on using alternative green approaches and design an eco-friendly and biocompatible strategy in a greener way to current cancer treatments and therapies (Gupta et al., 2022; Iqbal et al., 2017).

Plants have been used as medicinal agents since the origin of humankind and are still being used in developing countries as cures and preventive agents for cancer (Salmerón-Manzano et al., 2020). Approximately 35000 herbal phytochemicals are obtained from plant sources to create awareness of green therapy in the management, reduction, and minimization of side effects of modern chemotherapy and radiotherapy (Gupta et al., 2022). Medicinal agents of plant origin are being studied for their ability to inhibit the growth and initiate apoptosis of cancer cells. They have several advantages in terms of environmental availability, greater accessibility in terms of costs, and fewer toxic side effects (Salmerón-Manzano et al., 2020).

Phytochemicals are biologically active, naturally occurring chemical compounds present in different parts of the plants, including flower, pericarp, fruits, seeds, roots, rhizomes, stem, leaf, bark and perform several pharmacological functions (Iqbal et al., 2017).

They are known as secondary metabolites produced by plants. These metabolites are usually synthesized above all for self-defense against insects, pathogens, herbivores, ultraviolet exposure, and environmental hazards. In addition, they contribute to the plant's color, aroma, and flavour. These compounds differ from the essential nutrients, primary

metabolites, such as carbohydrates, proteins, fats, minerals, and vitamins that are needed for the day-to-day maintenance of the plants (Pagare et al., 2015).

Tens of thousands of phytochemicals have been identified so far, and an exact classification of phytochemicals have not been performed yet, due to the wide variety of them. The more common classification system divides phytochemicals into five chemically distinct groups, including phenolics, terpenes, terpenoids, organonitrides, and organosulfides (Egbuna et al., 2019).

Several plant products such as alkaloids, flavonoids, lignans, saponins, terpenes, taxanes, vitamins, minerals, glycosides, gums, oils, biomolecules and other primary and secondary metabolites play significant roles in inhibiting cancer cell activating proteins, enzymes and signaling pathways, in activating DNA repair mechanisms and in inducing antioxidant action thus showing strong anticancer effects (Iqbal et al., 2017).

This thesis aims to study the potential antitumoral activity of plant derived compounds through a green approach. The plants selected for this work are *Catalpa bignonioides* and a rare variety of *Phaseolus vulgaris*. Plant extracts have been analyzed for their chemical composition and their antitumoral activity has been evaluated *in vitro* by using colorectal cancer cell models.

I. Aim of the thesis

Cancer is one of the leading causes of death worldwide (Ferlay et al., 2020); it represents the second cause of death and its incidence and mortality are growing rapidly.

Among the various types of cancer, colorectal cancer is one of the most common neoplasms found in the Western world; overall it ranks third in terms of incidence and second in terms of mortality (Sung et al., 2021), with a much higher number of cases in industrialized countries than in those in transition (Bray, 2014). Although advances in surgery and chemotherapy have improved the survival and management of patients with colorectal cancer, side effects and resistance to therapy have shown the limits of current therapeutic strategies leading to the search for alternative treatments (Benarba and Pandiella, 2018).

To this end, medicinal plants certainly represent a promising source of anticancer bioactive molecules often associated with minimal side effects (Benarba et al., 2015). Many studies have therefore focused on their real use against different types of cancer, including colorectal cancer, demonstrating their efficacy in tumor treatment and studying the molecular mechanisms underlying the pharmacological activity (Benarba and Pandiella, 2018).

This thesis aims to study the antitumoral activity of plant derived compounds. To this purpose, two plants were selected which, on the basis of their content in active molecules and according to what reported in the literature, constitute excellent potential sources of anticancer compounds: *Catalpa bignonioides* and *Phaseolus vulgaris*.

Catalpa bignonioides Walt. is a plant belonging to the genus *Catalpa* Scop. of the Bignoniaceae family, already widely used in the medical practice of the indigenous cultures of South America (Rasadah and Houghton, 1998). Various studies have highlighted the promising anti-inflammatory, antinociceptive (Muñoz-Mingarro et al., 2003; Kim et al., 2004), and antioxidant (Dvorská et al., 2007) activity, and the potential use in the maintenance of glucose homeostasis (Oh et al., 2021) and in diabetes management (Bai et al., 2019) of the different compounds contained in its extracts.

Further studies conducted on some species belonging to the genus *Catalpa*, including *C. speciosa*, *C. ovata* and *C. bungei*, have revealed the presence, in their extracts, of molecules endowed with antitumor activity mainly represented by phenols (Elansary et al., 2019) and naphthoquinones (Fujiwara et al., 1998; Qin et al., 2022). However, despite

the similarity in terms of composition and properties among the various *Catalpa* species, studies regarding the antitumor activity of *C. bignonioides* are lacking.

In this work, three different extracts of *C. bignonioides* fruits collected in the Botanical Garden of the University of Paris, Faculty of Pharmacy were prepared; these extracts were analyzed for their chemical composition and for their antitumor activity.

To identify the main phytochemical constituents of *C. bignonioides* extracts, a liquid chromatography mass spectrometry (HPLC-DAD-MS) analysis was performed followed by HPTLC. The antitumoral activity was then evaluated through biochemical and functional assays in two different colorectal cancer cell models.

Phaseolus vulgaris is a food widely known and consumed all over the world due to its excellent biological and nutritional values. Numerous studies have shown that a consistent consumption of beans leads to beneficial effects for the human body. The most important biological activities include glycemic control, the improvement of insulin sensitivity and the increase of the concentration of HDL cholesterol (Bornet et al., 1987). Other studies have described the activity of *P. vulgaris* in tumorigenesis and tumor progression (Hayat et al., 2014). Among the many varieties of *Phaseolus vulgaris*, the one studied in this work is the Fagiola di Venanzio (FV), a rare species grown in a small area of the municipality of Murlo (Siena), in Tuscany.

In this work it has been determined the main phytochemical constituents of FV aqueous extracts by using high performance HPLC-DAD analysis, and then it has been studied the antitumoral potential in colorectal cancer cellular models.

In addition, to better evaluate the bioactivity of FV extracts it has been reproduced the normal condition of use reserved for beans by *in vitro* mimicking the traditional cooking procedures of beans (soaking and cooking in water) and their digestion by the gastrointestinal tract. Then, it has been evaluated the antitumoral activity of the different FV fractions obtained with this approach in colorectal cancer models.

II. Bibliographic part

1. Genus catalpa

In the order Lamiales, Catalpa Scop. is a genus of the family Bignoniaceae comprising just eight species, divided in two well-defined sections, *Catalpa* and *Macrocatalpa* Griseb. Section *Catalpa* is composed of four species of deciduous trees and is disjunctly distributed between temperate eastern Asia and eastern North America. *Catalpa bignonioides* is widely cultivated in all temperate areas of the world and is native to northern Florida, southern Georgia, southern Alabama, southern Mississippi, southern Louisiana, the eastern most edge of Texas, and the southern edge of Arkansas. *Catalpa speciosa* Teas is native to the middle Mississippi River and lower Ohio River valleys. Natural hybrids have been found in southeastern Missouri, where the two species have overlapping distribution ranges (Brown, 1920). *Catalpa bungei* C. A. Mey. sensu lato (s.l.), and *C. ovata* are distributed in central and northern China. Both species have been cultivated for millennia, especially in China, Japan, North America, Europe, and other temperate areas.

Catalpa fargesii Bureau and *C. fargesii* f. *duclouxii* (Don) Gilmour (or C. *duclouxii* Dode) are treated as synonyms of C. *bungei* s.l. by Olsen and Kirkbride (2017). However, the Flora of China accepted *C. fargesii* and treated *C. fargesii* f. *duclouxii* as a form of *C. fargesii*.

Four tropical, evergreen species of section *Macrocatalpa* Griseb. are distributed in the West Indies, from Jamaica across Cuba and Hispaniola to the Bahamas (Gentry, 1992), including *Catalpa purpurea* Griseb., *Catalpa macrocarpa* (A. Rich.) Ekman, *Catalpa longissima* (Jacq.) Dum. Cours, and *Catalpa brevipes* Urb. (Olsen and Kirkbride, 2017).

Species belonging to the *Bignoniaceae* have been widely used, for different purposes, in the medical practice of indigenous cultures of South America (Rasadah and Houghton, 1998). To date, interesting biological properties have been found in some extracts of the Catalpa species. For example, *C. ovata* fruit extracts have shown mutagenic activity against *Salmonella typhimurium* (Nozaka et al., 1989), while extracts obtained from the bark and stem have shown antitumor activity (Fujiwara et al., 1998). Regarding the properties of *C. bignonioides*, numerous uses have been reported depending on the part of the plant. In particular, the bark is used as a stimulating tonic in the form of syrup, the pod decoction is used for its emollient and relaxing properties, while the leaves have

proved to be useful for the treatment of ulcers. Further uses are those related to the treatment of respiratory diseases (decoction of pods and seeds), ophthalmia, skin affections and helminthic infections (Muñoz-Mingarro et al., 2003). Several studies have allowed the identification of the chemical composition of *C. bignonioides* methanol extract of its fruits fifteen compounds (Oh et al., 2021). The presence of different classes of compounds was observed in extracts of different parts of genus Catalpa, which could be related to the traditional uses of Bignoniaceae, including flavonoids, phenolic acids, phenols, triterpenes, fatty acids, iridoids, lignans, sterols, quinones, identified in "REAXYS" data base (hiips://www.reaxys.com).

1.1. *Catalpa bignonioides*

Catalpa bignonioides, also known as cigar tree, American catalpa and Indian bean tree, is a deciduous tree species of the family Bignoniaceae and is native to North, mainly in parts of Canada and the central and southern United States (Quan et al., 2022).

C. bignonioides is grown as an ornamental tree on streets and in gardens in almost all temperate climates around the world, and it is a medium-sized deciduous tree with a height of 15–18 m (Bozaci and Tağaç, 2022). Owing to its strong vigor, large leaves, and floral fragrance, as well as its ability to produce dense shade, *C. bignonioides* is mainly used as garden ornamental or street tree. Its flowers are white and bell-shaped, with yellow or lavender spots. The pods are similar to long beans and hang throughout the tree canopy, which is very attractive. There are different leaf colors, including green, yellow, and purple, as well as multicolored leaves. Attractive features of these trees are visible in three seasons, namely, its leaves in the spring, its flowers in the summer, and its fruits in autumn. The leaves are large and rough and have the ability to strongly absorb particles and heavy metals, such as lead and cadmium (Quan et al., 2022).

1.1. Botanical description

Catalpa bignonioides, commonly called Southern catalpa, is a medium-sized, deciduous tree that typically grows to 15–18 m tall with an irregular, broad-rounded crown. It is native to a relatively small area extending from central Mississippi, Alabama and Georgia south to the Florida panhandle (Missouri Botanical Garden, 2017).

The Southern catalpa is a perennial deciduous tree that blooms from May to June with a heavy load of flowers in the spring, followed by many leaves in the fall. In the winter, the

Southern catalpa develops many large seed pods. The tree has dense foliage in summer and porous foliage in winter. The leaves give off a disagreeable odour when crushed (Keeler, 1900).

The trunk up to 1 m diameter, with brown to grey bark, maturing into hard plates or ridges; it is short and supports long and straggling branches. The wood is light brown, sapwood nearly white; light, soft, coarse-grained, and durable in contact with the soil. The roots are fibrous and the branches are brittle, its juices are watery and bitter tasting (Missouri Botanical Garden, 2017).

The leaves are large, bright green and heart shaped, being 20-30 cm long and 15-20 cm broad. They secrete nectar, a most unusual characteristic for leaves, by means of groups of tiny glands in the axils of the primary veins (Seiler et al., 2016).

The flowers are 2.5-4 cm wide, trumpet shaped, white with yellow spots inside; they grow in panicles of 20-40 cm. The general effect of the flower cluster is pure white, but the individual corolla is spotted with purple and gold, and some of these spots are arranged in lines along a ridge, so as to lead directly to the nectar within. A single flower when, fully expanded, is 5 cm long and 40 mm wide. It is two-lipped and the lips are lobed, two lobes above and three below, as is not uncommon with such corollas (Missouri Botanical Garden, 2017).

The calyx is globular and pointed in the bud and splits into two, broadly ovate, entire lobes, green or light purple. The flower is perfect, possessing both stamens and pistils. The stamens are two, rarely four, inserted near the base of the corolla; the anthers are oblong, two-celled, opening longitudinally; the filaments are flattened and thread-like. The pistil is ovary superior and two-celled; the style is long, thread-like, with a two-lipped stigma (Keeler, 1900).

The fruit is a long, thin bean pod 20-40 cm long and 8-10 mm in diameter; it often stays attached to the tree during winter (Figure 1). The capsule is long and slender, nearly cylindrical; it contains numerous flat light brown seeds 25 mm long with two papery wings (Seiler et al., 2016).

Figure 1. Flower and fruits of *Catalpa bignonioides* Walt.
(https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:30194969-2)

1.2. Phytochemical composition

As mentioned above, previous phytochemical studies revealed that the extracts of genus Catalpa contained several classes of metabolites such as flavonoids, phenolic acids, phenols, triterpenes, fatty acids, iridoids, lignans, quinones (identified in REAXYS data base) and tannins (Rau, 1870; Tona et al., 1998).

Table 1 shows the phytochemical composition of *C. bignonioides*, including, flavonoids, iridoids, lignans, oligosaccharides, phenolic acids, phenolic glycoside, phenylethanoid diglycoside, the main classes identified in literature.

As shown in Table 1, iridoids are the largest class of compounds. Iridoids are an important class of cyclopentane[*c*]pyran skeleton metabolites and were detected in hight amounts in the extracts of genus Catalpa (Xu et al., 2014). They are a group of metabolites characterized by skeletons in which a six-membered ring containing an oxygen atom is fused to a cyclopentane ring. The term "iridoid" derives from a species of ants, *Iridomyrmex*, which utilize iridomyrmecin and iridodial in defensive secretions. Iridoids have been isolated from the secretion of several insects where they play a role in chemical defense and communication. They are irritants and deter most predators. Iridoid glycosides, aucubin and catalpol, present in *Plantago lanceolata* (Plantaginaceae) deter feeding by the beet armyworm herbivore *Spodoptera exigua* and the biotropic fungal pathogen *Diaporthe adunca* (Villaseñor, 2007).

Table 1. Bibliographic data concerning the phytochemical composition of *Catalpa bignonioides*.

Compounds	Molecular Formula	Molecular weight (exact)	precursor m/z $[M+H]^+$	UV λmax (nm)	*Catalpa bignonioides*				Ref.
Flavonoids					**Fruit**	**Petiole**	**Leaf**	**Root**	
5,6-Dihydroxy-7,4' -dimethoxyflavone-6-*O*-sophoroside	$C_{29}H_{34}O_{16}$	638.18469	639.1946	275, 335	✓				Oh et al., 2021
5,6-Dihydroxy-7,4'-dimethoxyflavone-6-*O*-*β*-D-glucopyranoside-(2→1')-*O*-[6'-benzoyl-*β*-D-glucopyranosyl-(1'→2'')]- *β*-D-glucopyranoside	$C_{42}H_{48}O_{22}$	904.26372	905.2669	266, 348	✓				Oh et al., 2021
Kaempferol 3-*O*-*β*-D-glucopyranoside	$C_{21}H_{20}O_{12}$	464.09548	465.1028	266, 348		✓			B. de Abreu et al., 2013
Isoquercetin	$C_{21}H_{20}O_{12}$	464.09548	465.1028	254, 354		✓			B. de Abreu et al., 2013
Kaempferol 3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	594.15847	595.1663	266, 348		✓			B. de Abreu et al., 2013
Quercetin 3-*O*-sambubioside	$C_{26}H_{28}O_{16}$	596.13773	597.1450	256, 356		✓			B. de Abreu et al., 2013
Quercetin 3-O-robinobioside	$C_{27}H_{30}O_{16}$	610.15338	611.1607	-		✓			B. de Abreu et al., 2013
Rutin	$C_{27}H_{30}O_{16}$	610.15338	611.1607	254, 354		✓			B. de Abreu et al., 2013
Luteolin 7-glucoside	$C_{21}H_{20}O_{12}$	448.10056	449.1078	-				✓	Harborne, 1967
6-Hydroxyluteolin	$C_{15}H_{10}O_7$	302.04265	303.0499	268, 364				✓	Harborne, 1967

Iridoids							
Catalpol*	$C_{15}H_{22}O_{10}$	362.12130	363,1286	210			Bai et al., 2019
Verproside**	$C_{22}H_{26}O_{13}$	498.13734	499.1446	224, 265			Park et al., 2009
Catalposide	$C_{22}H_{26}O_{12}$	482.14243	483.1497	215, 258	✓		Oh et al., 2021
Specioside	$C_{24}H_{28}O_{12}$	508.15808	509.1654	221, 310	✓		Oh et al., 2021
Picroside III	$C_{25}H_{30}O_{13}$	538.16864	539.1759	224, 328	✓	✓	Oh et al., 2021
Minecoside	$C_{25}H_{30}O_{13}$	538.16864	539.1759	224, 328	✓		Oh et al., 2021
6-*O-trans*-Feruloyl-5,7- bisdeoxycynanchoside	$C_{25}H_{32}O_{13}$	540.1843	541.1916	235, 303, 326		✓	Dal Piaz et al., 2013
6-*O-cis-p*-Isoferuloyl-5,7- bisdeoxycynanchoside	$C_{25}H_{32}O_{13}$	540.1843	541.1916	240, 295, 327		✓	Dal Piaz et al., 2013
6-*O-trans-p*-Isoferuloyl-5,7- bisdeoxycynanchoside	$C_{25}H_{32}O_{13}$	540.1843	541.1916	240, 295, 327		✓	Dal Piaz et al., 2013
6-*O-cis-p*-Coumaroyl-5,7- bisdeoxycynanchoside	$C_{24}H_{30}O_{12}$	510,1737	511.1810	230, 313		✓	Dal Piaz et al., 2013
6-*O-trans-p*-Coumaroyl-5,7- bisdeoxycynanchoside	$C_{24}H_{30}O_{12}$	510.1737	511.1810	230, 313		✓	Dal Piaz et al., 2013
6-*O-cis-p*-Methoxycinnamoyl-5,7-bisdeoxycynanchoside	$C_{25}H_{32}O_{12}$	524.1894	525.1967	225, 309		✓	Dal Piaz et al., 2013
6-*O-trans-p*-Methoxycinnamoyl-5,7-bisdeoxycynanchoside	$C_{25}H_{32}O_{12}$	524.1894	525.1967	225, 309		✓	Dal Piaz et al., 2013
6′-*O-p*-Hydroxybenzoyl-6-*O-p*-hydroxybenzoyl-5,7-bisdeoxycynanchoside	$C_{29}H_{32}O_{14}$	604.17921	605.1865	258, 320		✓	Dal Piaz et al., 2013
6-*O-p*-Hydroxybenzoylphelipaeside	$C_{22}H_{28}O_{12}$	484.15808	485.1654	263		✓	Dal Piaz et al., 2013
3,4-Dydrocatalposide	$C_{22}H_{28}O_{12}$	484.15808	485.1654	260		✓	Dal Piaz et al., 2013
6-O-(6′-*O-p*-Hydroxybenzoyl)-*β*-D-glucopyranosyl-des-p-hydroxybenzoyl-3-deoxycatalpin	$C_{22}H_{28}O_{11}$	468.16316	469.1704	257		✓	Dal Piaz et al., 2013

3-Methoxycatalpin	$C_{17}H_{20}O_7$	336.12090	337.1282	257		✓		Dal Piaz et al., 2013
Catalpin	$C_{16}H_{18}O_7$	322.10525	323.1125	-		✓		Dal Piaz et al., 2013
6-*O*-*p*-Hydroxybenzoyl-5,7-bisdeoxycynanchoside	$C_{22}H_{28}O_{12}$	484.15808	485.1654	-		✓		Dal Piaz et al., 2013
Des- *p*-hydroxybenzoyl-3-deoxycatalpin	$C_9H_{15}O_4$	187.0970	188.10431	-		✓		Dal Piaz et al., 2013
6'-*O*-*p*-Hydroxybenzoyl catalpol	$C_{22}H_{26}O_{12}$	482.1424	483.1497	215, 258			✓	Iwagawa et al., 1991
5,7-Bisdeoxycynanchoside	$C_{22}H_{28}O_{12}$	484.1581	485.1654	213, 258			✓	Iwagawa et al., 1991
5-*β*-Hydroxy-1-*O*-(6'-*O*-*p*-hydroxybenzoyl)-*β*-D-glucopyranosyl eucommiol	$C_{22}H_{30}O_{12}$	486.1737	487.1810	216, 258			✓	Iwagawa et al., 1991
Amphicoside	$C_{23}H_{28}O_{13}$	512.1530	513.1603	263, 293			✓	Iwagawa et al., 1991
Lignans								
Pinoresinol	$C_{20}H_{22}O_6$	358.14164	359.1489	232, 281	✓			Oh et al., 2021
Isolariciresinol	$C_{20}H_{24}O_6$	360.15729	361.1646	232sh, 284	✓	✓		Oh et al., 2021
(+)-Lariciresinol	$C_{20}H_{24}O_6$	360.15729	361.1646	231, 282	✓			Oh et al., 2021
5-Hydroxysesamin 5-*O*- *β*-D-glucopyranoside-(1→2)- [*β*-D-glucopyranosyl-(1→6)]- *β*-D -glucopyranoside	$C_{38}H_{48}O_{20}$	824.27389	825.2812	-		✓		B. de Abreu et al., 2013
Oligosaccharides								
1,6-di-*O*-*p*-Hydroxybenzoyl-*β*-D-glucopyranoside	$C_{20}H_{20}O_{10}$	420.10565	421.1129	246		✓		B. de Abreu et al., 2013
Benzyl *β*-D-glucopyranoside (benzyl *β*-D-glucoside)	$C_{13}H_{18}O_6$	270.11034	271.1176	258		✓		B. de Abreu et al., 2013

1-*O*-ethyl-6-(*p*-hydroxybenzoyl)- *β*-D-glucopyranoside	$C_{15}H_{17}O_8$	325.09234	326.0996	238		✓	B. de Abreu et al., 2013
1-*O*-*p*-Hydroxybenzoyl- *β*-D-glucopyranoside	$C_{13}H_{16}O_8$	300.08452	301.0918	242		✓	B. de Abreu et al., 2013
6-*p*-Hydroxybenzoyl-D-glucose (*α,β*)	$C_{13}H_{16}O_8$	300.08452	301.0918	260		✓	B. de Abreu et al., 2013
Phenolic acids							
4-Hydroxybenzoic acid	$C_7H_6O_3$	138.03169	139.0390	254	✓		Oh et al., 2021
Vanillic acid	$C_8H_8O_4$	168.04226	169.0495	270	✓		Oh et al., 2021
p-*C*oumaric acid	$C_9H_8O_3$	164.04734	165.0546	226, 312	✓		Oh et al., 2021
Phenolic glycoside							
1-Benzyl-[6-p-hydroxybenzoyl]- *β*-D-glucopyranosyl-(1→3)- *β*-D-glucopyranoside	$C_{26}H_{32}O_{13}$	552.1843	553.1916	257, 320sh		✓	B. de Abreu et al., 2013
Phenylethanoid diglycoside							
2-(4-hydroxyphenyl)ethyl[5-*O*-(4-hydroxybenzoyl)]- *O*- *β*-D-apiofuranosyl-(1→2)- *β*-D-glucopyranoside	$C_{26}H_{32}O_{13}$	552.1843	553.1916	210, 259			✓ Iwagawa et al., 1991

(*) Catalpol: widely found in plants of genus Catalpa, in which it was first discovered in 1962.
(**) Verproside: iridoid glucoside isolated from *Catalpa ovata*

The iridoids are widely distributed in the kingdom Plantae, specially with plant orders such as the Dipsacales, Gentianales, and Lamiales having significantly high concentrations of iridoids (Wang et al., 2020; Dinda, 2019).

The four major groups of iridoids are iridoid glycosides, non-glycosylated iridoids, secoiridoids, and bisiridoids. Iridoids have hemiacetal hydroxyl groups and are active in nature. Because of the unstable nature of the C1-OH group, iridoids often react with sugar to form glycosides. Three basic skeletons of iridoids are elucidated in Figure 2.

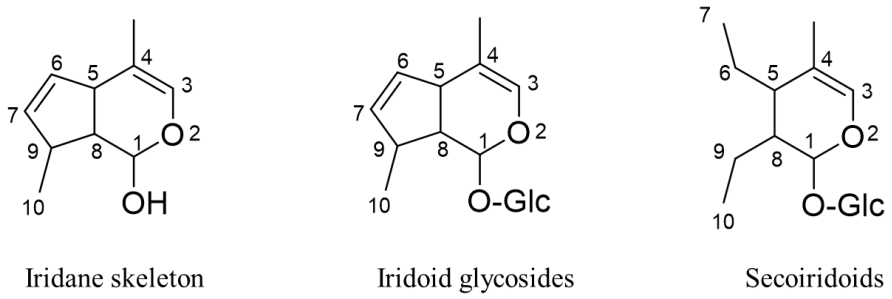


Figure 2. **Three basic skeletons of iridoids.** (Modified from Wang et al., 2020).

As of 2011, approximately 2057 iridoids had been isolated and identified from higher plants. Among them, catalpol and 6-*O*-substituted catalpol derivatives, are a class of the naturally occurring 7,8-epoxy cyclopentanoid iridoids. They are present in 50 genera of 14 families and are mainly located in the aerial parts or whole plants of *Bignoniaceae*, *Lamiaceae*, *Plantaginaceae* and *Scrophulariaceae* (Zhang et al., 2019). Their chemical structure is shown in Figure 3.

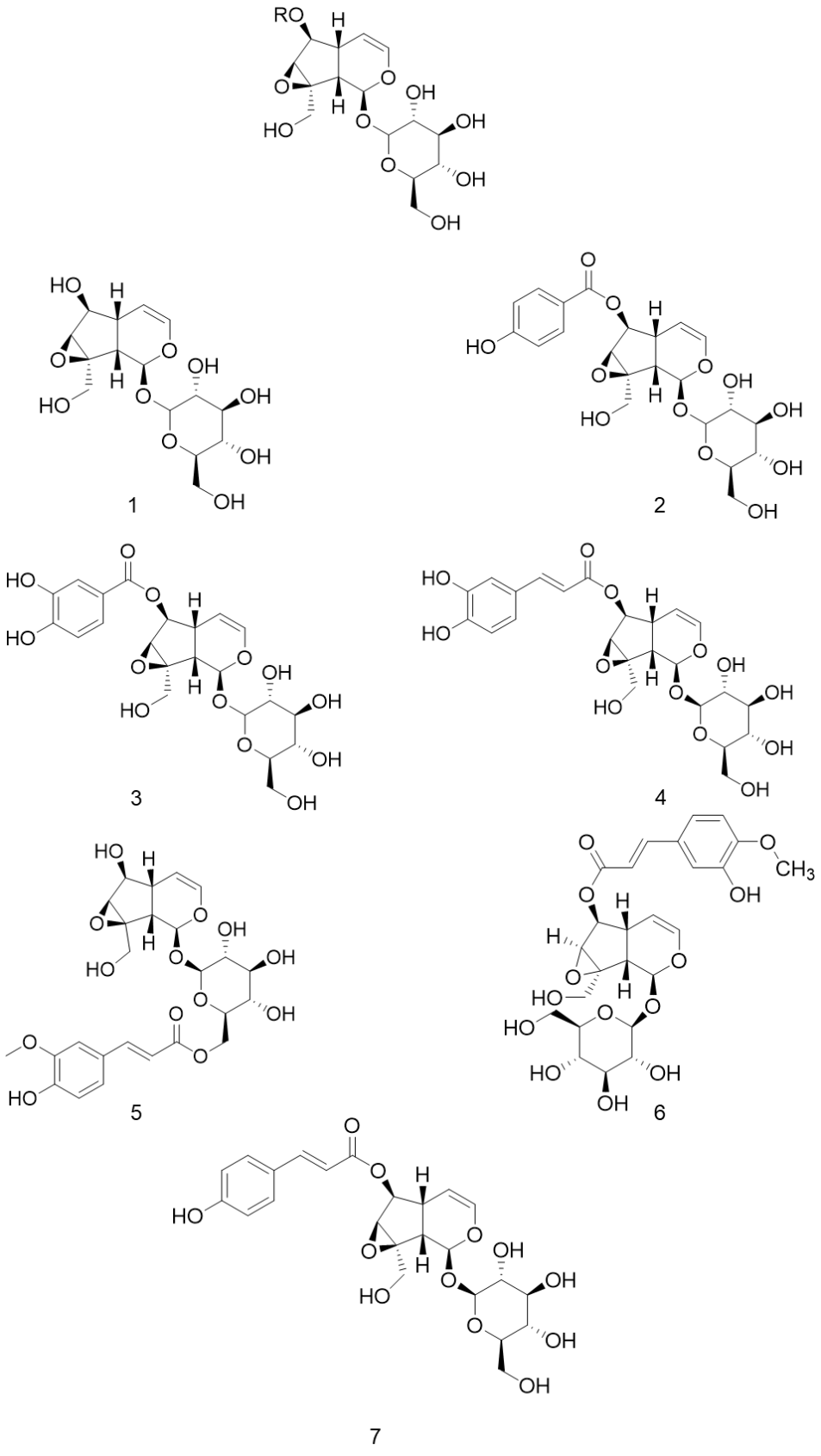


Figure 3. Chemical structure of 6-*O*-substituted catalpol derivatives. (1) catalpol; (2) catalposide; (3) verproside; (4) verminoside ; (5) picroside III; (6) minecoside; (7) specioside.

As described previously, the extracts of *Catalpa bignonioides* contain several classes of compounds, which could be linked to some of the traditional uses of *Bignoniaceae* (Muñoz-Mingarro et al., 2003).

Phenolic compounds, identified in various parts of *C. bignonioides*, include lignans, flavonoids and phenolic acids. Among the flavonoids, rutin, isoquercetin, 6-hydroxyluteolin and sugar derivatives of kampeferol and quercetin have been determined (De Abreu et al., 2013; Harborne, 1967). Phenolic acids have been identified in fruits of *C. bignonioides* and are represented by vanillic acid, *trans-p*-coumaric acid and 4-hydroxybenzoic acid. Lignans have also been found in the fruits of this plant and are represented by pinoresinol, isolariciresinol and (+)-lariciresinol (Oh et al., 2021).

Oligosaccharides are another class of compounds found in *C. bignonioides* petioles. The main components are derivatives of the *β-D*-glucopyranoside, *β-D*-glucoside, *α-D*-glucose and *β-D*-glucose (De Abreu et al., 2013).

Chisholm and Hopkins (1965) found that the seed oil of *Catalpa bignonioides* consisted of the following fatty acids: *trans*-9, *trans*-12-octadecadienoic, *trans*-9, *trans*-11, *cis*-13-octadecatrieiloic, palmitic, stearic, octadecenoic, linoleic.

1.3. Biological activities

1.3.1. Biological activity of iridoids

Many phytochemical studies reported that the extracts from Bignoniaceae species contained several iridoids (De Abreu et al., 2014). Tundis et al., reported that iridoids exhibit a wide range of anti-inflammatory, antioxidant, antitumor, hepatoprotective, antimicrobial, antinociceptive, neuroprotective, neuritogenic, angiogenic, anxiolytic, osteoporotic, and antiageing activities.

Numerous studies indicate that the main medicinal effects of Catalpa plants are attributable to iridoid glucosides (Kimura et al., 1963; Suzuki et al., 1991; Noro et al., 1992). The first study carried out on the presence of iridoids was published in 1888 by Claaseen (Claaseen, 1888) who isolated a bitter substance in the form of colorless needles from the bark and unripe fruits of *C. bignonioides*. The substance was later classified as a glucoside and given the name of catalpin. Fifty-five years later, Colin, Tauret and Chollet isolated the compound again from the same plant, naming it catalposide (Chollet,

1946), to which anti-inflammatory and diuretic properties have been attributed (An et al., 2002; Kim et al., 2004; Suzuki et al., 1968).

Furthermore, in the extracts of *C. bignonioides* there are other iridoids with biological properties, including catalpol, for which useful properties have been highlighted in the treatment and management of diabetes and its complications (Bai et al., 2019; Liu et al., 2023), (7R)-3-methoxy-hydroxyeucommic acid and minecoside, which are also known regulators of glucose homeostasis (Oh et al., 2021).

Catalposide, together with catalpol, represents the main component found both in the vegetative parts (leaves, wood, bark) and in the fruits of various species of Catalpa (Chollet, 1946; El-Naggar and Doskotch, 1980; Stephenson, 1982; Suzuki et al., 1991; Wysokińska and Światek, 1995). Studies conducted to demonstrate its biological properties revealed that catalposide isolated from the stem cortex of *C. ovata* inhibits the expression and production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), Interleukin-1β (IL-1 β) and Interleukin-6 (IL-6), through the inhibition of the translocation of p65, a subunit of Nuclear factor-kB (NF-kB), in RAW264 macrophages treated with lipopolysaccharides (LPS) (An et al., 2002).

Preliminary microarray tests also demonstrated that catalposide significantly reduced the expression of TNF-α -induced proinflammatory genes (e.g., Interleukin 8, IL-8), and the ERK 1/2 and p38 kinase pathways in *in vitro* colon cancer models (Kim et al., 2004). In addition, a protective effect of catalposide was demonstrated in mouse models of TNBS-induced colitis, an experimental model of inflammatory bowel disease (IBD). In particular, catalposide has been shown to be active through the inhibition of the NF-kB and mitogen-activated protein kinase (MAPK) signaling pathways, thus reducing the expression of various pro-inflammatory genes (Kim et al., 2004). In conclusion, thanks to this study, it has been demonstrated that the administration of catalposide can have a beneficial effect for the treatment of IBD in humans, justifying the wide use of *C. ovata* extract for the treatment of inflammatory colon disease in Korea.

Catalpol represents one of the main iridoid glucosides present in plants of the genus Catalpa, in which it was discovered for the first time in 1962 (Tang and Eisenbrand, 1992). As an iridoid glucoside with a polar structure, catalpol is soluble in water (Bai et al., 2019), unstable at high temperatures (Dongyan et al., 2014), and rapidly degrades at 100°C (2-6 h) (Dang et al., 2017). Furthermore, it is less stable in acidic than in alkaline environments (Liu et al., 2014). Recent studies aimed at establishing the biological

properties of catalpol have demonstrated how its multiplicity of action makes it useful for the treatment and management of diabetes. The results obtained using diabetic mouse models treated with catalpol revealed that it is able to restore correct glycemic levels, lipid profile and improve glucose tolerance (Bai et al., 2019). Furthermore, as evidenced by further studies, catalpol showed a hypoglycemic effect in streptozotocin-induced C57BL/6J diabetic mice (Xu et al., 2015; Li et al., 2014) through the reduction of blood glucose (FBG) and fasting insulin.

The actions of catalpol extend to various tissues, including liver, pancreatic tissue, adipose tissue, skeletal muscle tissue (figure 4).

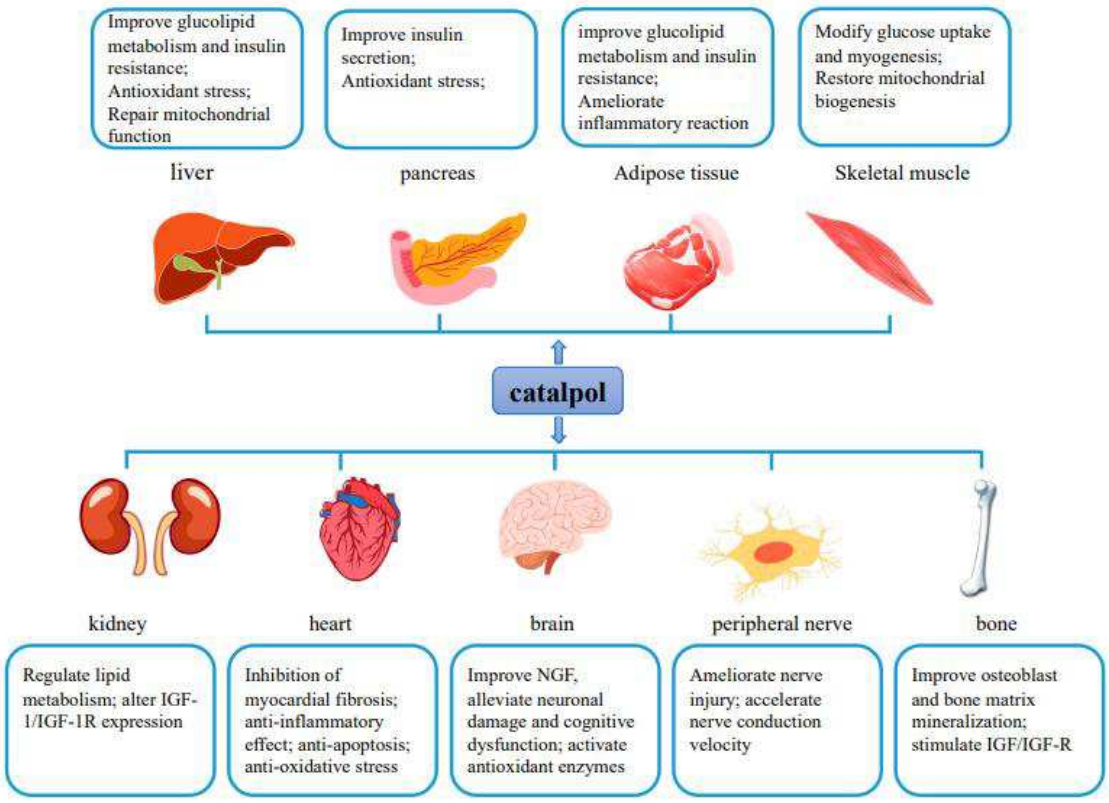


Figure 4. Activity of catalpol on various tissues and systems. (Bai et al., 2019)

Catalpol has been shown to exert a beneficial effect on the diabetic liver by promoting glucose utilization and glycogen synthesis, as well as lipid metabolism and improving mitochondrial function. The mechanisms underlying these effects may be involved in the regulation of the AMPK/NOX4/PI3K/Akt signaling pathway and in the modulation of multiple genes or protein expression, such as PEPCK, GSK3β, ACC, HMGCR, MFN1, FIS1, DRP1 and β- endorphins (Bai Y et al., 2019). At the pancreatic level, as

demonstrated by experiments conducted on diabetic mice, it is able to exert a protective action on the tissue by increasing insulin secretion, upregulating the expression of AMPK-a1 (Zou et al., 2018) and by antioxidant activity (Zhu et al., 2016).

The protective effect of catalpol on diabetic adipose tissue stems from its ability to reduce inflammation, mitigate oxidation, and improve glycolipid metabolism through suppression of NF-κB activation and MAPK phosphorylation as well as through the reduction of inflammatory reactions induced by advanced glycation end products (AGEs) (Zhou et al., 2015; Choi et al., 2013).

Furthermore, catalpol has been shown to exert its antidiabetic effect by promoting myogenesis and mitochondrial biogenesis in skeletal muscle by contributing to glucose uptake and consequently leading to blood glucose normalization (Xu et al., 2018). The mechanisms underlying these effects may be related to the regulation of PGC-1α expression and the PI3K/Akt signaling pathway (Bai et al., 2019).

In addition to the beneficial and protective effects on the various tissues, it has been demonstrated that catalpol also carries out an activity against the main complications associated with diabetes, attributable to diabetic nephropathies, cardiomyopathies, encephalopathies, diabetic osteoporosis and neuropathies (Bai et al., 2019). Catalpol may preserve diabetic renal function through regulation of lipid metabolism and modulation of IGF1 expression. At the cardiac level it exerts cardiovascular protective effects by preventing the proliferation of myofibroblasts and by inhibiting the apoptosis of cardiomyocytes thanks to its anti-inflammatory and antioxidant characteristics. Furthermore, numerous studies have shown that it exerts a therapeutic effect on myocardial dysfunction and ischemia-reperfusion injury after myocardial infarction (Bi et al., 2018; Huang et al., 2013; Pavlovic et al., 2007). Catalpol has also been reported to prevent the development of diabetic encephalopathy through upregulating GSH-Px, SOD and CAT activity and decreasing MDA content (Wang et al., 2010), and to improve hippocampal injury and attenuate cognitive dysfunction through increased expressions of nerve growth factor (NGF), protein kinase Cγ (PKCγ), and caveolin-1 (Cav-1) (Zhou et al., 2014).

In relation to diabetic peripheral neuropathy, catalpol demonstrates a therapeutic effect in the prevention of this pathology (Liu, 2006; Zhou, 2007) since the results obtained from various studies suggest that it could improve nerve conduction velocity and preserve the histological structures of the root ganglion dorsal and sciatic nerve. Furthermore, catalpol

could induce upregulation of IGF-1 and activation of PI3K/Akt signaling pathway. Finally, the activity against diabetic osteoporosis is demonstrated by the action that catalpol exerts by increasing the activity of osteoblast differentiation and mineralization of the bone matrix. The mechanisms underlying these effects may be associated with inhibition of PPARγ and GSK-3β and activation of IGF/β-catenin and PI3K/Akt/ mammalian target of rapamycin (mTOR) signaling (Zhang, 2016).

Thanks to the results obtained from these studies, it has been demonstrated that catalpol, a natural, safe and non-toxic compound, can play a leading role in the management of diabetes and its complications.

In addition to catalpol, other iridoids including (7R)-3-methoxyhydroxyeucommic acid and minecoside, together with a flavone glycoside, 5,6-dihydroxy-7,4'dimethoxyflavone-6-*O*-*β*-D-glucopyranoside, can be employed in the regulation of glucose homeostasis and therefore as an alternative for the treatment of diabetes. In particular, these compounds exhibit significant inhibitory activity on *α*-glucosidase and on the GSIS effect, or on glucose-mediated insulin secretion. Furthermore, the GSIS effects of these three active compounds were supported by the increased expressions of PPAR-γ, IRS-2, PI3K, Akt, and PDX-1 (Oh et al., 2021).

As previously reported, fatty acids are also present in *C. bignonioides* extracts, mainly represented by catalpic acid that is an isomer of conjugated linoleic acid containing trans-9, trans-11, cis-13 double bonds. It is present in high concentrations in the seeds of plants of the genus Catalpa, including *C. bignonioides*, where it makes up over 60% of the seed oil. Studies conducted on mice fed diets supplemented with catalpic acid have shown that this compound decreases abdominal fat deposition, triglyceride concentrations and regulates glucose and insulin homeostasis, promotes HDL cholesterol increase and modulates gene expression of white adipose tissue. These changes have been associated with an upregulation of PPARα and its responsive genes represented by SCD1 and ECH (Hontecillas et al., 2008). In addition to iridoid glucosides and fatty acids, the presence of phenols, terpenes, alkaloids, tannins, saponins and quinones in the extracts of *C. bignonioides* was also taken into consideration (Muñoz-Mingarro et al., 2003), and studies have been conducted on their possible biological activities. Both methanolic extracts obtained from freeze-dried material from corollas, calyxes, inflorescences, vale of capsules, hypertrophic placenta and leaves, and aqueous extracts of leaves, pods and seeds have been used (Muñoz-Mingarro et al., 2003). The highest content of phenols,

flavones and flavonols was found in the methanolic extracts of the leaves. These also show the highest antioxidant activity in both inhibition of tyrosine nitration and DPPH (2,2-diphenyl-1-picryl-hydrazylhydrate) reduction (Dvorská et al., 2007). Encouraging results were also obtained from the analysis of the activities of the aqueous extracts of *C. bignonioides* for which a very low toxicity was demonstrated following tests conducted against the HepG2 cell line and promising anti-inflammatory and antinociceptive activities attributed to saponins, sterols and phenolic compounds. In particular, steroid saponins, which possess hydro-aromatic ring systems similar to those of steroids, could play a significant role (Gupta et al., 1969).

Many of the characteristics and properties described for *C. bignonioides*, including antioxidant and anti-inflammatory activity, as well as the high content of phenolic compounds in leaf extracts, are shared among various species belonging to the genus Catalpa, such as *C. ovata* (Kim et al., 2019), *C. bungei* and *C. speciosa* (Xu et al., 2014). Further studies conducted on these species have highlighted, in addition to the already mentioned activities, promising antitumor properties attributed to phenols and naphthoquinones present in these plants. The anticancer activity of *C. speciosa* is mainly related to its phenolic compounds which include ferulic acid, phydroxybenzoic acid, vanillic acid and caffeic acid (Elansary et al., 2019). In particular, it has been shown that ferulic acid is able to inhibit the epithelial-mesenchymal transition (EMT) in breast cancer cells (Zhang et al., 2016) and to induce cell cycle arrest in models of cervical cancer cells (Gao et al., 2018).

Studies conducted on *C. ovata* (Fujiwara et al., 1998) and *C. bungei* (Qin et al., 2022) have demonstrated the role of naphthoquinones in the antitumor action. Previous studies on the chemical composition of the Catalpa genus had already highlighted the antitumor action of *β*-lapachone, a quinone that induces death in some human breast cancer cell lines, and of *α*-lapachone, another anticancer compound isolated from the same genus of plants and distinguished from *β*-lapachone by its 1,4-naphthoquinone structure. Eleven additional *α*-lapachone derivatives were subsequently isolated from *C. bungei*, for which antiproliferative activity was demonstrated in tumor cell models (Qin et al., 2022). However, despite the similarities in terms of composition and activity among the various Catalpa species, studies on the antitumor activity of *C. bignonioides* are lacking.

1.4.2. Biological activity of other classes

Scientific evidence has shown that flavonoids induce several health benefits in humans, and a diet rich in these compounds can help prevent some chronic diseases. They can scavenge free radicals and act as antioxidants is indubitably the most relevant (Dias et al., 2021). The antioxidant action mechanisms of flavonoids can be by the (a) direct scavenging of ROS, (b) inhibition of ROS formation through the chelation of trace elements (e.g., quercetin has iron-chelating and iron-stabilizing properties), or inhibition of the enzymes that participate in the generation of free radicals (e.g., glutathione S-transferase, microsomal monooxygenase, mitochondrial succinoxidase, NADH oxidase, and xanthine oxidase), and (c) activation of antioxidant defenses (e.g., upregulation of antioxidant enzymes with radical scavenging ability) (Kumar and Pandey, 2013; Agrawal, 2011).

Most of the flavonoids appear as glycosides, and the number and position of connections with the sugar affect the antioxidant properties of the flavonoid. However, aglycone forms have a higher antioxidant capacity, but their availability is lower (Sandoval, et al., 2020). Some flavonoids, such as quercetin, rutin, catechin, genisten and cyanidin have been demonstrated to exhibit anti-inflammatory functions during *in vitro* and *in vivo* experiments and in clinical studies. The anti-inflammatory activities of flavonoids also have an important impact on cancer development. Flavonoids have been reported to inhibit tumor cell proliferation by inhibition of ROS formation and repression of the enzymes xanthine oxidase, cyclooxygenase-2, and 5-lipoxygenase, implicated in tumor promotion and development (Dias et al., 2021).

Flavonoids exert a wide range of anticancer effects. Kaempferol possesses antiproliferative and apoptosis activity in human osteosarcoma and breast (MCF-7), stomach (SGC-7901), and lung (A549) carcinoma cells (Kozlowska and Szostak-Wegierek, 2017).

Phenolic acids are the most prominent class of bioactive chemicals grouped under phenolic compounds present in different parts of the plant. They are the aromatic secondary metabolites imparting color, flavor, astringency, and harshness, which contribute to the typical organoleptic characteristics of the foods (Rashmi and Negi, 2020). Phenolic acids contribute to the overall health improvement, primarily because of antioxidant and anti-inflammatory actions (Tresserra-Rimbau et al., 2014); help in the

prevention of cardiovascular diseases and various cancers (Goleniowski et al., 2013); protect against oxidative damage diseases; and exhibit antimicrobial, antimutagenic, hypoglycemic and anti-platelet aggregating activities (Saxena et al., 2012).

They are classified as hydroxybenzoic acids and hydroxycinnamic acids, because of their two distinctive carbon frameworks as well as depending on positioning and number of the hydroxyl groups on the aromatic ring. Gallic acid, p-hydroxybenzoic acid, salicylic acid, ellagic acid, gentisic acid, protocatechuic acid, syringic acid, and vanillic acid are the major hydroxybenzoic acids. Among the hydroxycinnamic acid and its derivatives, p-coumaric acid, cinnamic acid, caffeic acid, ferulic acid, sinapic acids, isoferulic acid, and p-hydroxycinnamic acid are the most common (Rashmi and Negi, 2020).

Previous studies have shown that phenolic acids have an antidiabetic activity because they are able to inhibit α-amylase and α-glucosidase by vanillic acid, syringic acid and *O*-coumaric acid (Mojica et al, 2015). Their anti-obesity and cardioprotective action is shown by the greater reduction of total cholesterol, triglycerides, free fatty acids, and phospholipids by p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid and hydroxycinnamic acid (Zhu et al., 2012). Ferulic and *p*-coumaric exhibit anti-bacterial activity (Amarowicz et al., 2008), whereas chlorogenic and caffeic acids are claimed to be anti-diabetic and anti-hypertensive (Ranilla et al., 2010). Caffeic acid and chlorogenic acids inhibit cancers of stomach and colon (Kurata et al., 2007).

Lignans are traditionally defined as a class of secondary metabolites that are derived from the oxidative dimerization of two or more phenylpropanoid units (Barker, 2019). Most lignans occur freely in plants, but a small proportion of them co-exist with sugars to form glycosides in wood and resin of plants (Zhang et al., 2014). Despite their common biosynthetic origins, they boast a vast structural diversity. It is also well-established that this class of compounds exhibit a range of potent biological activities (Barker, 2019).

Traditionally, health benefits attributed to lignans have included a lowered risk of heart disease, menopausal symptoms, osteoporosis, and breast cancer. Rodriguez-Garcia et al. present a review that focuses on the potential health benefits attributable to the consumption of different diets containing naturally lignan-rich foods. Current evidence endorses lignans as human health-promoting molecules and, therefore, dietary intake of lignan-rich foods could be a useful way to bolster the prevention of chronic illness, such as certain types of cancers and cardiovascular disease (Barker, 2019).

Fatty acids are major components of triacylglycerols, phospholipids, and other complex lipids. Triacylglycerols, and hence fatty acids, are the main contributors to dietary fat in humans. A major metabolic role of fatty acids is as a source of energy because of their oxidation. Fatty acids are good energy sources and can be used for energy generation by most aerobic tissues but not the brain, providing an alternative to glucose. An important functional role of fatty acids is as components of cell membrane phospholipids.
Several fatty acids can regulate the expression or activity of transcription factors, meaning that those fatty acids play a role in controlling gene expression and protein production by cells. These effects enable fatty acids to regulate metabolic processes such as fatty acid synthesis and oxidation and lipoprotein assembly and clearance, insulin sensitivity, and inflammation. Thus, it is evident that fatty acids, even those commonly consumed in the diet, have a range of general and specific biological activities that act to influence cell and tissue metabolism, function, and responsiveness to hormonal and other signals. Through these effects, fatty acids influence health well-being and disease risk (Calder PC, 2015).

Oligosaccharides and their esters, a significant group of phytochemical compounds, are widely distributed in the roots, rhizomes, stems, barks, leaves, aerial, and whole parts of plants. They not only serve as the energy storage components, but also play a vital role in the treatment of diseases.
These compounds have a wide variety of structure types because of the assembly of different monosaccharide units, the combination of various linking styles and the existence of kinds of substituents. And more promising biological activities associated with some of the oligosaccharides and their esters have been discovered. *In vitro* and *in vivo* investigations have demonstrated that they displayed antioxidant, antidepressant, anti-inflammatory, neuroprotective, cerebral protective, antidiabetic, cytotoxic, antineoplastic, plant growth regulatory, and immunopotentiating activities, and so forth (Chen et al., 2015).

2. Genus Phaseolus

Common bean is the most important edible legume for direct consumption and production in the world; it was first cultivated in Peru and Mexico around 8000 years ago (Ganesan and Xu, 2017). Among the highest consumers of common beans are Brazil (19.7%), India (19.7%), Mexico (7.7%), the United States (6.6%), Tanzania (2.7%), and Uganda (2.7%). In Italy, about 12 thousand tons were produced on an area of 6411 hectares, a production that has grown strongly in recent years thanks to the interest that this food is receiving (FAOSTAT, 2018).

2.1. Botanical description and taxonomy

Cultivated *P.vulgaris* has a tap root system with lateral roots located in the first 15 cm of soil. The roots are colonized by bacteria of the genus Rhizobium, which produces irregular root nodules (Purseglove, 1968). The stems are protected hairy, with hair length and density dependent on the cultivar. However, short, hooked hairs are always present on the youngest stem spots (Debouck and Hidalgo, 1986; Singh et al., 1991; Lackey, 1981; Freytag and Debouck, 2002). Hair plays a role in both disease resistance and as a defense against insects. There is evidence that hairs interrupt the production of fungal spores, which allow for the reduction of secondary inoculum (e.g. bean rust, Uromyces appendiculatus), and actually injure insects (e.g. leafhoppers, Empoasca fabae), resulting in a reduction in predation (Mmbaga and Steadman, 1992; Pillemer and Tingey, 1978).
The leaves are trifoliate and alternate on the stems, generally they are entire and hairy, with small stipules (Purseglove, 1968; Wortmann, 2006) and, although they differ between the different cultivars, in most cases they have a broad base and a flattened tip (Singh et al., 1991).
The pods are narrow, usually containing 4-6 seeds, but in some cases up to 12 seeds per pod can be achieved. Seeds are produced in a wide variety of colours, depending on the cultivar, (Purseglove, 1968; Wortmann, 2006) and their sizes can vary considerably.
Numerous species of Phaseolus are grown throughout the world, primarily for their seeds (beans), which are harvested when ripe and marketed as dry produce.
Most legume seeds have a similar morphological structure and they have three major components: the seed coat, the cotyledons, and the embryo axis. In most dry seeds, they account for 8–20%, 80–90%, and 1–2% of the seed weight, respectively. Many nutrients

are present in cotyledons. Typical seed structure and various anatomical parts of legume seeds are shown in Figure 5. (Encyclopedia of Food Sciences and Nutrition, 2016).
The outermost layer of the seed is the seed coat or head. The outer seed structure includes the hilum, micropyle, and raphe. The hilum is a scar-like structure (usually oval in shape) near the centre edge where the seed leaves the stem. The micropyle is the small opening in the seed coat where the pollen tube originally enters the valve. The raphe is the crest on the side of the hilum opposite the micropyle and represents the base of the stalk which fuses with the maturing seed coat. In most legumes, the endosperm is short-lived and shrinks into a thin layer that surrounds the cotyledons or embryos (Lam et al., 2003).

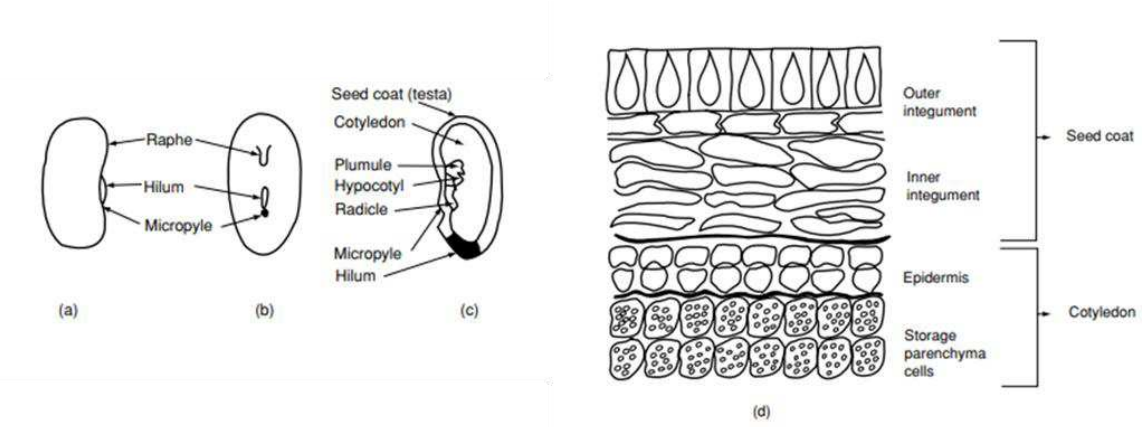


Figure 5. Dry bean *Phaseolus vulgaris* seed. **(a)** external side view, **(b)** external face or edge view (viewing at hilum side), **(c)** cross-section (one cotyledon removed), and **(d)** detailed cross-section across the seed coat and cotyledon. Reproduced from Encyclopedia of Food Sciences and Nutrition, 2nd edn. (2003), p. 407, Elsevier Ltd.

P. vulgaris is part of the legume family and its taxonomic hierarchy is reported below:

Order: *Fabales*
Family: *Fabaceae*
Genus: *Phaseolus* L.
Species: *Phaseolus vulgaris* L.

The genus *Phaseolus* includes approximately 80 cultivated and wild species, but *P.vulgaris* is the most widely cultivated species. In addition to *P. vulgaris*, four other species of Phaseolus are mainly cultivated: *P. dumosus*, *P. coccineus*, *P. acutifolius* and *P. lunatus* (Chacón et al., 2007; Broughton et al., 2003; Bellucci et al., 2014; Delgado-Salinas et al., 2006). *P. vulgaris* shares many of the features characterising the family, but

two features distinguish the entire *Phaseolus* genus from the rest of the family: the keel of the flower terminates in a coil, having from one to two turns (Purseglove, 1968; Gentry, 1969; Bailey, 1975), and uncinate hairs are present on both vegetative and reproductive structures of the plant (Freytag and Debouck, 2002).

2.2. *Phaseolus vulgaris*

Common beans (*Phaseolus vulgaris* L.) are annual plants, cultivated in temperate and semitropical regions for their edible dry seeds that are variously called navy beans, kidney beans, red beans, black beans, pinto beans, and cranberry beans (Ganesan and Xu, 2017). They do not differ mostly in their nutritional compositions; they differ slightly in taste, texture and cooking times.

Navy beans are small, white-skinned, oval-shaped beans. They were used in the US Navy diet during the 19th century hence their name. Diets containing navy beans exerted beneficial effects during experimental colitis by reducing both local and systemic inflammatory biomarkers. Emerging evidence supports the efficacy of navy beans in regulating serum cholesterol and lipid profiles and in inhibiting the incidence and recurrence of adenomatous polyps or precancerous growths, thereby preventing colorectal cancer.

Kidney beans are large-sized, firm textured, red/pink glossy skinned and kidney-shaped beans. They have the potential to reduce glycemic index in experimental diabetes and the ability to attenuate colonic inflammation in healthy mice.

Red beans are small, soft red textured, oval-shaped beans. They exert an anti-inflammatory response and have health-promoting potential with anti-fungal, immunomodulatory, anti-proliferative and apoptosis-inducing activities in tumor cells.

Black beans are known as turtle beans and are sweet in taste, soft texture, medium-sized, and oval-shaped beans. These coats are an excellent source of anthocyanins and other phenolics with the potential to be used as natural food colorants with exceptional anti-diabetic potential.

Pinto beans are medium-sized, brown-skinned, oval-shaped beans. Hemagglutinins, defensins isolated from pinto beans, possess anti-fungal, anti-diabetic and anti-tumor activities.

Cranberry beans, also called Roman beans, are red creamy textured, medium-sized and oval-shaped beans. They are rich in phenolic compounds and non-digestible fermentable

components, which may help alleviate experimental colitis and mitigate the severity of other gut barrier-associated pathologies (Ganesan and Xu, 2017).

2.3. Phytochemical composition

Common beans play a vital role in the diet and provide numerous health benefits as they are a rich source of nutrients. Health benefits of beans are generally attributes to their high content of proteins, carbohydrates, dietary fiber, low saturated fat content, vitamins, and minerals (Uebersax et al., 2023). In addition to these major components, beans also contain a rich variety of phytochemicals, antioxidant activity, and an extensive array of flavonoids such as anthocyanins, flavonoids (kaempferol, pelargonidin, cyanidin, delphinidin), proanthocyanidins, flavonols, phenolic acids (chlorogenic acid, syringic acid, caffeic acid), and isoflavones (Hayat et al., 2014). Sufficient amounts of polyphenols in the dried beans act as potent anti-oxidants (Ganesan and Xu, 2017). Regular intake of these dried beans containing total and soluble fiber as well as resistant starches have reduced glycemic index in the human. Studies have also suggested that diets that include beans reduce low-density lipoprotein (LDL), increase high-density lipoprotein (HDL) levels and positively affect risk factors for metabolic syndrome, thereby decreasing the risk of cardiovascular diseases (CVD) obesity, and diabetes (Mullins and Arjmandi, 2021).

2.3.1. Carbohydrates

Carbohydrates represent the major component in *P. vulgaris*, accounting up to 50–60% of the dry matter depending on the bean variety. A 100 g portion (approximately ½ cup) of most types of cooked dry beans provides an average of 110-143 kcal. (Sathe, 2016)

Starch and nonstarch polysaccharides constitute the major components of these carbohydrates along with considerable amounts of carbohydrate derivates such as mono-, di-, and oligosaccharides. Common beans contain slow-digesting carbohydrates and a high percentage of non-digestible carbohydrates, which can be fermented in the large intestine, and include starch, resistant soluble and insoluble dietary fiber, and non-digestible oligosaccharides (Suárez-Martínez et al., 2015).

Non-digestible carbohydrates are resistant to hydrolysis by human enzymes and are called resistant starch; they consist of insoluble, soluble dietary fibers and non-digestible

oligosaccharides that are not hydrolyzed in the small intestine but ferment thanks to the microflora of the large intestine (Hayat et al., 2014).

Dietary fiber may be classified into soluble and non-soluble fraction, based on solubility during extraction and isolation in an enzymatic solution at controlled pH (Kutos et al., 2003; Shiga and Lajolo, 2006). Soluble fiber includes oligosaccharides, fructooligosaccharides, pectins, β-glycans, galactomannan gum, alginate, and psyllium, while its non-soluble counterpart contains mainly cellulose, hemicellulose, resistant starch and lignin (Perry et al., 2016).

The fermentation of resistant starch leads to the production of short-chain fatty acids such as acetic, butyric and propionic acid whose concentration and distribution depend on both the microflora and the content of carbohydrates in the intestine (Hayat et al., 2014). Butyrate, a four-carbon fatty acid, is physiologically relevant to the colon epithelium because it promotes the normal proliferation of the intestinal mucosa. Furthermore, it induces apoptosis, inhibits proliferation, and leads to a highly differentiated phenotype in human colon cancer cells.

The digestibility of starch is also influenced by several factors including the amylose/amylopectin ratio, the molecular structure of the amylopectin, the length of the amylose chain, the degree of crystallinity, and the size of the granules; in addition, there are other factors called anti-nutritional factors that greatly affect digestibility. Resistant starch is becoming a topic of interest because it has many beneficial health effects by protecting against tumorigenesis through various mechanisms.

Beans contain large amounts of resistant starch. This bioactive compound, along with dietary fiber, gives the bean the ability to lower the glycemic index compared to other carbohydrate-rich foods, thus producing great health benefits. The glycemic index for beans is between 29 and 38, while for brown rice is 50 and for oat flakes is 55 (Messina, 2014).

Beans require a relatively long cooking time to soften the tissues, be palatable and reduce anti-nutritional factors. During cooking, several physicochemical changes that occur in dietary fiber cause the solubilization and degradation of pectic polysaccharides. Such changes may improve the hypocholesterolaemic or glycemic response during intestinal transit. Complex changes in dietary fiber have been observed due to thermal processing of beans, which depends on the cultivar and processing method (Tiwari et al., 2011).

2.3.2. Proteins

Common beans are an excellent source of proteins that play an important role in human nutrition by complementing other foods such as wheat and other cereals (Hayat et al., 2014). Intake of beans along with cereals offers the best strategy to combat the problem of protein malnutrition. The protein content in beans is equal to that of meat, ranging from 20-30%. One portion (90g or ½ cup of cooked beans) provides 7 to 8 g of protein, nearly 15% of the recommended dietary intake of protein for a 70 kg adult (Suárez-Martínez et al., 2015).

Globulins and albumins constitute the major protein fractions, while prolamin and glutelin exist as minor fractions. Contrary to other legume proteins, beans contain high amounts of glutelins 20-30% versus 7-15% in other legumes (Hayat et al., 2014).

Dry common beans also contain great amounts of essential amino acids including lysine which is deficient in cereal grains. Values for histidine, isoleucine, lysine, phenylalanine, threonine, and valine are slightly variable between species and arginine can be detected in some variations. digestibility of bean proteins is about 79%; the amino acid score is 0.78 and protein digestibility is between 0.57-0.68 (FAO/WHO, 1991).

2.3.3. Lipids

In *Phaseolus* beans, the lipid content is very low, approximately 2%. The major lipid components in beans are triacylglycerols (30-50% of total) and phospholipids (25-35% of total), while minor amounts of diacylglycerols, hydrocarbons, steryl esters and hydrocarbons may also be present (Hayat et al., 2014). Common beans are also considered an essential source of unsaturated fatty acids, comprising 61% of the total fatty acids with palmitic, oleic, and linoleic acids being the dominant fatty acids. Linolenic acid is dominant among unsaturated fatty acids consisting of 43.1% of the fatty acids in beans (Grela and Gunter, 1995).

2.2.4. Lectins

Lectins are proteins with high affinity for carbohydrates and carbohydrate-containing biomolecules, including sugars, modified sugars, proteins, and lipids. This high specificity allows lectins to affect a broad range of biological processes (Chan et al., 2016). Generally, they are classified into four groups based on their affinity for glucose/mannose, galactose, N-acetyl-D-galactosamine, L-fructose, or sialic acid.

Several types of lectins have been shown to induce cancer cell death, suggesting they may have an application for cancer treatment. Cancer cells secrete or express glycoconjugates with abnormal structure, and lectins can detect such changes. Based on this property, they can be used for cancer diagnosis or in some specific treatments for this disease (Pervin et al., 2015). The lectins contained in the common bean have been reported to reduce the growth of non-Hodgkin's lymphoma, a tumor of lymphoid tissue that invades lymph nodes, spleen, and other organs (Ulloa et al., 2011; Pervin et al., 2015). They can also be used as tumor markers because they identify those cells undergoing the first stages of differentiation towards tumor cells (Guzamàn et al., 2002). Pervin et al. reported the inhibition of the proliferation of breast cancer cell line MCF7, hepatoma cell line HepG2, and nasopharyngeal carcinoma CNE1 and CNE2 induced by the presence of these compounds contained in the common bean (Pervin et al., 2015).

Lectins can bind to the intestinal epithelium and interfere with nutrient absorption. Bean lectins are inactivated by cooking for at least 15 min at atmospheric pressure or under pressure for 7.5 min, while neither dry heat nor conventional microwave ovens have been found to be effective in destroying the common bean lectins (Lajolo and Genovese, 2002).

2.3.5. Minerals and vitamins

Beans are an essential source of minerals and vitamins and are considered superior to grains as a source of micronutrients (Welch et al., 2000). Beans have the highest level of mineral content compared to other legumes and contain iron, zinc, copper, phosphorus, and aluminum, while other minerals are also found in appreciable quantities (Broughton et al., 2003). The calcium in beans has a higher bioavailability than magnesium or potassium. The average mineral concentrations are 18 mg/kg for copper, 60 mg/kg for iron, 23 mg/kg for manganese, 29 mg/kg for zinc, and 234 mg/kg for sulfur. The concentration of iron and zinc in the seeds of cooked beans is influenced by pressure cooking and the previous steeping. However, other minerals such as P, K, Ca, S, Cu, and Mn are unaffected by the firing process. Although Fe content can vary significantly between varieties (for example, white kidney beans contain twice as much Fe as black), a half-cup serving provides nearly 10 percent of the recommended daily intake. However, most of the iron in legumes is closely related to phytates. Processes such as maceration, germination and fermentation have been shown to significantly reduce the content of these compounds.

Legumes are also a source of selenium, which plays a fundamental role in the prevention of breast, esophagus and stomach cancers, thanks to its ability to inhibit the development of cancer cells.

Vitamins are organic compounds that cannot be synthesized by humans and therefore must be taken to prevent metabolic disorders, although vitamin deficiency syndromes such as scurvy, beriberi and pellagra are now rare in Western societies. Specific clinical subgroups remain at risk: for example, the elderly are at particular risk for vitamin B12 and D deficiencies, alcohol-dependent individuals are at risk for folate, B6, B12 and thiamine deficiencies, and hospitalized patients are at risk of folate and other water-soluble vitamins deficiency (Fairfield et al., 2002).

The common bean is one of the main sources of vitamins B, in particular, they contain thiamin, riboflavin, niacin and folic acid (Guzmàn et al., 2002; Hayat et al., 2014).

Folic acid is a potential nutraceutical; a 100 g portion of common bean can satisfy the daily requirement (0.18-0.4 mg/day) of folic acid for both adults and children (Augustin et al., 2000; Campos-Vega et al., 2010).

The common bean also contributes significant amounts of thiamine to the diet; in fact, a portion of about 100g contains the recommended dietary dose for adults (1.2 mg/day). Finally, dried beans contain vitamin B6 for about 13% of the DRI (Dietary Reference Intake) (1.8 mg/day) (Camara et al., 2013).

The retention values of water-soluble vitamins during cooking among bean classes range on average between 70% and 75% (Olaniperkun et al., 2015). Davey et al. reported that vitamins are lost during heat treatment because they are highly sensitive to oxidation (Davey et al., 2000). Table 2 shows the vitamin content of raw and cooked pinto beans and indicates that heat treatment decreases the content of water-soluble vitamins. Vitamin C is the most affected by the cooking process, with a decrease of 87%, followed by thiamine which decreases by 73% (Câmaraet al., 2013).

Table 2. Vitamin content of raw and cooked pinto beans. (Câmara et al., 2013).

	Value for 100 g	
	Raw	Cooked *
Vitamin C (total ascorbic acid)	6.30 mg	0.80 mg
Thiamine	0.71 mg	0.19 mg
Riboflavin	0.21 mg	0.06 mg
Niacin	1.17 mg	0.32 mg
Vitamin B_6 (Pyridoxine)	0.47 mg	0.23 mg
Folate, DFE	0.53 mg	0.17 mg
Vitamin E (alpha-tocopherol)	0.21 mg	0.94 mg
Vitamin E (alpha-tocopherol)	0.21 mg	0.94 mg
Vitamin K (phylloquinone)	5.6 µg	3.5 µg

2.2.6. Phenolic compounds

Dry common beans contain many phenolic compounds that constitute a group of heterogeneous molecules both from a structural and a functional point of view, including phenolic acids, tannins and flavonoids belonging to this group.

Polyphenols are bioactive compounds widely known for their antioxidant properties, thanks to which they play a very important role in decreasing the risk of cardiovascular disease, diabetes, some types of cancer, Alzheimer's disease and Parkinson's disease. The antioxidant properties of these compounds reside in their ability to neutralize free radicals and chelate transition metals, thus counteracting the initiation and propagation of oxidative processes (Huberet al., 2016).

Studies have demonstrated that phenolic compounds are predominantly located in the bean's seed coat than in the cotyledon and testa. The content of the phenolic compounds is about 145 mg/g and represents about 11% of the total seed. The phenolic compounds in the seeds are flavones, monomers, and oligomers of flavanols, flavanones, isoflavonoids, anthocyanins, chalcones, and dihydrochalcones. However, the phenolic acids and non-flavonoid phenolic compounds (hydroxybenzoic and hydroxycinnamic acid) are mainly found in the cotyledons of the bean. Based on their chemical structure, they are a highly diverse group ranging from simple molecules such as phenolic acids to complex polymers such as tannins and lignin. The testa of the beans contains greater quantities of proanthocyanidins and anthocyanins. Condensed tannins (10.65 mg catechin equivalents/g) and cyanidin 3-glucoside (3.75 mg catechin equivalents/g) are also mainly present in the bean’s seed coats.

De Mejìa et al. observed that the largest amounts of polyphenolic compounds are found in the bean's seed coat. In another study, Almanza-Aguilera et al., demonstrated the presence of a high content of phenolic compounds in the raw seed, and a significant reduction of these following cooking processes.

The isolation and characterization of the phenolic compounds of the bean date back to the early 60s. Four main anthocyanins were extracted from the coating: delphinidin 3-glucoside, petunidin 3-glucoside, malvidin 3-glucoside and 3,5-diglucoside; subsequently flavonoids were also identified, including quercetin, myricetin, cyanidin, procyanidin, naringenin, catechin, hesperetin and kaempferol. Significant flavonoid glucosides are apigenin 7-*O*-glucoside, quercetin 3-*O*-glucoside, myricetin 3-*O*-glucoside, naringenin 7-*O*-glucoside, quercetin 4-*O*-galactoside and kaempferol 3-*O*-glucoside (Messina, 1999).

Raw and cooked beans contain phenolic acids, mainly derived from benzoic acid (vanillic, *p*-hydroxybenzoic and gallic acids) and cinnamic acid (ferulic, *p*-coumaric and chlorogenic acids) (Figure 6). Among them, ferulic acid is the predominant phenolic acid in dried common beans (Wallace et al., 2015). Studies have shown that cooking common beans at a high temperature does not change the phenolic acid content.

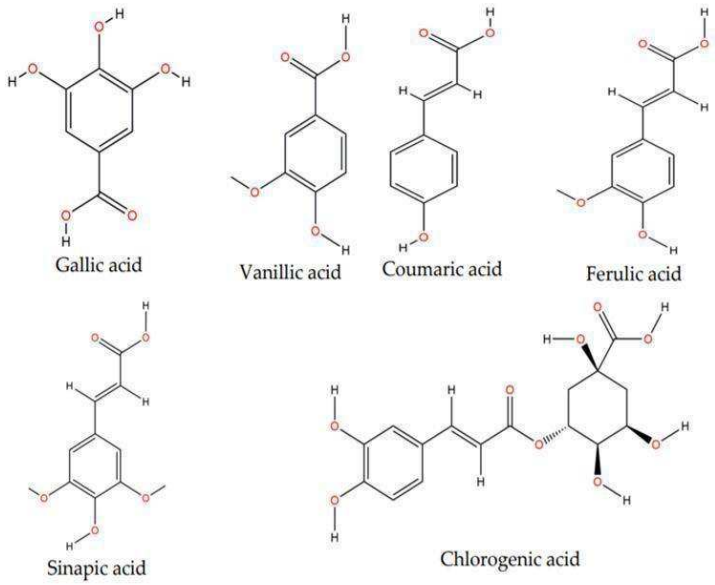


Figure 6. Chemical structure of the main phenolic acids present in beans.

Phenolic acids have been found to exhibit anthelmintic activity and suppress liver fibrosis in chronic liver disease (Huber et al., 2016). Some studies have reported that gallic acid has bacteriostatic properties, counteracts the onset of melanoma, and works as an antioxidant. Gallic acid has also been proposed for the treatment of brain tumors. Chlorogenic acid protects against neurotoxins by decreasing apoptosis induced by beta-amyloid peptide. It also displays anticholinesterase, anti-amnesic and radical scavenger activities. Ferulic acid shows beneficial health effects such as antioxidant, anti-inflammatory and immunostimulant. It also promotes the degradation of recombinant amyloid-beta peptide. Caffeic acid exhibits a neuroprotective effect against beta-induced toxicity by inhibiting calcium efflux and tau protein phosphorylation. It also protects neurons from oxidative stress-induced cytotoxicity and, together with coumaric acid, may induce neuroprotective effects against Parkinson's disease similar to what was previously observed with flavonoids, catechins and quercetin (Rauter et al., 2012).

Flavonoids have two aromatic rings connected by three carbon atoms, forming an oxygenated heterocycle. They are classified into six subclasses according to their heterocycle: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechins and proanthocyanidins or condensed tannins). The main flavonoids contained in both raw and cooked beans are catechin, kaempferol, quercetin, myricetin and procyanidin (Huber et al., 2016) (Figure 7).

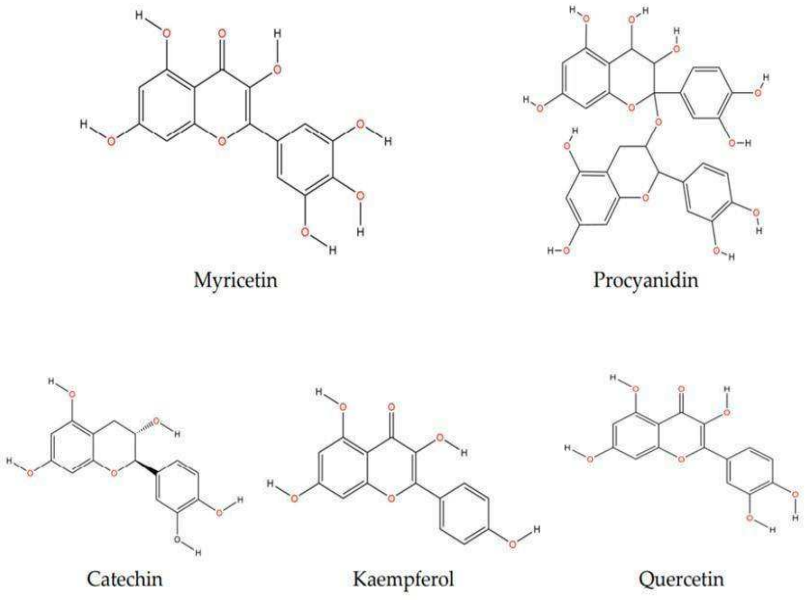


Figure 7. Chemical structure of the major flavonoids found in beans.

The consumption of flavonoids has been inversely correlated with lung cancer and the risk of cardiovascular disease (Dìaz-Batalla et al., 2006). The main effect of these compounds is the antioxidant capacity, while other mechanisms of action include the modulation of detoxifying enzymes and the inhibition of cell proliferation. Flavonoids have also been shown to prevent platelet aggregation, induce muscle relaxation and, together with proteoglycans, show an inhibitory effect on allergic symptoms. Additionally, flavonoids such as procyanidin B1 and resveratrol can increase brain capacity and longevity (Drago et al., 2006).

The color of the seed coat in the various types of common beans is based on the presence of polyphenols including anthocyanins, flavonols glucosides, and condensed tannins. In particular, a lower anthocyanin content was found in the yellow or white common beans than in the dark-colored ones (Takeoka et al., 1997). The light yellow or pink seed coat of beans are generally based on the presence of condensed tannins.

Tannins have antioxidant, anti-mutagenic, anti-carcinogenic properties and act as a free radical scavenger. The concentration of tannins is higher in the skin of the seed than in the cotyledons. Together with oxalates and phytates they are considered antinutritional factors because they affect the bioavailability of nutrients. Beans contain moderate amounts of phytic acid which represents 1 to 5% of the weight of the seed. It has been recognized as an antinutrient because it is not digested and because it has the ability to reduce the bioavailability of divalent cations bound to proteins or starch, such as iron and zinc, thanks to the formation of complexes with non-absorbable metal cations in the gastrointestinal tract. This same property has an essential biological function for plants as it provides phosphorus and minerals essential for their growth and participates in cellular signalling and response against pathogens.

Phytic acid is reduced after the process of maceration, germination and heat, which promotes greater mineral extractability and bioavailability (Carvalho et al., 2012). Extrusion cooking is a processing method widely used to improve the nutritional value of legumes, mainly in order to decrease heat-labile anti-nutritional compounds. Protein content does not appear to be affected by this treatment (Marzolo et al., 1993).

Phytic acid has been reported to attenuate the risk of cancer, primarily of the breast and colon, an effect likely due to its antioxidant capacity (Guzman et al., 2002; Ulloa et al., 2011).

2.4. Biological activities

The inclusion of beans in the diet has received increased attention because of their beneficial physiological effects in the prevention of cardiovascular diseases, obesity, diabetes mellitus and cancers (Mullins and Arjmandi, 2021). According to the Dietary Guidelines for Americans (2005), the consumption of half a cup (130 g) of beans and other legumes several times per week has been associated with beneficial physiological effects; the total recommendation per week is 3 ½ cups.

2.4.1. Cardiovascular Diseases

Several epidemiological and clinical studies have observed that the risk of developing of coronary heart and cardiovascular diseases is lower among adults who frequently consume legumes (Finley et al., 2007). In an epidemiological trial, men and women consuming four times or more legumes per week decreased their risks of coronary heart diseases and cardiovascular diseases by up to 22 and 11%, respectively, as compared to those with one serving per week (Flight and Clifton, 2006). Higher legume intakes were associated with lower body mass index, blood pressure, serum total cholesterol, and a lower incidence of diabetes mellitus, compared with lower legume intakes. In particular, the consumption of common beans is considered beneficial for healthy individuals as well as those preconditioned for metabolic syndrome by lowering serum total cholesterol and LDL cholesterol (Anderson and Major, 2002). Bazzano et al. (2001) investigated that consumption of at least 3/4 cups of cooked beans resulted in a 38% decreased risk of myocardial infarction. In another study, hypercholesterolemic men were given half a cup daily intake of baked beans for a period of 8 weeks, which resulted in reductions of serum LDL cholesterol and total cholesterol by 5 and 6%, respectively (Winham and Hutchins, 2007). Finley et al. (2007) also observed a significant decline in serum total cholesterol and LDL cholesterol in healthy individuals who were given a 130 g daily intake of dried, cooked pinto beans, concluding that consumption of beans four times per week decreases the risk of heart diseases by 20%.

Resistant starch (RS) and dietary fiber content of beans are mainly responsible for delaying the degree of glucose as fuels, changing fat utilization, and controlling appetite through increased satiety, thus lowering the risk of cardiovascular diseases (Anderson et al., 2002; Park et al., 2004). Fermentation of fiber as well as RS by bacteria in the large intestine results in the generation of specific short-chain fatty acids (SCFA), propionate being dominant, which alter metabolic pathways resulting in reduced serum cholesterol

(Pereira et al., 2003). Thus, increased production of SCFA by fermentation of RS is an underlying reason for the protective benefits of the consumption of dry beans (Finley et al., 2007). The cholesterol-lowering effect of dietary fiber has been ascribed to its ability to restrain the intestinal absorption of neutral steroids and bile acids and total steroid excretions (Moundras et al., 1997). The hypocholesterolemic effect can also be achieved by regular ingestion of beans that reduce the need to rely on animal proteins by replacing it with plant proteins (Marcello, 2006).

2.4.2. Diabetes Mellitus

The consumption of common beans has a protective role in the development of type-2 diabetes mellitus (DM2). Epidemiological studies show that consumption of three or more servings of whole grain foods per week reduces the risk of diabetes mellitus by 20–35%, as compared to less consumption. The reduced digestibility of bean carbohydrates due to the presence of viscous soluble dietary fiber and high amylose and resistant starch contents together with the production of short-chain fatty acids prevents elevated glucose levels, thus ultimately resulting in reduced insulinemic and glycemic responses. Due to the slow release of carbohydrates, beans are considered as low glycemic index (GI) foods (Campos-Vega et al., 2010).

Several studies indicate that the consumption of low glycemic index foods is useful in the reduction of diabetes mellitus and obesity (Jenkins, 2007). It has been observed that a fall of 10% in the glycemic index of a diet results in a 30% increase in insulin sensitivity. In US adults, bean consumers presented a 23% lower risk of obesity, and lower systolic blood pressure (Foster-Powell et al., 2002).

Starch in beans is slowly digested and attenuates postprandial response to insulin (Hangen and Bennink, 2002). In another study, low glycemic index starch diets were found to increase glucose oxidation indicating enhanced peripheral insulin action as well as glucose utilization (Feregrino-Pérez et al., 2008).

High amylose content in beans is considered an important factor to affect glucose metabolism. In an experiment wherestarches with different amylose/amylopectin ratios were introduced in mixed meals, meal with higher amylose starch showed higher glycemic response (Behall and Howe, 1995).

The α-amylase inhibitor isoform 1 (α-AI1) has been extracted and used in diverse commercial products against obesity and diabetes in humans (Barrett and Udani, 2011)

A *P. vulgaris* extract, prepared to contain *α*-amylase inhibitors and phytohemagglutinin, was compared to a standard medication with metformin in adult male Wistar rats. It was observed that extracts reduced postprandial glycemia in a similar way as metformin (Carai et al., 2009).

2.4.3. Antioxidant and anti-inflammatory activity

The most widespread flavonoids in beans are proanthocyanidins (Reynoso-Camacho et al., 2006). These compounds can restrain the development of initiating free radical species by chelating metal ions or inhibiting enzymes that are involved in the radical production process (Rocha-Guzmán et al., 2007). Phenolic compounds restrain the formation of superoxide anion as well as the production of reactive oxygen species by inhibiting key enzymes such as protein kinase, xanthine oxidase, lipoxygenase, cyclyoxygenase, S-transferase, glutathione, and NADH oxidase (Oomah et al., 2010).

In addition, phenolic compounds have antimutagenic activity by inhibiting the potential mutagens such as aflatoxins, nitroarenes, and polycyclic aromatic hydrocarbons (Cardador-Martinez et al., 2002). The ability of flavonoids to complex with metal ions plays an important role in their antioxidant activity. There is a specific relationship between flavonoid structures and their antioxidant activity; as larger, the number of hydroxyl groups in the flavonoid nucleus, the greater would be the antioxidant activity (Cao et al., 1997). Flavonoids in dry beans such as quercetins are all glycosylated at the 3-position, which greatly reduces their metal complexing ability as the chelation of the 3-hydroxy-4- keto group is the strongest metal complexing group (Letan, 1966). The most imperative structural attribute for antioxidant activity is the B-ring ortho 3',4' -dihydroxy orientation. No antioxidant activity was observed in kaempferol 3-*O*-glucoside having a single B-4-hydroxyl substitution, while antioxidant activity was observed in kaempferol 3-*O*-neohesperidosideing in a 1,1-diphenly-2-picrylhydrazyl (DPPH) free radical assay (Gamez et al., 1998). A specific relationship also exists between flavonoid structure and their antimutagenic activities, as the antimutagenic effect depends on the position and number of phenolic hydroxyl groups and blocking of these groups by acetylation or alkylation decreases antimutagenic activities (Rocha-Guzmán et al., 2007). The antioxidant activity of flavonoid compounds from beans has been reported both *in vitro* as well as *in vivo* (Reynoso-Camacho et al., 2006).

2.4.4. Anticancer activity

Beans are a fundamental food of human nutrition, they contain numerous bioactive substances, including enzyme inhibitors, lectins, phytates, oligosaccharides, and phenolic compounds, as well as non-digestible compounds that play an important metabolic role in humans and animals that consume them frequently.

Fermentation of dietary fibers in the large intestine modifies the colonic microbiota species and composition and exhibits several health-promoting properties related to potential anticancer activities, lipid metabolism, anti-inflammatory effects, and other immune effects (Campos-Vega et al., 2009). Many of these phenomena can be linked to their digestion and the production of short-chain fatty acids (SCFA) by bacteria in the large intestine (Feregrino-Pérez et al., 2008). Metabolism of the non-digestible fraction of the common bean by the human intestinal flora produces SCFAs such as acetate, propionate, and butyrate. The latter induce differentiation, growth arrest, and apoptosis in colon cancer cells, stimulate the physiological pattern of proliferation in the basal crypt of the colon while reducing the number and size of aberrant crypt foci (ACF), which are the first detectable neoplastic lesions in the colon (Canani et al., 2011).

SCFAs are used as a short-term test to identify modulators of colon carcinogenesis. In several studies, it has been reported that SCFAs modulate cancer by inhibiting some genes involved in cell proliferation and inducing cell cycle arrest or leading to colonocyte apoptosis (Mahyar-Roemer et al., 2001).

Butyrate increases β-catenin signaling, cell cycle regulatory protein expression p21 independently of p53 expression and regulates the expression of caspase-3 active subunits; moreover, it also causes a concomitant decrease in BCL-2 expression and an increase in BAX gene expression via mitochondrial protein (Jan et al., 2002).

Phaseolus vulgaris L. cultivar Bayo Madero does not induce ACF formation and has demonstrated a chemoprotective effect against azoxymethane (AOM) treatment, suggesting that consumption of common beans prevents the development of colon cancer in the early phase (Vergara-Castañeda et al., 2010). It hase been shown that treatments with cooked bean and non-digestible fraction significantly reduced the activity of β-glucuronidase, indicating a strong probiotic effect exerted by these compounds (Feregrino-Perez et al., 2008). These results suggest that the suppression of ACF is strongly influenced by the decrease of β-glucuronidase activity in the colon, probably by

altering the metabolic activities of the intestinal microflora which prevent the release of AOM in the colon.

In studies on rat models, it was observed that, in rats fed with beans, the incidence of breast cancer decreased by up to 70% (Thompson et al., 2008). This effect appeared to be due to the promotion of a pro-apoptotic environment, which induces an arrest of the G1/S transition of the cell cycle and a downregulation of mTOR activity (protein kinase that regulates growth, proliferation, motility and cell survival) during breast carcinogenesis (Laplante and Sabatini, 2009).

Since the effect of consuming a food may involve synergy between its components, studying the role of beans in diet, cancer prevention and control may require a systemic perspective. In a study conducted by Matthew et al. consumption of beans has been shown to reduce circulating glucose, insulin and IGF-1. This effect was also confirmed in other studies on breast carcinogenesis, indicating the influence of dietary bean consumption on glucose availability and the insulin/IGF-1 system (Thompson et al., 2012).

As the demand for *de novo* lipid biosynthesis increases in carcinomas to maintain high cell proliferation rates as part of metabolic reprogramming, changes in lipid levels in physiological and metabolic processes are amplified in tumors. Lipid metabolism is regulated in part by AMP-activated protein kinase (AMPK), whose activity is upregulated in bean-fed rats, and by mTOR, which affects proteins such as SREBP-1 (response element binding protein of sterols) (Laplante and Sabatini, 2009). AMPK is a known regulator of lipid metabolism through its effects on acetyl-CoA carboxylase (ACC), where activated AMPK increases the phosphorylation of ACC.

Although further work is needed to determine the mechanisms explaining the impaired lipid metabolism by beans, particularly regarding the influence of mTOR signaling on lipid biosynthesis rates in tumors, the effects of bean consumption on metabolism, growth factor signaling and energy homeostasis are of considerable interest and are the subject of numerous studies.

2.5. *Phaseolus vulgaris* L. var. Venanzio

Phaseolus vulgaris L. var. Venanzio, named “Fagiola di Venanzio” (FV), grows in a restricted area of the municipality of Murlo (Siena, Tuscany). FV has been cultivated since the mid-nineteenth century by the Burresi family on their farm located near Murlo and has been recognized as a specific variety in 2017 (N. VE_145 20-12-2017, Regione

Toscana, Italy). FV beans are characterized by a white seed coat color, with a relatively small size, very flattened and elliptical-wide shape (Figure 8).

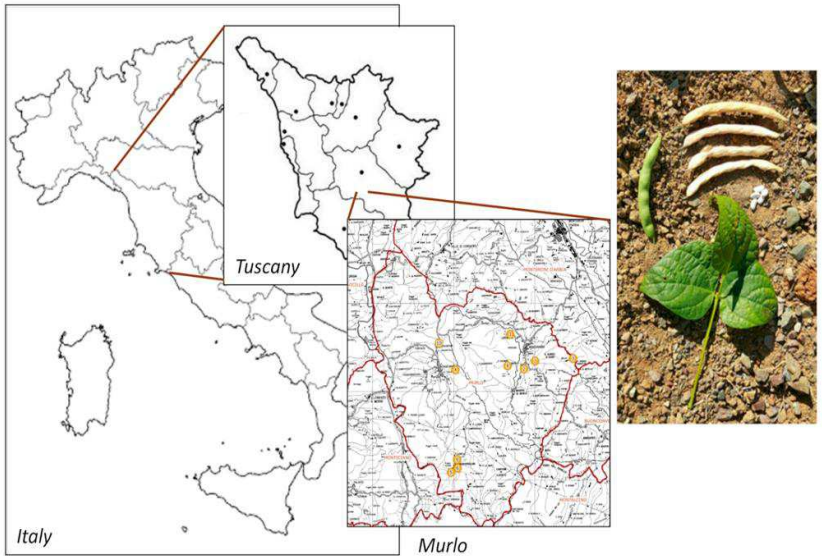


Figure 8. Production area of Fagiola di Venanzio (FV). Colored circles indicate the micro areas within Murlo municipality where FV is cultivated (Finetti et al., 2020).

In a study conducted by Finetti et al., FV beans showed to be rich in polyphenols, including phenolic acids and hydroxycinnamic derivatives. In addition, aqueous extracts of FV exhibited antiradical activity *in vitro* and reduced ROS (reactive oxygen species) production promoted by interleukin 1β (IL-1β) in two different models of colon cancer cells. It has also been shown that FV extracts are able to reduce the expression of the inflammatory marker COX-2, the activation of NFkB and ERK 1/2 MAPK, and the growth of colon cancer cells promoted by IL-1β, a well-known pro-inflammatory cytokine able to mimic inflammatory conditions that occur in the intestinal tract and that may drive the development of such inflammatory chronic diseases as cancer, or such inflammatory bowel diseases (IBD) as ulcerative colitis and Crohn's disease (Finetti et al., 2020). These results demonstrated that FV extracts are able to reverse the effects of IL-1β on colon cancer cells and suggest a potential role in preventing the alteration of the molecular processes that characterize the inflammatory microenvironment that can lead to cancer and other chronic diseases. Based on these experimental evidences, FV represents a promising source of bioactive compounds and possesses important nutraceutical properties.

3. Cancer

The term neoplasm or tumor indicates a mass of tissue that grows in excess and in uncoordinated way in relation to normal tissues and represents, to date, one of the leading causes of death and a major barrier to increasing life expectancy in every country in the world (Bray et al., 2021).

According to World Health Organization (WHO) estimates in 2019 (WHO, 2020) cancer was the first or second leading cause of death before the age of 70 in 112 out of 183 countries and ranks third or fourth in 23 countries (Sung et al., 2021). From these data, it is possible to deduce that the incidence and mortality of cancer are growing rapidly worldwide, manifesting both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, many of which are associated with socioeconomic development (Omran, 1971; Gersten and Wilmoth, 2002). Female breast cancer is the most commonly diagnosed cancer (11.7% of total cases), followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancer. From a mortality point of view, lung cancer is the leading cause of cancer death (18.0% of total cancer deaths), followed by colorectal (9.4%), liver (8 .3%), stomach (7.7%) and female breast (6.9%) cancer (Sung et al., 2021) (figure 9).

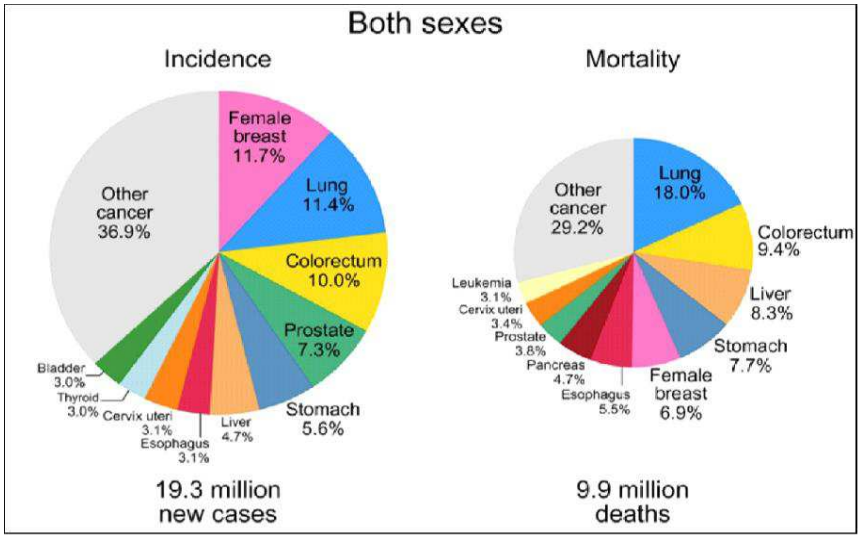


Figure 9. Distribution of cases and deaths for the top ten most common cancers in 2020. (Sung et al., 2021).

Neoplasms can be classified in various ways, one of the best known being the distinction between benign and malignant growths, where benign tumors do not consist of cancerous cells. The cells of the benign tumor multiply more than necessary but do not invade other tissues, i.e. they do not give rise to metastases, remain well-defined, and maintain the characteristics and functions of the tissue of origin. Further classifications may concern the area in which the tumor develops, the type of cells from which they are made up, the aggressiveness or the stage.

The underlying causes of neoformations are different and still a reason for study today; they are attributable to some genetic mutations, hereditary factors, exposure to carcinogens (physical agents, ionizing radiation), as well as infections, and incorrect nutrition. Statistically, 5-10% of all cancer cases can be attributed to genetic defects, while the remaining 90-95% have their roots in lifestyle and environment. Risk factors include cigarette smoking, diet (fried foods, red meat), alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity (Anand et al., 2008).

Carcinogenesis is a multistage process involving mutations in genes that play a role both in maintaining the balance between cell proliferation and apoptosis, i.e. in maintaining a stable cell mass (number) and in regulating complex metabolic pathways, which ensure the functioning and structural integrity of cells and tissues (Derelanko et al., 2001; Kontomanolis et al., 2021). This damage can be the result of endogenous processes such as errors in DNA replication, chemical instability of some of its bases, or attack by free radicals generated during metabolism. Damage can also result from interactions with external agents, including ionizing radiation, UV rays, and chemical carcinogens.

Although cells possess defense mechanisms to repair this damage, for various reasons errors occur and permanent changes are introduced in the genome (mutations). Thus, genes involved in the normal homeostatic control mechanisms of cell proliferation and cell death undergo mutational damage resulting in the activation of genes that stimulate proliferation or protection against cell death, oncogenes, and the inactivation of genes that would normally inhibit proliferating, tumor suppressor genes. Once these controls have been passed, the cancer cell must overcome replicative senescence, become immortal, and must obtain an adequate supply of nutrients and oxygen to maintain this high proliferation rate (Bertram, 2000).

As proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2000), the hallmarks of cancer comprise six biological capabilities acquired during the multiphase development of human tumors. These hallmarks that help us better understand the complexity of tumor pathology include: supporting proliferative signaling, evading growth suppressants, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (figure 10).

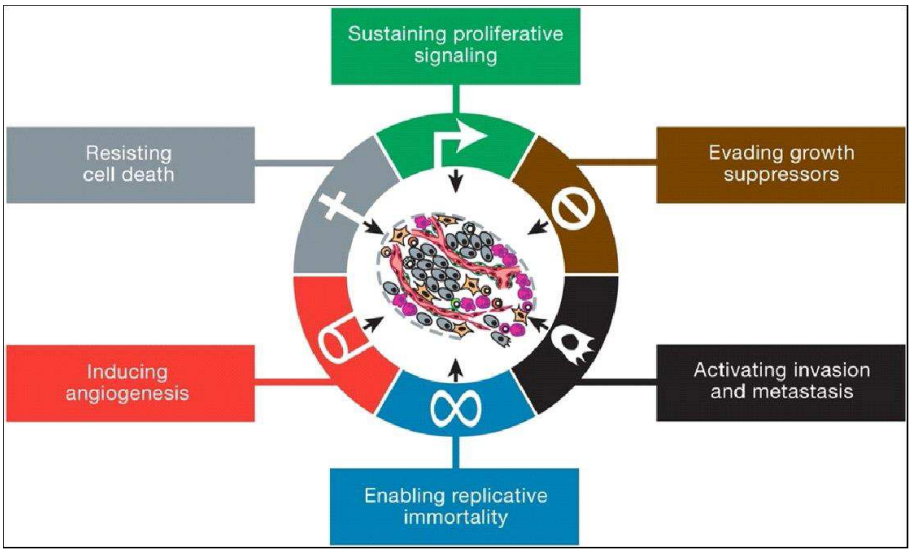


Figure 10. **Cancer "Hallmarks".** (Hanahan e Weinberg, 2000)

Subsequently, the same authors introduced two further distinctive signs, namely the reprogramming of energy metabolism and the evasion of immune destruction together with two characteristics that motivate the acquisition of these functions, represented by genomic instability in tumor cells and by the role of inflammation in tumor promotion (Hanahan and Weinberg, 2011).

- Sustaining proliferative signaling: it certainly represents the fundamental characteristic of cancer cells. Normally, in order to maintain homeostasis of cell number, tissue architecture and function, cells carefully control the production and release of signals that promote the cycle of cell growth and division. Cancer cells, however, are able to deregulate these signals and acquire the ability to sustain proliferative signaling in a number of alternative ways. Through autocrine proliferative stimulation they can produce growth factors to which they respond through the expression of similar receptors; send signals to stimulate normal cells within the stroma to produce growth factors; increase

the levels of receptor proteins on the surface of tumor cells making them hyperactive to otherwise limiting amounts of growth factor and promote ligand binding; activate signaling pathways downstream of these receptors overcoming the need to stimulate these pathways via ligand-mediated activation of the receptor.

- Evading growth suppressors: cancer cells have to circumvent the actions of tumor suppressor genes that negatively regulate cell proliferation. The two prototype tumor suppressors encode the proteins RB (retinoblastoma-associated) and TP53 which, operating in a complementary manner, regulate cell proliferation or, alternatively, activate senescence and apoptotic programs. The RB protein integrates extracellular and intracellular signals thus regulating the progression or deciding the block of the cell growth and division cycle (Burkhart and Sage, 2008; Deshpande et al., 2005; Sherr and McCormick, 2002). TP53, receiving input from stress sensors and intracellular abnormalities, can disrupt cell cycle progression or in case of excessive damage, trigger apoptosis.

- Resisting cell death: the concept that apoptotic programmed cell death serves as a natural barrier to cancer has been established by several studies conducted in recent years (Adams and Cory, 2007; Lowe et al., 2004; Evan and Littlewood, 1998). Apoptosis is activated in response to various physiological stresses or as a result of anticancer therapy. The mechanism of apoptosis is composed of upstream and downstream regulators which in turn are divided into two main circuits that receive and process intra- and extracellular signals. One of the processes used by cancer cells to limit apoptosis involves the loss of the tumor suppressor function TP53, which eliminates this apoptosis-inducing sensor. Alternatively, tumors may increase the expression of antiapoptotic regulators (Bcl-2, Bcl-Xl) or survival signals (Igf 1/2), by downregulating proapoptotic factors (Bax, Bim, Puma) or by sabotaging the activation of the upstream extrinsic ligand-induced apoptotic pathway (Adams and Cory, 2007).

- Enabling replicative immortality: to generate tumors, tumor cells must be able to replicate indefinitely achieving immortality. Cells generally exhibit a limited number of cycles of cell growth and division made possible by the activation of senescence, a state in which cells are viable but not proliferative, and by the crisis leading to cell death. Studies show that telomeres, which protect the ends of chromosomes, are involved in the capacity for unrestricted proliferation (Blasco, 2005; Shay and Wright, 2000). In non-immortal cells, telomeres progressively shorten, losing the ability to protect the ends of

chromosomal DNAs and this triggers the defensive mechanisms of senescence and crisis. Conversely, in immortal cells, telomerases, specialized in adding telomere segments to the ends of telomeric DNA, are highly expressed allowing to counteract the erosion of telomeres and re-exist the induction of defense mechanisms.

- Inducing angiogenesis: as with normal tissues, tumors also require sources of sustenance in the form of nutrients and oxygen. These needs are met through tumor-associated neovascularization generated by the process of angiogenesis. During the development of the tumor an "angiogenic switch" remains always active causing the vascular system, normally quiescent, to continuously develop new vessels that help to support the expansion of neoplastic growth (Hanahan and Folkman, 1996). These angiogenic regulators are mostly represented by vascular endothelial growth factor A (VEGF-A), as an inducer of angiogenesis, and thrombospondin-1 (TSP-1) as an inhibitor.
- Activating invasion and metastasis: the multistep process of invasion and metastasis has been schematized as a sequence of steps, often referred to as the invasion-metastasis cascade (Talmadge and Fidler, 2010; Fidler, 2003). This scheme involves a series of cellular biological changes starting with local invasion, followed by entry of tumor cells into nearby blood and lymphatic vessels, transit of cells through the lymphatic and vascular system followed by extravasation into distant tissue parenchyma, the formation of small nodules of tumor cells (micrometastases) and finally the growth of micrometastatic lesions into macroscopic tumors, a step called "colonization".
- Reprogramming of energy metabolism: tumor development involves not only a dysregulation of cell proliferation but also adjustments of energy metabolism in order to fuel cell growth and division. Under aerobic conditions, normal cells degrade glucose first to pyruvate, through glycolysis, and then in the mitochondria pyruvate is oxidized to carbon dioxide. In anaerobic conditions, glycolysis is favored and only small quantities of pyruvate are sent to the mitochondria. Otto Warburg observed an anomalous feature of the energy metabolism of tumor cells (Warburg, 1930) showing that tumor cells, even in the presence of oxygen, can reprogram their energy metabolism by limiting it to glycolysis, leading to a state defined as "aerobic glycolysis".
- Evasion of immune destruction: the immune system also plays an important role in controlling tumor proliferation; in particular, cells and tissues are monitored by the immune system whose surveillance is responsible for the recognition and elimination of many tumor cells.

- Genomic instability in cancer cells: the acquisition of the various distinctive signs largely depends on a succession of alterations in the genome of cancer cells. Some mutant genotypes confer a selective advantage to cell clones by allowing them to grow and dominate at the local tissue level. Consequently, tumor progression can be represented as a succession of clonal expansions, each of which is triggered by the random acquisition of an enabling mutant genotype. The ability of genome maintenance systems to detect and resolve defects in DNA ensures that spontaneous mutation rates are generally very low; however, flaws can occur in these control mechanisms. Defects in genome maintenance and repair are selectively beneficial and therefore determinant in tumor progression, if only because they accelerate the rate at which evolving precancerous cells can accumulate favorable genotypes.
- Inflammation in tumor promotion: inflammation may contribute multiple distinctive capabilities by delivering bioactive molecules to the tumor microenvironment, including growth factors that support proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular enzymes that facilitate cell angiogenesis, invasion and metastasis (De Nardo et al., 2010; Grivennikov et al., 2010; Qian et al., 2010; Karnoub and Weinberg, 2007). Furthermore, during inflammation, chemicals such as reactive oxygen species can be released, which are actively mutagenic to neighbouring cancer cells, accelerating their genetic evolution towards states of increased malignancy (Grivennikov et al., 2010). As such, inflammation can be considered an enabling characteristic for the acquisition of the hallmarks of cancer.

3.1. Colorectal cancer

Colorectal cancer is one of the most common malignancies found in the Western world; overall it ranks third in terms of incidence and second in terms of mortality (Sung et al., 2021). It can be considered as an indicator of socioeconomic development as incidence rates are much higher in industrialized countries than in transition countries (Bray, 2014; Fidler et al., 2016). Approximately 250,000 new cases of colon cancer are diagnosed in Europe each year, representing 9% of all malignancies. The incidence is slightly higher in Western and Northern Europe than in Southern and Eastern Europe (Labianca et al., 2010).

In some Western countries, however, a decline in the incidence of colorectal cancer has been observed attributed to changes in people's lifestyles towards healthier lifestyle

choices, and also thanks to the adoption of screenings whose the main goal is to detect 90% of sporadic cases of colorectal cancer, most of which occur in people over the age of 50 (Bernard and Paul, 2003).

The main histological type of colorectal cancer is represented by adenocarcinoma, which constitutes 90-95% of all tumors of the large intestine. Colloid or mucinous adenocarcinomas, defined by large amounts of extracellular mucin retained within the tumor, account for approximately 17% of these tumors. Other variants of epithelial tumors include squamous cell carcinomas, adenosquamous carcinomas, also called adenocanthomas, and undifferentiated carcinomas that lack certain features such as glandular structures or mucous secretions. The latter are further divided into carcinoma simplex, medullary and trabecular carcinoma. Rarer forms are represented by the signet ring cell carcinoma (2-4%) characterized by a greater amount of mucin at the intracellular level which causes alterations at the nucleus level. Other types of tumors that can be found in the large intestine are gastrointestinal stromal tumors (GIST), haematopoietic and lymphoid neoplasms, carcinoid and non-epithelial tumors such as leiomyosarcomas (Labianca et al., 2010).

Colorectal cancers are also classified according to the depth of local invasion (T stage), lymph node involvement (N stage), and the presence of distant metastases (M stage). This classification according to TNM stage and Union International Contre le Cancer (UICC) provides valuable prognostic information and guides therapeutic decisions.

The main treatments for colorectal cancer patients are surgery, chemotherapy, and radiation therapy.

Colorectal cancer occurs in most cases in a sporadic form and only in 5% of cases has a genetic origin (Kwak EL et al., 2007). Major risk factors identified include a family history of colorectal cancer, inflammatory bowel disease (Crohn's disease and ulcerative colitis), smoking, excessive alcohol consumption, high consumption of red and processed meat, obesity, and diabetes (Brenner et al. al., 2014), indicating that diet certainly represents one of the most relevant etiological factors in the onset of this type of tumor. On the other hand, the consumption of foods containing dietary fiber, as well as garlic, milk and calcium demonstrate a protective effect. Vegetables, fruits, foods containing folate, as well as fish, foods containing vitamin D and also selenium protect against colorectal cancer (Labianca et al., 2010). Known protective factors also include physical activity, the use of estrogen-progestin hormone replacement therapy and aspirin which

show a reduction in cancer risk of the order of 20-30%. Additional data suggest that infections with *Helicobacter pylori*, *Fusobacterium spp* and other potentially infectious agents could be associated with an increased risk of colorectal cancer (Brenner et al., 2014).

In addition to the risk factors mentioned above, the hereditary component is also involved in the onset of this type of cancer. Hereditary forms such as familial adenomatous polyposis and hereditary non-polyposis colon cancer (Lynch syndrome), resulting from known genetic mutations, account for approximately 5% of all colorectal cancers (Burn et al., 2013).

Colorectal cancer is the type of complex cancer best understood in terms of molecular mechanisms involved, where the first step in carcinogenesis is the development of specific types of neoplastic polyps in the colonic mucosa (Cappell, 2008). This process begins with an aberrant crypt, which evolves into a precursor neoplastic lesion (a polyp) and eventually progresses to colorectal cancer over a 10–15-year period. The cell of origin of most colorectal cancers is hypothesized to be a stem cell, which is the result of a progressive accumulation of genetic and epigenetic alterations that inactivate tumor suppressor genes and activate oncogenes (Dekker et al., 2019).

Tumor development is a multistage process that is linked to two major distinct pathways: the traditional adenoma-carcinoma pathway (referred to as the chromosome instability sequence) and the serrated neoplasm pathway (Dekker et al., 2019). Mutations in the adenomatous polyposis (APC) gene are an early event in the multiphase process of colorectal cancer formation and occur in over 70% of colorectal adenomas. The adenoma-carcinoma sequence is further promoted by activating mutations in the KRAS oncogene and inactivating mutations in the TP53 tumor suppressor gene. These characteristic genetic mutations are often accompanied by chromosomal instability, i.e. changes in the number and structures of chromosomes (Brenner et al., 2014).

Serrated adenoma appears to transform into colon cancer through a different pathway than conventional adenomas and result in a recognizable form of colon cancer. Unlike conventional adenomas, serrated adenomas often have genetic BRAF mutations and show extensive DNA methylation but lack APC gene mutations.

Dentate adenoma is a precursor lesion of high microsatellite instability (MSI-H) colorectal cancer, which accounts for approximately 15% of sporadic colon cancer. Like serrated adenomas, MSI-H colon cancers exhibit BRAF gene mutations and extensive

DNA methylation but generally lack mutations in the APC gene or in the KRAS oncogene (Cappell, 2008).

Studies show that in some types of tumors, including colorectal cancer, the inflammatory component (Terzić et al., 2010) as well as the alteration of some signaling pathways, for example the one regulated by EGF (epidermal growth factor) (Krasinskas, 2011), contribute to tumor development and survival. The involvement of inflammation in tumorigenesis was already hypothesized by Rudolf Virchow in 1863, when he noted the presence of leukocytes in neoplastic tissues (Singh et al., 2019). To date, tumor-related inflammation represents one of the hallmarks of cancer (Hanahan and Weinberg, 2011).

Generally, inflammation is the body's response to tissue damage, whether caused by physical injury, ischemic injury, infection, exposure to toxins, or other types of trauma, which causes cellular changes and immune responses that result in cell repair and proliferation in damaged tissues. If the inflammatory cause persists or if some control mechanisms fail to arrest the process, these inflammatory responses become chronic. In this case, mutations and cell proliferation can occur, often creating an environment conducive to the development of cancer.

Numerous studies demonstrate how chronic inflammation is linked to various steps involved in tumorigenesis, including cell transformation, promotion, survival, proliferation, invasion, angiogenesis and finally metastasis (Mantovani, 2005). To further investigate the relationship between inflammation and cancer and further tumor development associated with inflammation, studies were conducted on the specific roles of regulatory molecules involved in this process.

The chronic inflammatory microenvironment is dominated by macrophages which, together with other leukocytes, generate high levels of reactive oxygen and nitrogen species to fight infection (Maeda et al., 1998). Inflammatory cytokines, represented by TNF, IL-1, IL-6, and chemokines, produced by tumor cells and/or tumor-associated leukocytes and platelets, may also directly contribute to malignant progression (Smyth et al., 2004).

In the progression of colon cancer and in the therapy associated with it, an important role is also played by EGF. In fact, it represents a growth factor that plays an important role in regulating cell proliferation, growth and differentiation, through binding to its own EGFR receptor (Krasinskas, 2011). Mutations, gene amplification, and protein overexpression of various elements of the EGF signaling pathway not only contribute to

carcinogenesis but also impact prognosis and provide specific targets for colon cancer therapeutic intervention.

EGFR is a membrane receptor belonging to the ErbB family of receptors. In normal cells, the EGFR signaling cascade begins with ligand binding to the receptor; up to eleven different ligands can bind to these receptors, including EGF (Hynes and Lane, 2005). Ligand binding induces receptor dimerization with the formation of homodimers and heterodimers, which leads to the activation of tyrosine kinase activity and cross-phosphorylation of tyrosine residues present in the intracellular domain of the receptor. These events induce the activation of multiple signal transduction pathways, including the Ras/Raf/MEF/ERK (Extra-cellular signal Regulated Kinase) pathway and the PI3K/PDK1/Akt pathway (Jorissen et al., 2003), and the consequent activation of various transcription factors that influence cellular responses such as proliferation, migration, differentiation and apoptosis (Citri and Yarden, 2006).

III. Personal work

1. Materials and methods

1.1. Chemicals and Reagents

All chemical solvents were of analytical grade. LC-MS quality grade methanol, acetic acid, sulfuric acid, ethyl acetate, formic acid, isopropanol, 1-butanol, toluene, dichloromethane and acetonitrile, were purchased from Carlo Erba (Val de Reuil, France).

Standard compounds, quercetin (purity > 99%), rutin (purity > 99%), verbascoside (purity > 90%), minecoside (purity > 90%), catalpol (purity > 90%), and picroside III (purity > 98%) were purchased from Extrasynthese (Genay, France). The pure catalposide was isolated from the fruits of *Catalpa bignonioides* and identified by NMR and mass spectroscopy analyses.

Vanillin and magnesium chloride hexahydrate ($MgCl_2$) and 2-aminoethyl diphenylborinate (NP) were purchased from Sigma Aldrich, a subsidiary of Merck KGaA (Darmstadt, Germany); polyethylene glycol 4000 (PEG) was purchased from Ferak (Berlin, Germany).

1.2. Plant materials

Fruits of *C. bignonioides* were collected in the Botanical Garden of the Université Paris Cité, Faculté de Pharmacie de Paris (Latitude: 48°51′24″ N, Longitude: 2°21′07″ E) in September 2017. Fruits were randomly selected and examined for integrity and absence of dust and insects' contamination. Samples were harvested at maturity stage, defined by visual colour and size measurement. The fresh fruits were weighted and then stored frozen.

Phaseolus vulgaris L. var. "Fagiola di Venanzio" (FV) dried seeds (beans), were harvested in the municipality of Murlo (Siena, Tuscany, Italy, Latitude 43°10′16″32 N, longitude 11°23′32″28 E) and were identified by botanists in the Siena University Botanical Garden. Four samples of FV provided by different growers were used (Table 3).

Table 3. "Fagiola di Venanzio" samples.

Grower	#Sample
Società Agricola Aiellino	1
Nicola Ulivieri	2
Burresi family	3
Azienda Agricola Podere Vignali	4

1.3. Preparation of plant extracts

Frozen fresh fruits of *C. bignonioides* (1.0 kg) were extracted with 4 L of boiling water for 2 h and then, the solution was filtered on cotton. The extraction procedure with boiling water, followed by filtration, was performed three other times with 2 L of boiling water. The filtrates of the extracts were combined and subjected to evaporation and lyophilisation to finally yield an aqueous brown extract (eaC).

EaC, dissolved in 1 L of water, was extracted with 1 L of pure n-BuOH and then, with 0.5 L of water-saturated n-BuOH for three times. After evaporation of n-BuOH, the extract was in a paste form. The organic paste was solubilized in 200 mL of water and then, lyophilized obtaining a solid butanolic brown extract (eohC).

At the end of the evaporation of n-BuOH, the paste was triturated in 600 mL of dichloromethane (DCM) under stirring for 1h. The obtained precipitate was filtered, rinsed with DCM and dried until to yield a yellow solid DCM extract (edC).

To prepare the FV aqueous extract, 10 g of beans were manually grinded and briefly soaked in water at 50 °C; after discarding the liquid, a maceration has been performed at 35 °C with 100 mL of distilled water for 48h. The extract was adjusted to a 1:10 final drug:extract ratio.

To simulate the traditional cooking procedures of the beans and their digestion by the gastrointestinal tract, the INFOGEST method has been applied (Brodkorb et al., 2019):

a) Over/night (o/n) soaking of the beans with water

b) Soaking water recovery (fraction AA)

c) Added cooking water

d) Cooking for 3h and cooking water recovery (fraction AC)

e) *In vitro* digestion (fraction Dig):

-Oral phase: 5 g of cooked beans were mechanically whisked for 1 min in a 1% w/w NaCl solution (5 mL, pH=4.5) containing 75 UI/mL α-amylase, thus mimicking the very brief mastication occurring in the oral cavity after the consumption of a beans puree;

- Gastric phase: the "oral bolus" was mixed with a solution of pepsin, NaCl, 1M HCl (pH 1.8) and water. This mixture was incubated for 2h at 37 °C with stirring;

-Intestinal phase: the gastric chyme was mixed with $NaHCO_3$ to bring the pH back to 7.0 and intestinal digestive enzymes such as pancreatin and bile were added. The resulting mixture was further incubated for 2h at 37 °C under gentle stirring. The fraction thus obtained (Dig) was used for subsequent investigations.

1.4. High performance liquid chromatography (HPLC) analysis

Before analyses, dry extracts of *C. bignonioides* were dissolved in MeOH before being analysed in HPLC-DAD-MS and in HPLC (U-3000, Thermo) coupled to an ESI-QTOF-MS (Maxis II, Bruker) (Cb1, 10.6 g/L; Cb2, 5.1 g/L and Cb3, 2.7 g/L) and filtered through Econo filter Nylon 13 mm 0.2 µm (Agilent, Les Ulis, France).

Qualitative analyses of *Catalpa bignoniodes* extracts were performed using an HPLC (U-3000, Thermo) coupled to an ESI-QTOF-MS (Maxis II, Bruker) on a C18 column (Acclaim RSLC polar advantage II, 100 × 2.1 mm, 2.2 µm) at 35 °C. The mobile phase was a mixture of 0.1% formic acid, 10% methanol and water (phase A), and 0.1% formic acid and acetonitrile (phase B). The elution gradient was: 0 to 2 min 95% A; 2 to 7 min, 95 to 85% A; 7 to 15 min, from 85 to 50% A; 15 to 18 min, 50 to 20% A; 18 to 19 min, 20% and 19 to 21 min, 20 to 95% A. The injection volume was 2 µL and the flow rate was 0.3 mL min^{-1}. Chromatograms were obtained at 240, 270, 340, and 510 nm. Mass spectra were acquired in positive mode by using the following parameters: ESI 3500 V, *m/z* 50-1200, MS 2 Hz.

Quantitative analyses of *C. bignonioides* extracts was performed using an HPLC-DAD-MS ThermoScientific Dionex U3000 (Thermo-Dionex, Les Ulis, France) including a quaternary pump (LPG-3400 SD), an autosampler thermostat (WPS-3000TSL), a column thermostat (TCC-3000SD) and a Diode Array Detector (DAD-3000) (Thermo-Dionex, Les Ulis, France) on line with a quadripole mass spectrometer (Surveyor MSQ plus System, Thermo-Dionex, Les Ulis, France). The analytical column was a C18 Acclaim

Polar Advantage II (ThermoScientific, Courtabooeuf, France), 100 x 2.1 mm, 3 µm thermostated at 35 °C during the analyses.

Two chromatographic mobile phases were employed for a gradient elution as follows: solvent A: 0.1% formic acid in ultrapure water containing 10% LC-MS grade methanol (*v/v*), solvent B: 0.1% formic acid in acetonitrile LC-MS grade (*v/v*). In the mobile phase, the gradient was applied as follow: at the time of injection and for 45 min, 80:20 between 45 and 50 min analysis time, then to 20:80 between 50 and 60 min and finally to 95:5 between 60 and 65 min; this *ratio* is maintained until to initial conditions. The pump flow rate was set at 0.6 mL/min; the sample injection volume was 10 µL. Detection at specific wave lengths 210, 254, 280, and 350 nm, were used to record the chromatograms. The chromatographic effluent carried by a stream of nitrogen was directed into the electrospray ionization source of the mass spectrometer (MS). The MS was operated in the positive and negative ionization modes with the following operating conditions: ion spray voltage 3 kV, curtain gas 50 psi, Q energy was 70 V, cone voltage 50 V, desolvatation temperature 500 °C, and ion energy 0.8 V. Chromeleon version 6.8 software, provided by ThermoScientific Dionex, (Les Ulis, France) was used for obtaining results.

HPLC-DAD analysis of FV extracts was performed by using a Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak® C18 column, 10 µm, 125 Å, 3.9 mm × 300 mm column (Waters Corporation, Milford, MA, USA).

Water solutions containing 0.1 % (*v*/*v*) formic acid (A) and 0.1% (*v*/*v*) acetonitrile (B) were used as mobile phase. The following program was applied: B from 10% at 0 min to 35% at 20 min, then B 50% at 25 min ; flux was set at 0.8 mL/min. Chromatograms were recorded at 254, 280, 330 and 350 nm. Analyses were performed using 10 µL of FV extract; gallic acid, chlorogenic acid, caffeic acid, catechin, genistein, daidzein, quercetin and kaempferol (Sigma Aldrich) were used as external standards. Calibration curves were established using reference standards ranging from 0.008 mg/mL to 0.500 mg/mL. The correlation coefficient (R^2) of each curve was >0.99.

1.5. HPLC-DAD-DPPH (2,2-Diphenyl-1-picrylhydrazyl) of FV extract

To evaluate the different role of FV polyphenols in exerting antiradical activity, the HPLC-DAD run described above was repeated after having incubated the FV extract with a 1×10^{-2} M DPPH (2,2-diphenyl-1-picrylhydrazyl) methanolic solution for 15 min. Each chromatogram peak area was compared before and after the DPPH reaction. Ascorbic acid was used to validate the test.

1.6. Quantification of catalposide in *C. bignoniodes* extracts

To evaluate the catapolside dosage, an HPLC analysis was performed using the same conditions described above (HPLC-DAD-MS ThermoScientific Dionex U3000). The method used to quantify catalposide was internal calibration. Initially, a calibration curve was constructed with six different concentrations of pure catalposide (pC), isolated and characterized from the fruits of *C. bignonioides* in the Pharmacognosy laboratory. At the six different concentrations of pure catalposide C^0= 0.950 g/L; C^1= 0.8 g/L; C^2= 0.5 g/L; C^3= 0.4 g/L; C^4= 0.2 g/L; C^5= 0.04 g/L an internal standard was added, i.e. a quantity of commercial quercetin at a concentration of 10.0 g/L. The regression equation and an R^2 were obtained from the ratio of the peak areas of the standard compound catalposide and internal standard compound quercetin respectively. Then a further HPLC analysis was performed keeping the same conditions used previously. A quantity of quercetin at a concentration of 10.0 g/L was added to the aqueous extract with a concentration of 10.0 g/L, to the butanolic one 5.0 g/L and to the one in dichloromethane 2.5 g/L. From the ratio of the areas of the catalposide and of the quercetin present in the three extracts and from the regretion equation previously obtained, the catalposide in each of the three extracts was quantified. To validate the method, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated.

1.7. High performance thin-layer chromatography (HPTLC) analysis of *C. bignonioides* extracts

General procedure for the preparation of sample solutions analysed by HPTLC: Each compound was weighed, dissolved in MeOH, sonicated and then filtered through a 13 mm 0.2 µm Econo Nylon filter (Agilent, Les Ulis, France).

HPTLC analyses were performed on 20 × 10 cm HPTLC glass plates silica gel 60 F_{254} (Merck KGaA) with an HPTLC system (CAMAG) controlled by vision CATS 3.0 software and consisting of Automatic TLC Sampler ATS 4, Automatic Developing Chamber ADC 2, TLC Plate Heater, Derivatizer, Immersion device II, Visualizer 2.

5.0 µL of each sample were applied at a rate of 150 nL/s as 5 mm band, 8 mm apart and 8 mm from the lower edge, resulting in 11 tracks with the first one at 28 mm from the left edge of the plate. After conditioning for 10 min at 33% relative humidity (saturated $MgCl_2$ solution), the plates were developed in a saturated chamber (20 min, with filter paper) to a distance of 70 mm from the lower edge of the plate, using nine different mobile phases. After the development, the first two plates were derivatised with 2 mL of vanillin sulfuric acid spraying reagent. The derivatised plates were heated (CAMAG TLC Plate Heater III) at 100 °C for 3 min until colour developed.

The other two plates were derivatised with 2 mL of NP/PEG reagent and the last plate was derivatised with 2 mL of DPPH reagent 0.05% solution in methanol (50 mg of DPPH reagent were added in 100 mL of methanol).

Digital images of the plates were recorded in ultraviolet (UV) 254 nm (short-wave UV), UV 366 nm (long-wave UV) and white light after derivatization.

1.8. Determination of polyphenol, soluble carbohydrate and protein contents of FV extracts

Total polyphenols content (TPP) of FV extracts was evaluated by Folin–Ciocalteau (FC) colorimetric assay, optimizing the procedure reported by Biagi et al., 2019. Briefly, 100 µL of extract were diluted to 3 mL with distilled water; 500 µL FC reactive (Sigma-Aldrich, Milan, Italy, 1:10 diluted with water) were added and the mixture was gently shaken for 1 min. A quantity of 1000 µL of 30% *w*/*v* sodium carbonate water solution was added and, after incubation for 1h in the dark at RT, absorbance of samples was read at 750 nm, using distilled water as blank. Gallic acid (Sigma-Aldrich) was used as a reference standard. A calibration curve was created using gallic acid 5000 to 78 mg/L.

Total soluble carbohydrates of extract were quantified using the acid phenol assay described for the first time by Dubois et al., 1951, with optimization of the method. A quantity of 100 µL of the supernatant was added to 190 µL of water and 100 µL of a 6% *w*/*v* phenol (Sigma-Aldrich) water solution. The solution was gently shaken for 30 s and 500 µL of concentrated sulfuric acid (Sigma-Aldrich) were added. The mixture was

heated at 80 °C for 15 min and cooled to RT. Absorbance was read at 490 nm. D-glucose (Sigma-Aldrich) was used from 80 to 1.25 mg/L as reference standard. Total saccharide content was calculated, interpolating the data on the calibration curve of D-glucose.

Total proteins of extracts were determined spectrophotometrically using the BCA protein assay kit (Euroclone, Milan, Italy). Briefly, 2 µL of different dilutions of FV extract were added to 100 µL of bicinchoninic acid and, after incubation at 37 °C for 30 min, the absorbance was measured at 562 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Protein concentration was determined and reported with reference to standards of a bovine serum albumin (BSA).

1.9. Cell culture

The cell culture procedures and the experiments conducted were performed under sterile conditions, using sterile reagents and materials and working under a Safemate Eco+ hood.

HCT-116, colorectal carcinoma cells, (ATCC, Rockville, MD, USA) and FU (human dermal-derived fibroblasts) (American Type Culture Collection, ATCC, Rockville, MD, USA) were cultured in DMEM (Euroclone) supplemented with 10% fetal bovine serum (FBS, Euroclone), 100 U/mL penicillin/streptomycin (Euroclone), and 4 mM L-glutamine (Euroclone).

HT-29, colorectal adenocarcinoma cells, (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 (Euroclone) medium supplemented with 10% FBS with 100 U/mL penicillin/streptomycin. Both cell lines were grown at 37 °C and 5% CO_2.

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Lonza, Basel, Switzerland). All experiments were performed on low passage cell cultures. Cells were grown on gelatin-coated dishes in Endothelial Growth Medium (EGM-2) (EBM-2, FBS 10%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000) (Lonza) at 37 °C and 5% CO_2.

1.10. Determination of ROS levels

3.5 or 2.5 x 10^3 cell/well (HT-29, HCT-116 or HUVEC) were seeded in 96-multiwell plates and, after adherence, were maintained for 18h in medium without phenol red (0.1% serum) with different concentrations of the extract (10 and 50 µM) and then exposed to IL-1β (10 ng/mL) for 24h. $DCFH_2$-DA (2,-7-dichlorodihydrofluorescein diacetate) (Invitrogen, Milan, Italy) was added (10 µM, 1h) and intracellular levels of ROS were

evaluated photometrically with a microplate reader (excitation/emission 495/527) (EnVision, PerkinElmer).

1.11. Trypan Blue assay

HT-29 cells were cultured in microtubes at a cell density of about 5.0 x 10^5 cells/mL and treated with the sample, in medium containing 0.1% serum, at the concentration of 100 µg/mL, for a total volume of 300 µL per microtube.

After 48h of incubation, Trypan Blue solution (0.4%) (1:10) was added to each experimental point. After 3 min, 8 µL of suspension were withdrawn and the cells were counted using the Thoma chamber. The result was expressed as the number of dead cells in relation to the total number of cells.

1.12. MTT assay

HT-29 cells were plated at a density of 3.5 x 10^3 cells per well in RPMI-1640, while HCT-116 and fibroblasts (FU) were plated at a density of 2.5 x 10^3 cells per well, in DMEM, using 96-multiwell plates, with final volume 100 µL/well. 10% FBS was also added to both media. After 24h in the incubator at 37°C and 5% CO_2, time which allowed the cells to adhere, the culture medium was replaced by adding medium with 0.1% FBS, to synchronize the cells.

The cells were then the cells were subjected to the following treatments:

- extracts (aqueous or butanolic) of *C. bignonioides* at five different concentrations (0.01, 0.1, 1, 10, 100 µg/mL) or catalposide at four concentrations (0.01, 0.1, 1, 10 µg/mL) for 48h or
- FV aqueous extract (5, 10, 50 and 100 µM) and then exposed to IL-1β (10 ng/mL) for 24h or
- FV fractions, AA, AC and Dig, (1, 10, 100 µg/mL) for 48h.

The cells were left in the incubator for 48h after which the medium was removed and replaced with 100 µL/well of 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) solution in phenol red-free DMEM. The plates were incubated for 4h at 37°C and 5% CO_2.

After incubation, the medium was removed, and cells were incubated for 4h with fresh medium in the presence of 1.2 mM MTT. The MTT solution was then removed and 100 µL of DMSO were added to each well to dissolve the blue formazan crystals. The

absorbance of the formazan dye was measured at 570 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Data were expressed as a percentage of the basal control.

At the end of the treatment, the culture medium was removed and 100 µL of MTT solution, 0.5 mg/mL in phenol red-free DMEM were added to each well. The plates were kept in the incubator for 4h. Subsequently, once the MTT solution was removed, the formazan salts were solubilized in 50 µL of DMSO. Finally, the absorbance values at 540 nm of the solution thus obtained were measured (Envision 2015, PerkinElmer). The results were expressed as a percentage of the control conditions.

1.13. Clonogenic assay

For *Catalpa bignonioides,* HT-29 cells were counted and plated in 6-well multiplates at a concentration of 750 cells/well in the respective culture medium containing 10% FBS. After 24h, necessary for cell adhesion, the medium was removed and replaced with medium containing 0.1% FBS. After a further 24h the medium was removed, new 1% FBS medium was added and the cells were first pretreated for 1h with aqueous extract or butanol extract of *C. bignonioides*, at concentrations of 10 and 100 µg/mL or with catalposide at concentrations of 1 and 10 µg/mL. Where indicated, cells were treated with 25 ng/mL EGF or 10 ng/mL IL-1β. Subsequently the plates were left in an incubator for 10 days.

For *Phaseolus vulgaris* L. var. Venanzio, HT-29 cells were counted and plated in 24-well plates, at a concentration of 300 cells/well, adding the respective culture media, both supplemented with 10% FBS. After 24h, the medium was removed, a new 1% FBS medium was added and the cells were treated with FV samples AA, AC or Dig at concentrations of 1, 10 and 100 µg/mL. Subsequently the plates were left inside the incubator for 10 days.

At the end of the incubation, cells were fixed with methanol (+4°C, o/n) and stained with Blue for Fast Staining (Panoptic n°3) DC, Eosin for Fast Staining (Panoptic n°2) DC (Panreac).

1.14. Preparation of protein extracts

HT-29 cells were plated at a density of 3.5 x 10^3 cells/well, while HUVEC were seeded in 6-multiwell plates at a density of 3.0 x 10^5 cells/well in medium at 10% FBS and kept

for 24h inside the incubator to allow adhesion. After 24h, the medium was removed and replaced with new 0.1% FBS culture medium, in order to synchronize all the cells, and left in the incubator for another 24h.

Subsequently the cells the cells were subjected to the following treatments:

-1h pretreatment with the aqueous or butanolic extracts of *C. bignonioides* at a concentration of 100 µg/mL or with catalposide at a concentration of 10 µg/mL. Then the cells were treated with 25 ng/mL EGF or 10 ng/mL IL-1β for 15 min or 48 h, depending on the type of signal to be studied, or

- FV aqueous extract of FV fractions for 18h (10 and 50 µM) and then exposed to IL-1β (10 ng/mL), or

-FV samples for 48h at concentrations of 1, 10, 100 µg/mL.

At the end of the treatment, the cells were placed on ice, the medium was removed, and the plates were washed twice with cold PBS to remove excess medium. 70 µl of a solution formed by RIPA buffer added with protease and phosphatase inhibitors (Lysis buffer) were then added to each well. The cells were removed mechanically, and the lysates obtained were collected in 1.5 mL microtubes. The collected samples centrifuged at 13,000 g at 4°C for 15 min. The supernatant was then collected, and the protein assay was performed by the colorimetric method of BCA (bicinchoninic acid).

The value of the protein concentration protein was obtained from a standard curve previously constructed using samples of bovine serum albumin (BSA) (Euroclone) at known concentration. The BCA assay was performed using 96-well plates and a commercial kit (Euroclone). The protein extracts were subsequently analyzed by SDS-PAGE and Western blotting.

1.15. SDS-PAGE and Western blotting analysis

Aliquots of cell extract supernatants containing an equal amount of proteins (50 µg) were treated with Laemmli buffer, boiled for 10 min, resolved on 4–20% stain-free gel and then blotted onto a nitrocellulose membrane using Semidry Electro-blotter System (Galileo Bioscience, Cambridge, MA, USA).

The blots were blocked with 5% defatted dry milk (Euroclone) in tris-buffered saline (TBS) containing 0.5% tween 20 for 1h, at RT, and incubated overnight at 4 °C, under stirring, with appropriate dilutions of the primary antibody. Subsequently, membranes were incubated for 1h with the appropriate horseradish peroxidase (HRP)-conjugated

secondary antibodies. Proteins were then visualized by an enhanced chemiluminescence detection system (Euroclone) using the ImageQuant Las 4000 instrument (GE Healthcare Life of Science). The primary antibodies used in the present study included: anti-p-NFkB (Cell Signaling Technology), anti-GSH (Virogen, Watertown, MA, USA), anti-ERK 1/2 (Cell Signaling Technology), anti-COX-2 (Cell Signaling Technology), anti-Caspase-3 (Cell Signaling Technology). The bands of the proteins of interest were normalized by incubating the membranes overnight at 4°C with anti- β-actin (Invitrogen), or anti-GAPDH (Cell Signaling Technology). The results obtained were processed using the Image J software, which evaluates the optical density of the immunoreactive bands.

2. Results and discussion

2.1. *Catalpa bignonioides*

2.1.1 Chemical composition of *C. bignonioides* fruit extracts

Catalpa bignonioides grows in the Botanical Garden of the Université Paris Cité, Faculté de Pharmacie de Paris. As described in the bibliographical part, several phytochemical studies revealed that *C. bignonioides* extracts contained flavonoids, iridoids, lignans, oligosaccharides, phenolic acids, phenolic glycoside, phenylethanoid diglycoside.

To identify the major phytochemical constituents of *C. bignonioides* fruit extracts, an HPLC coupled to an ESI-QTOF-MS analysis was performed. Table 4 shows the compounds present in the three extracts, identified on the basis of the UV spectra, and of the molecular weight (ion m/z $[M+H]^+$). Compound identification was also established by fragment ions and confirmed by error values < 10 ppm and precision value < 20 mSigma. HPLC coupled to an ESI-QTOF-MS analysis of extracts showed the presence of flavonoids, iridoids, phenylpropanoid glycosydes, phenolic acids, oligosaccharides and terpenoid quinones (naphthoquinones) as main constituents of fruits. There are some differences between the three extracts in the number of compounds identified, depending on the nature of the solvent and of the molecule itself.

The compounds identified with the HPLC-ESI-QTOF-MS analysis are in agreement with the phytochemical composition of the extracts of genus Catalpa. Many studies on the characterization of *C. ovata*, *C. speciosa* and *C. bungei* extracts reveled the presence the following main classes: naphthoquinone, phenolic acids, iridoids, oligosaccharides, lignans, flavonoids, phenylpropanoid glicosydes and triterpenes.

Some of these compounds are present in the extracts of *C. bignonioides* as reported in Table 1.

Table 4. Identification of chemical compounds in *Catalpa bignonioides* fruit using ESI-QTOF-MS technique.

Compounds	Molecular Formula	Molecular weight (exact)	Pseudo-molecular Ion $[M+H]^+$	err [ppm]	mSigma	Score (%)/RT	Fragments Ions $[+H]^+$	*Catalpa bignonioides* fruits			Ref.
								eaC	eohC	edC	
Flavonoids											
Quercetin coumaroylglucoside (Helichrysoside isomer)	$C_{30}H_{26}O_{14}$	610.13226	611.1374	2.8	9.7	100/9.2	303.0489	✓	✓	✓	Datta et al. 2000
Quercetin 3-O-(6"-feruloyl)-*β*-D-galactopyranoside	$C_{31}H_{28}O_{15}$	640.14282	641.1494	1.1	8.8	100/8.9	-	✓	✓		Datta et al. 2000
Isoquercetin	$C_{21}H_{20}O_{12}$	464.09548	465.1019	1.9	2.7	100/7.7	303.0484	✓	✓		B. de Abreu et al., 2013
Iridoids											
Catalpol	$C_{15}H_{22}O_{10}$	363.30340	363.1065	2.4	6.5	100/7.9	163.0389	✓			Wang et al., 2016
Catalposide	$C_{22}H_{26}O_{12}$	482.14243	483.1485	2.5	11.5	100/7.8	321.0945 303.0857	✓	✓	✓	Oh et al., 2021
6-*O*-*p*-Hydroxybenzoylphelipaeside	$C_{22}H_{28}O_{12}$	484.15808	485.1685	1.1	6.0	100/7.4	287.0909	✓	✓	✓	Dal Piaz et al., 2013
Verproside	$C_{22}H_{26}O_{13}$	498.13734	499.1438	4.7	15.8	100/7.1	137.0234 319.0814	✓	✓	✓	Park et al., 2009
Compound A*	$C_{22}H_{28}O_{13}$	500.1530	501.1583	3.5	10.9	100/6.6	321.0958 483.1483	✓	✓	✓	Iwagawa et al., 1991
Verminoside	$C_{24}H_{28}O_{13}$	524.15299	525.1587	3.1	15.9	100/6.0	163.0389	✓	✓		Han et al., 2015
6-*O*-*cis*(*trans*)-*p*-Coumaroyl-5,7-bisdeoxycynanchoside	$C_{24}H_{30}O_{12}$	510.17373	511.1794	3.2	12.7	100/8.2	-	✓	✓	✓	Dal Piaz et al., 2013

Minecoside*	$C_{25}H_{30}O_{13}$	538.16864	539.1745	2.6	18.6	100/8.3	377.1226 177.0543	✓	✓	✓	Oh et al., 2021
Picroside III*	$C_{25}H_{30}O_{13}$	538.16864	539.1743	3.1	8.9	100/8.4	377.1252 177.0545	✓	✓	✓	Oh et al., 2021
Specioside	$C_{24}H_{28}O_{12}$	508.15808	509.1645	1.8	6.8	100/8.6	-	✓	✓	✓	Oh et al., 2021
Oligosaccharides											
6-*O*-(*p*-Hydroxybenzoyl)-D-glucopyranose	$C_{13}H_{16}O_8$	300.08452	301.0909	3.9	4.5	100/4.5	139.0389	✓	✓		Tang et al., 2016
Phenolic acid											
p-Coumaric acid	$C_9H_8O_3$	164.04734	165.0546	0.2	13.2	100/1.3	-	✓	✓		Oh et al., 2021
Phenylpropanoid glycosides											
Martynoside	$C_{31}H_{40}O_{15}$	652.23672	653.2421	2.9	16.0	100/8.9	177.0544	✓	✓	✓	Tang et al., 2016
Caffeolyl phenylethanoid glycoside isomer (Verbascoside type)	$C_{29}H_{36}O_{15}$	624.20542	625.2113	2.3	8.5	100/8.1	163.0389	✓	✓	✓	Tang et al., 2016
Terpenoid quinones (naphthoquinones)											
9-Methoxy-4-oxo-α-lapachone	$C_{16}H_{14}O_5$	286.08412	287.0910	1.6	13.3	100/8.5	-	✓			Park et al., 2010

(*) Compound A : 4-Hydroxy-benzoic acid (1S,4aR,5S,6R,7S,7aS)-6,7-dihydroxy-7-hydroxymethyl-1-((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydro-cyclopenta[c]pyran-5-yl ester

The analysis of chromatograms allowed the identification of seventeen compounds in the extracts of *C. bignonioides* fruits using HPLC-DAD-MS technique, as reported in Figure 11.

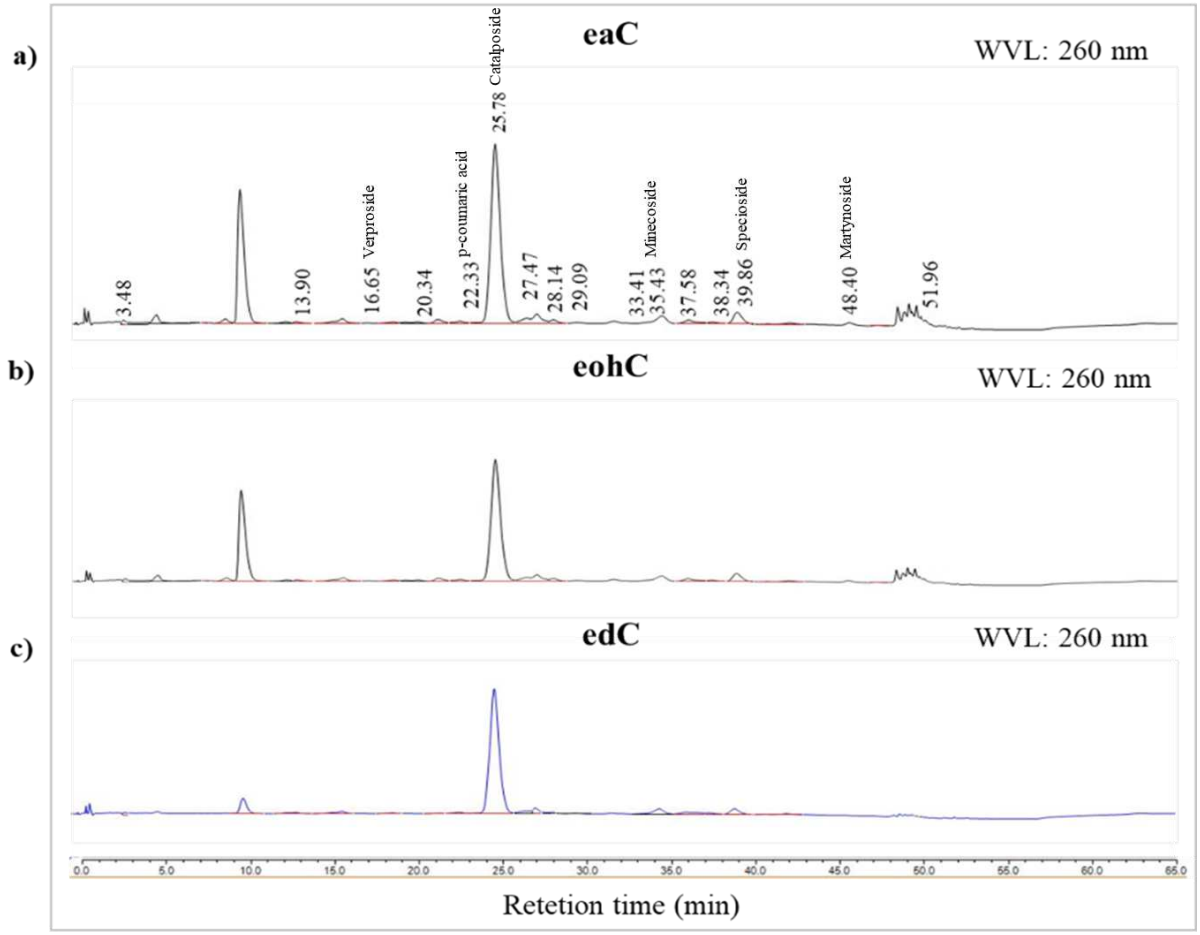


Figure 11. **HPLC-DAD-MS profile of compounds identified in *C. bignonioides* extracts**. **(a)** eaC= 100.8 g/L; **(b)** eohC= 12.1 g/L; **(c)** edC= 12.6 g/L.

As reported in Table 5, all the compounds are present in the aqueous and butanol extracts with the exception of 9-methoxy-4-oxo-*α*-lapachone, which is present only in the aqueous extract. Eleven compounds were identified and characterized in the edC extract. The smaller number of molecules found in the edC extract depends on the characteristics of the extraction solvent. Based on the literature data, iridoids are the compounds mostly present in *C. bignonioides* fruits; in particular, the following have been identified: catalposide, 6-*O*-p-hydroxybenzoylphelipaeside, verproside, verminoside, 6-*O*-*cis/trans*-*p*-coumaroyl-5,7-bisdeoxycynanchoside, minecoside, picroside III and specioside.

Table 5. Retention time (Rt) and compounds identified in *C. bignonioides* extracts using HPLC-DAD-MS technique.

Peaks	Rt	Identification	MH^- /M+45*	UV λmax (nm)	*Catalpa bignonioides* fruits		
					eaC	eohC	edC
1	3.48	6-*O*-(*p*-Hydroxybenzoyl)-D-glucopyranose	299/344	215, 263	✓	✓	✓
2	10.83	Compound A**	499/545	257	✓	✓	✓
3	13.90	6-*O*-*p*-Hydroxybenzoylphelipaeside	483/529	226, 260	✓	✓	✓
4	16.65	Verproside	497/543	224, 265	✓	✓	✓
5	20.34	Quercetin 3-*O*-(6"-feruloyl)-β-D-galactopyranoside	639/685	255, 358	✓	✓	
6	22.33	*p*-Coumaric acid	163/209	202, 227, 310	✓	✓	
7	25.78	Catalposide	481/527	216, 261	✓	✓	✓
8	27.47	Isoquercetin	463/509	254, 354	✓	✓	
9	28.14	Verbascoside isomer	623/669	224, 330	✓	✓	✓
10	29.09	Verminoside	523/569	224, 328	✓	✓	
11	33.41	6-*O*-*cis/trans*-*p*-coumaroyl-5,7-bisdeoxycynanchoside	509/555	224, 302	✓	✓	✓
12	35.43	Minecoside***	537/583	224, 328	✓	✓	✓
13	37.58	Picroside III***	537/583	224, 328	✓	✓	✓
14	38.34	Helichrysoside isomer	609/655	258, 355	✓	✓	✓
15	39.86	Specioside	507/553	230, 315	✓	✓	✓
16	48.40	Martynoside	651/697	224, 323	✓	✓	✓
17	51.96	9-Methoxy-4-oxo-α-lapachone	285/331	217, 282, 394	✓		

(*) Formic acid adduct in ESI-

(**) Compound A : 4-Hydroxy-benzoic acid (1S,4aR,5S,6R,7S,7aS)-6,7-dihydroxy-7-hydroxymethyl-1-((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydro-cyclopenta[c]pyran-5-yl ester

(***) interchangeable

The peak of catalposide (Retetion Time, RT= 25.78 min), as shown in Figure 11, is the one with the largest area of all the others in the chromatogram.

To determine the dosage of the catalposide within each extract, an HPLC-DAD-MS analysis was conducted by adding quercetin as the internal standard in the three extracts of *C. bignonioides* fruits. Initially, a calibration curve was constructed by injecting an internal standard, in particular a solution of quercetin with concentration of 10.0 g/L, at six different known concentrations of pure catalposide: C^0= 0.950 g/L; C^1= 0.8 g/L; C^2= 0.5 g/L; C^3= 0.4 g/L; C^4= 0.2 g/L; C^5= 0.04 g/L (Figure 12) and figure 13.

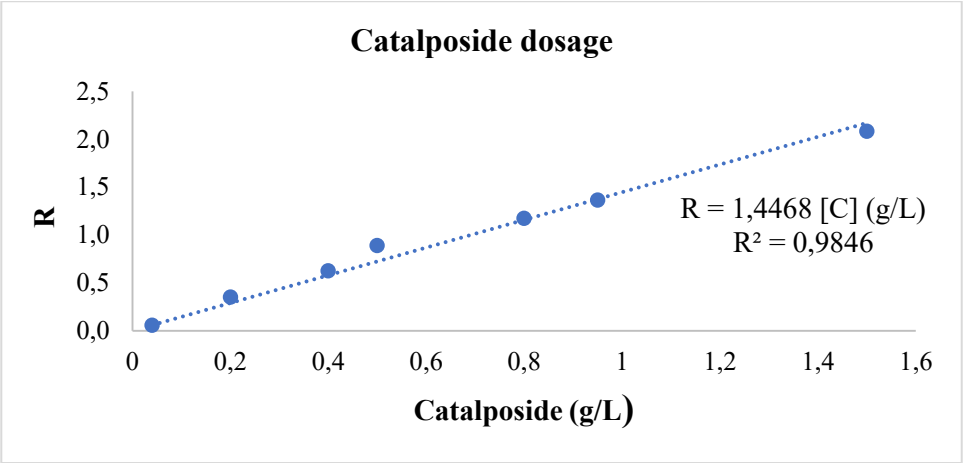


Figure 12. **Internal calibration of catalposide.** R is the ratio of the chromatographic area of catalposide to that of quercetin and C is the mass concentration of catalposide. The dispersions values include: ±0.007, ±0.02, ±0.2, ±0.2, ±0.2, ±0.2, ±0,3.

From the calibration curve, the equation is R = 1.4468 x $C_{[Catalposide]}$ (g/L), it has been obtained the following value of R^2 = 0.9846.

Then, the concentration and the percentage of catalposide in each extract was determined by adding the standard solution of quercetin (10.0 g/L) in the three extracts with the following concentrations: eaC= 10.0 g/L; eohC= 5.0 g/L; edC= 2.5 g/L). Catalposide concentrations in the various extracts are above the determined limit of quantification (0.007 ± 0.005) for a 5% shared risk. The dosage of catalposide results were as follows (Figures 13b, c, d): the highest amount of caltalposide for a 5% shared risk, is found in the edC extract (45±3 %, figure 13d), then in the eohC extract (28±6 %, figure 13c) and finally in the eaC extract (11±2 %, figure 13b).

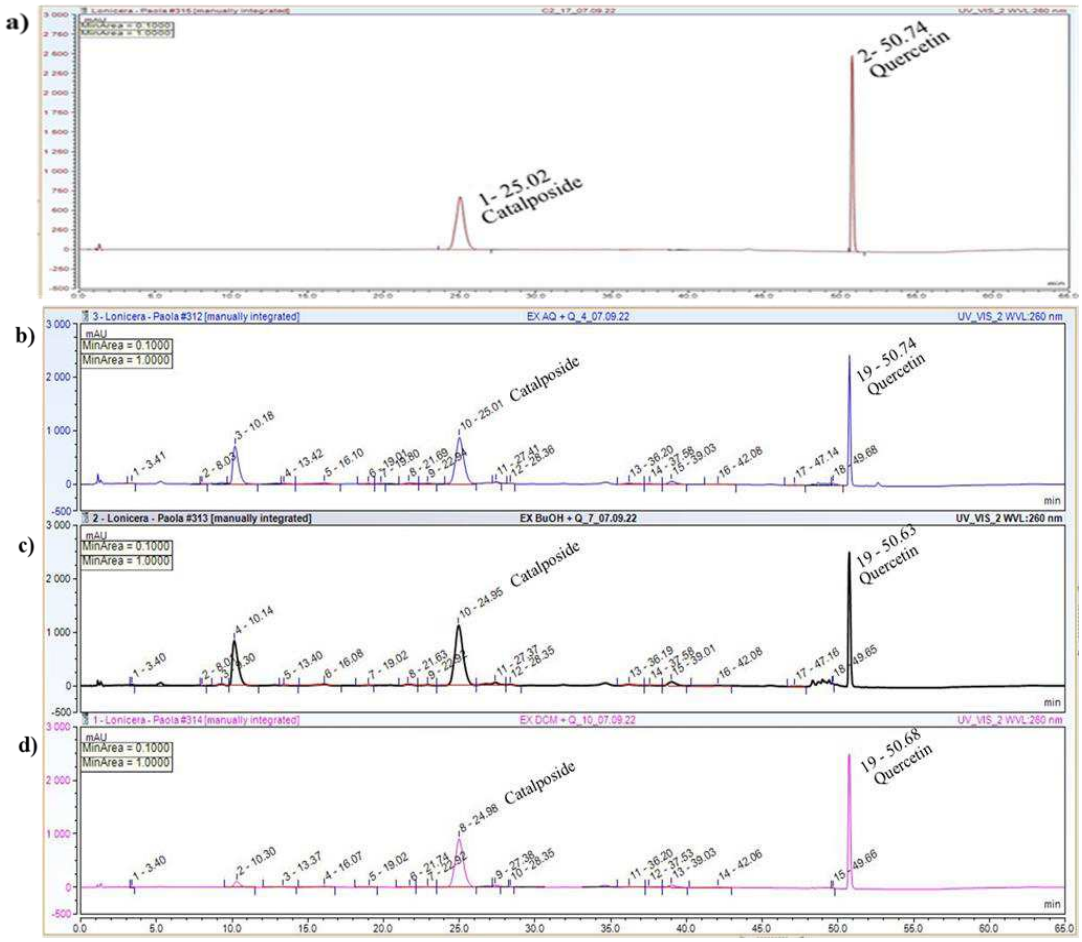
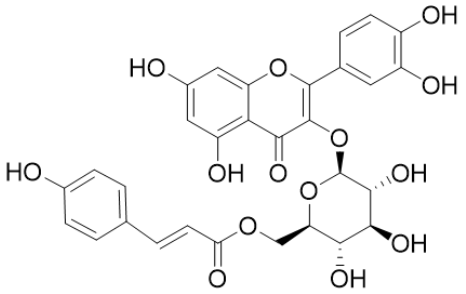


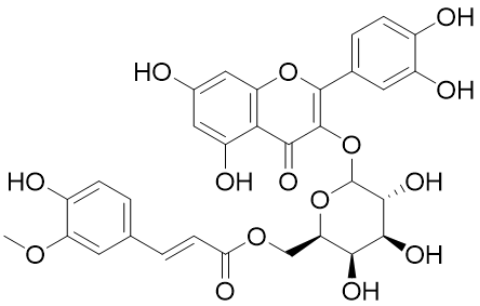
Figure 13. HPLC-DAD-MS analysis for the quantification of catalposide in the three extracts. **(a)** Chromatogram of pure catalposide C1= 0.8 g/L with the internal standard, a solution of quercetin with concentration of 10.0 g/L. **(b)** Chromatogram of eaC= 10.0 g/L with quercetin 10.0 g/L. **(c)** Chromatogram of eohC= 5.0 g/L with quercetin 10.0 g/L. **(d)** Chromatogram of edC= 2.5 g/L with quercetin 10.0 g/L.

Figure 14 shows the chemical structures of the compounds identified in the three extracts of *C. bignonioides* fruits; they are divided into the main subclasses of phytochemicals.

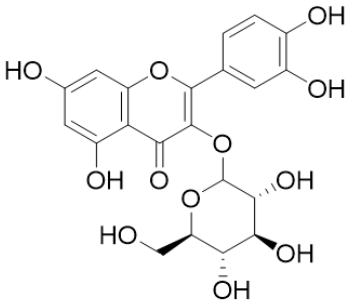
Flavonoids



Helichrysoside

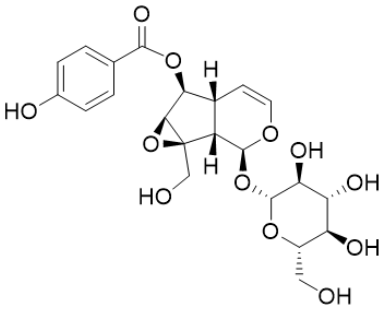


Quercetin 3-O-(6''-feruloyl)-β-D-galactopyranoside

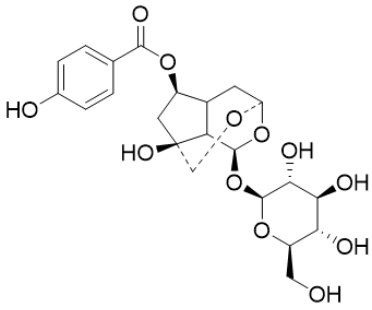


Isoquercetin

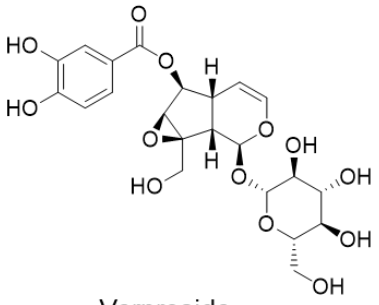
Iridoids



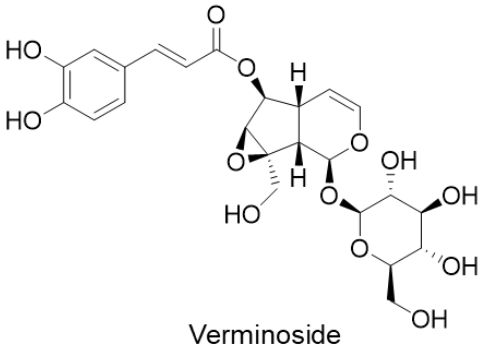
Catalposide



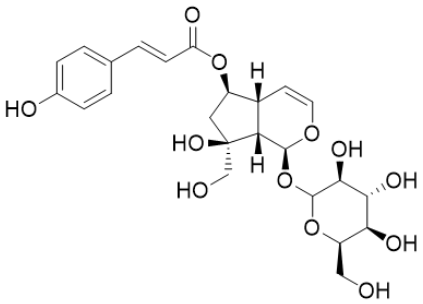
6-O-p-hydroxybenzoylphelipaeside



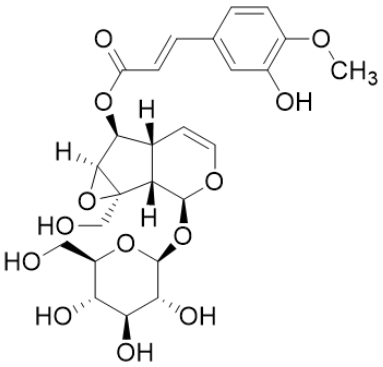
Verproside



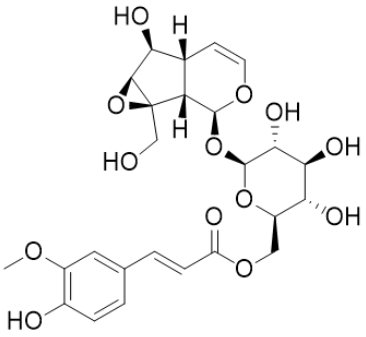
Verminoside



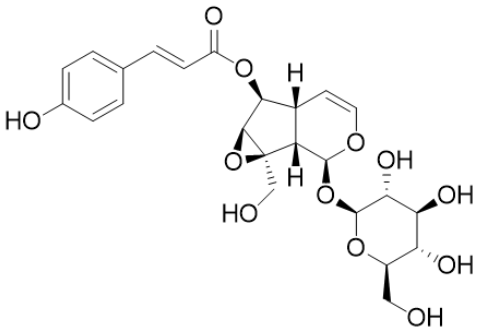
6-O-trans-p-coumaroyl-5,7-bisdeoxycynanchoside



Minecoside



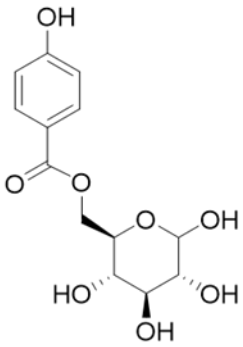
Picroside III



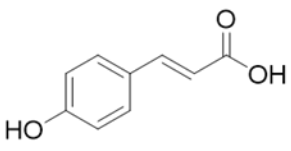
Specioside

Oligosaccharides

Phenolic acid

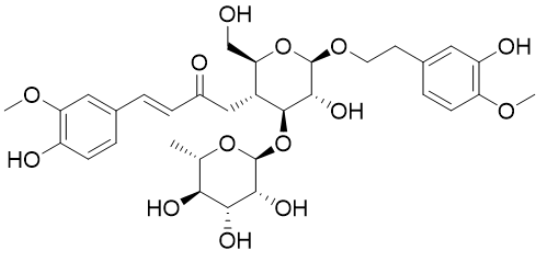


6-O-(p-hydroxybenzoyl)-D-glucopyranose

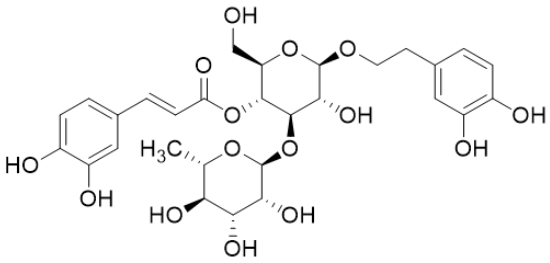


p-coumaric acid

Phenylpropanoid glycosdes

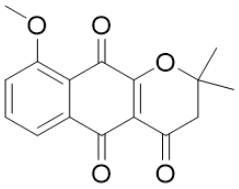


Martynoside



Verbascoside

Terpenoid quinones (naphthoquinones)



9-methoxy-4-oxo-α-lapachone

Figure 14. The chemical structure of the compounds identified in *Catalpa bignonioides* fruits.

2.1.2. HPTLC analyses

Previous high performance thin layer chromatography (HPTLC) methods have been developed to detect plant components (Waksmundzka-Hajnos et al., 2008; Wagner et al., 2016).

Do et al. selected a set of nine mobile phases (MPs), covering different polarities and selectivities of development solvents and the most suitable detection modes, in order to generate a complete set of HPTLC data on a collection of 19 iridoids for identification and the evaluation of important plants and herbal products.

Phytochemical profiling of medical plant by HPTLC can be detected with different derivatization reagents, including vanillin and Natural Product-polyethylene glycol (NP/PEG) reagent that can give preliminary information on the composition and biological activity of investigated extracts (Choma and Nikolaichuk, 2022).

In light of these bibliographic data, HPTLC was performed to confirm the presence of iridoids and other compounds identified by HPLC analysis in the three extracts.

Four reference compounds, including the iridoids catalpol, catalposide, minecoside and picroside III, the flavonoid rutin and the phenylpropanoid glycoside verbascoside were chosen as positive or negative standards in accordance with the previously obtained results.

Two mobile phases (MP1 and MP2) reported in the literature by Do et al., were used. MP1 was composed of AcOEt:MeOH:H_2O / 77:15:8 (V:V:V) with a polarity index of 4.95 and MP2 from CH_3Cl:MeOH:H_2O / 8:2:0.1 (V:V:V) with a polarity index of 4.40.

HPTLC analyses were performed on 20 × 10 cm HPTLC glass plates silica gel 60 F_{254} (Merck KGaA) with an HPTLC system (CAMAG).

In the first three positions of each plate were loaded, from left to right: the aqueous extract (eaC) 100.8 g/L, the butanol extract (eohC) 12.1 g/L and the dichloromethane extract (edC) 12.6 g/L. Then the reference compounds were loaded: catalposide 1.6 g/L, minecoside (TG5-146-B), catalpol 1.6 g/L, picroside III 1.0 g/L, verbascoside 1.4 g/L, rutin 1.1 g/L, ursolic acid 1.1 g/L and 4-hydroxybenzoic acid 1.3 g/L.

The first two analyzes were performed with the two different mobile phases MP1 and MP2 and after chromatography the plates were scanned at 254 nm (Figure 15a), at 366 nm (Figure 15b) and then analyzed with the vanillin sulfuric reagent (Figure 15c).

Many derivatization reagents, such as vanillin sulfuric and Natural Product-polyethylene glycol (NP/PEG) reagents, can give preliminary information on the composition and biological activity of investigated plant extracts (Choma and Nikolaichuk, 2022).

At different polarity of eluants, MP1 and MP2 were able to separate the compounds in the three extracts. However, iridoids such as catalposide, minecoside and picroside III with a similar structure and polarity showed almost aquivalent Rf with these eluents.

The catalpol, on the other hand, is located in the lower part of the plate, due to its more polar nature to catalposide. In figure 15a, at the top right of the plate, in the position 11, the fingerprint of 4-hydroxybenzoic acid can be seen under UV 254 nm. HPTLC showed this compound present in the three extracts, as its imprint can be seen at the same height.

In figures 15a, 15b, and 15c, in the lower right part of the plates with the MP2, it is possible to note the imprint of the rutin (spot 9) and verbascoside (spot 8) references. Thanks to the MP2 eluant, the HPTLC highlights the presence of verbascoside or one of its isomers in extracts. Concerning the rutin, figure 15c do not show its presence. All HPTLC reveal that catalposide and its derivatives are the main compounds.

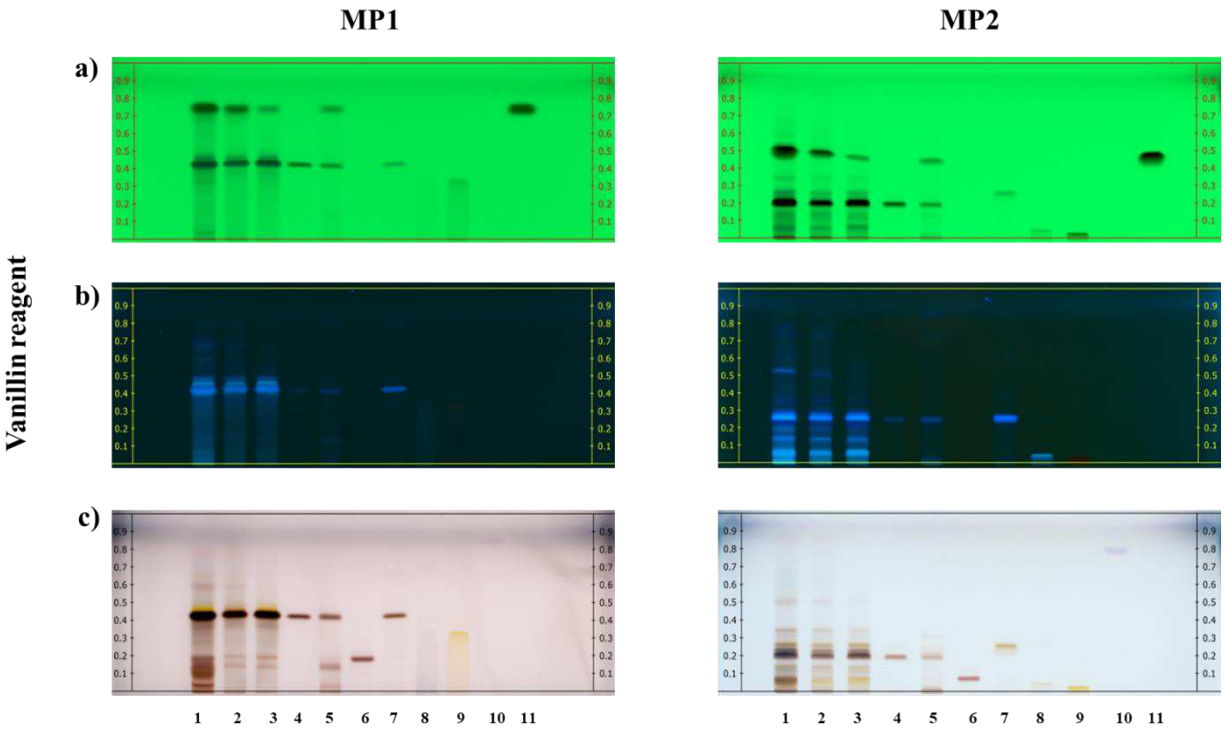


Figure 15. HPTLC fingerprints developed with vanillin reagent. (a) UV 254 nm prior derivatization; **(b)** UV 366 nm; **(c)** white light after derivatization with vanillin reagent. From left to right: (1) eaC 100.8 g/L; (2) eohC 12.1 g/L ; (3) edC 12.6 g/L; (4) catalposide 1.6 g/L; (5) minecoside (TG5-146-B); (6) catalpol 1.6 g/L; (7) picroside III 1.0 g/L; (8) verbascoside 1.4 g/L; (9) rutin 1.1 g/L; (10) ursolic acid 1.1 g/L; (11) 4-hydroxybenzoic acid 1.3 g/L.

Moreover, two analyzes were also performed with the same mobile phases MP1 and MP2 and plates were scanned analyzed with NP/PEG reagent. (Figure 16b). NP-PEG reagent can detect the presence of polyphenols and phenols. Different colors of fluorescent fingerprints, obtained as a result of derivatization with NP-PEG, allow characterizing the type of compounds in a sample. In particular, red fingerprints in NP-PEG correspond to chlorophylls, purple to anthocyanins, intense blue to phenol carboxylic acids, yellow/orange to flavanols, and green to flavanones.

These analyses show that rutin is not detected at the concentrations of the various samples studied and confirm the HPLC coupled to an ESI-QTOF-MS results. No orange spots corresponding to rutin were detected in the 3 extracts.

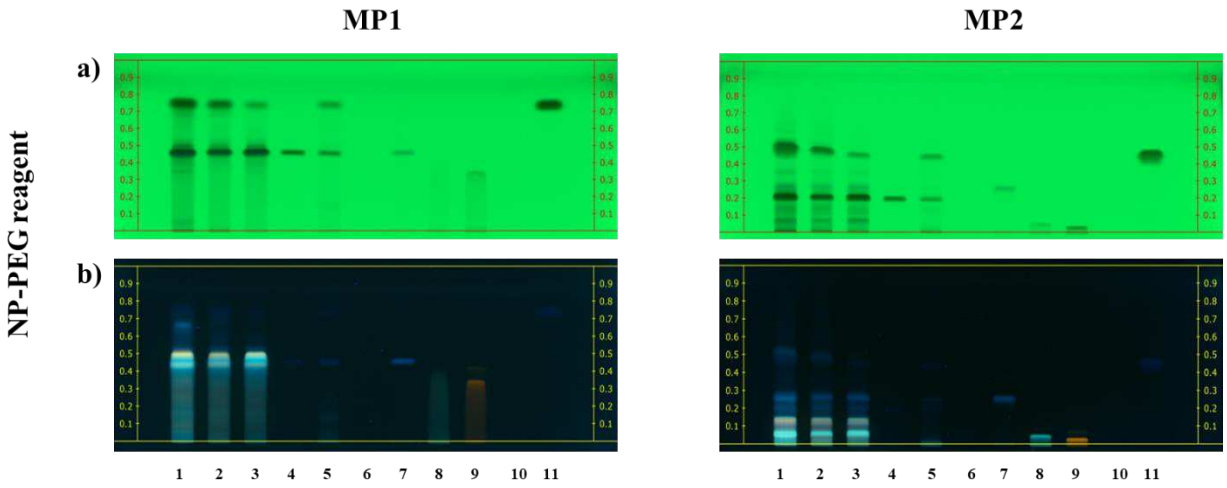


Figure 16. HPTLC fingerprints developed with NP-PEG reagent. (a) UV 254 nm prior derivatization; (b) white light after derivatization with NP-PEG reagent. From left to right: (1) eaC 100.8 g/L; (2) eohC 12.1 g/L ; (3) edC 12.6 g/L; (4) catalposide 1.6 g/L; (5) minecoside (TG5-146-B); (6) catalpol 1.6 g/L; (7) picroside III 1.0 g/L; (8) verbascoside 1.4 g/L; (9) rutin 1.1 g/L; (10) ursolic acid 1.1 g/L; (11) 4-hydroxybenzoic acid 1.3 g/L.

Compounds being detected using NP-PEG usually have antioxidant properties. This can be confirmed by derivatization with DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, which is used to detect antioxidants.

HPTLC analysis coupled with DPPH derivatisation reagent is a novel approach to the analysis of antioxidant activity of natural products (Islam et al., 2021). DPPH* is a stable free radical, which is sensitive to reaction with Lewis bases. It is characterised by an intense purple colour (absorbance at 515–520 nm), which is lost upon reaction with oxidising reagents. In particular, constituents which can rapidly decrease the absorbance of DPPH* by donating a hydrogen atom are considered good antioxidants. However, a significant advantage of the assay is that DPPH* reacts even with weak antioxidants if sufficient reaction time is given.

To explore the possible antioxidant effect of the three extracts, HPTLC analysis coupled with DPPH reagent was performed with MP1 conditions. In the first three positions of the plate were loaded, from left to right: the aqueous extract (eaC) 100.8 g/L, the butanol extract (eohC) 12.1 g/L and the dichloromethane extract (edC) 12.6 g/L. Then the reference compounds were loaded: catalposide 1.6 g/L, minecoside (TG5-146-B), catalpol 1.6 g/L, picroside III 1.0 g/L, verbascoside 1.4 g /L, rutin 1.1 g/L, ursolic acid 1.1 g/L and 4-hydroxybenzoic acid 1.3 g/L. As can be seen in Figure 17, the antioxidant activity of the three extracts was confirmed by several yellow bands appearing on the purple background in their tracks. In particular, there are more bands that are intensive in each path of the three extracts and it is present at the same Rf value 0.48 corresponding catalposide and their derivatives. EaC and eohC have a band at the Rf value

0.68 could be corresponding for 4-hydroxybenzoic acid (spot 11). Surprisingly, nonetheless, standard 4-hydroxybenzoic acid has no antioxidant effect.

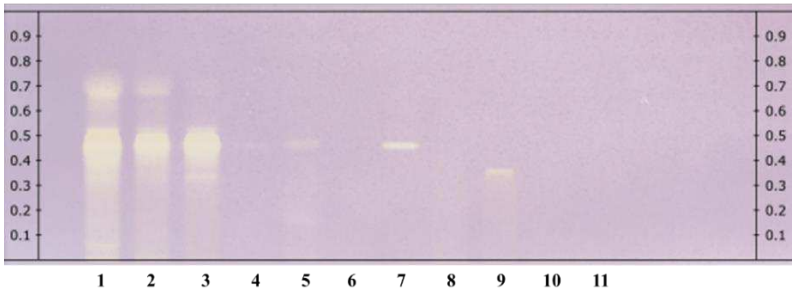


Figure 17. HPTLC fingerprints developed with DPPH reagent. From left to right: (1) eaC 100.8 g/L; (2) eohC 12.1 g/L ; (3) edC 12.6 g/L; (4) catalposide 1.6 g/L; (5) minecoside (TG5-146-B); (6) catalpol 1.6 g/L; (7) picroside III 1.0 g/L; (8) verbascoside 1.4 g/L; (9) rutin 1.1 g/L; (10) ursolic acid 1.1 g/L; (11) 4-hydroxybenzoic acid 1.3 g/L.

2.1.3. *In vitro* antitumor activity

Various studies have highlighted the promising anti-inflammatory, antinociceptive (Muñoz-Mingarro et al., 2003; Kim et al., 2004), antioxidant activities (Dvorská et al., 2007), as well as the involvement in glucose homeostasis (Oh et al., 2021) and diabetes management (Bai et al., 2019), of the different compounds contained in C. *bignonioides* extracts. Further studies conducted on other species of *Catalpa* including, *C. ovata*, *C. speciosa* and *C. bungei*, have demonstrated promising antitumor activities linked to the presence of phenols and naphthoquinones in the extracts of these plants (Fujiwara et al.,1998; Elansary et al., 2019; Qin et al., 2022).

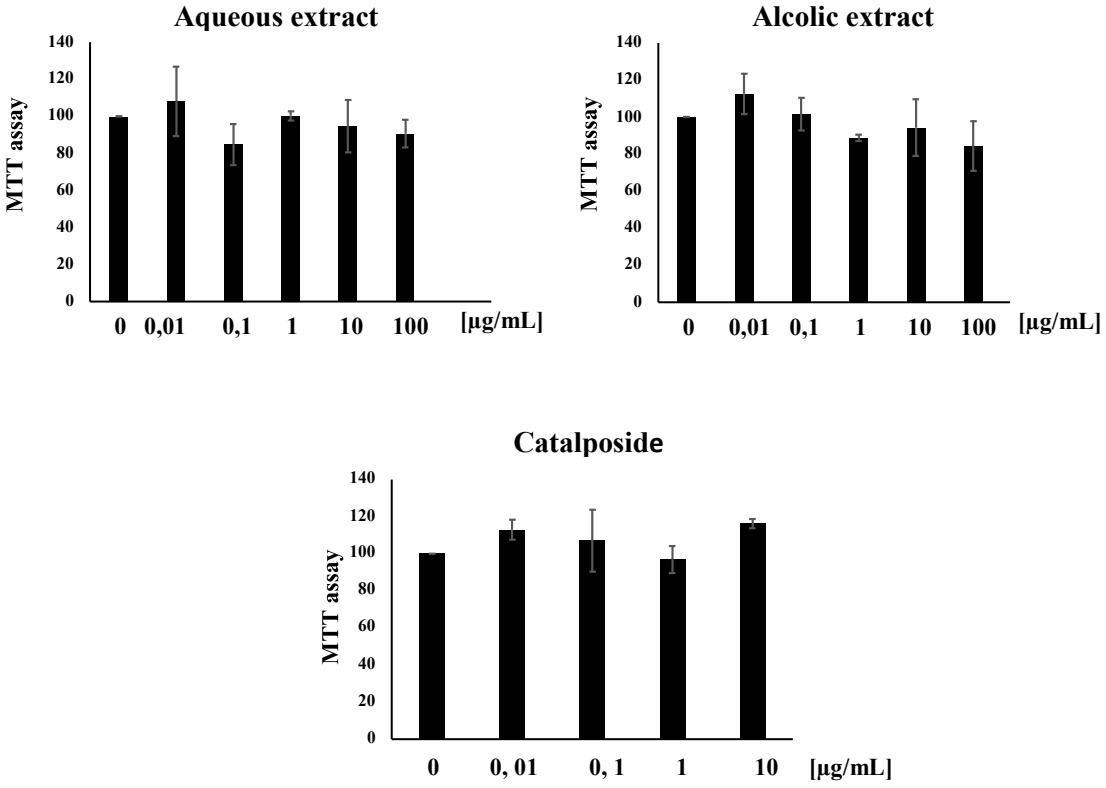
It has been demonstrated that inflammation (Terzić et al., 2010) as well as the alteration of some signaling pathways, including that of EGF (Krasinskas, 2011), contribute to the development of colorectal cancer, representing a specific target for colon cancer therapeutic intervention.

In this work, it has been evaluated the antitumor potential of *C. bignonioides* extracts by determing their effect on the modulation of cell growth, the IL-1β and EGF-induced ERK 1/2 activation, and the expression of enzymes related to inflammation in colon cancer cell models (HT-29 and HCT-116).

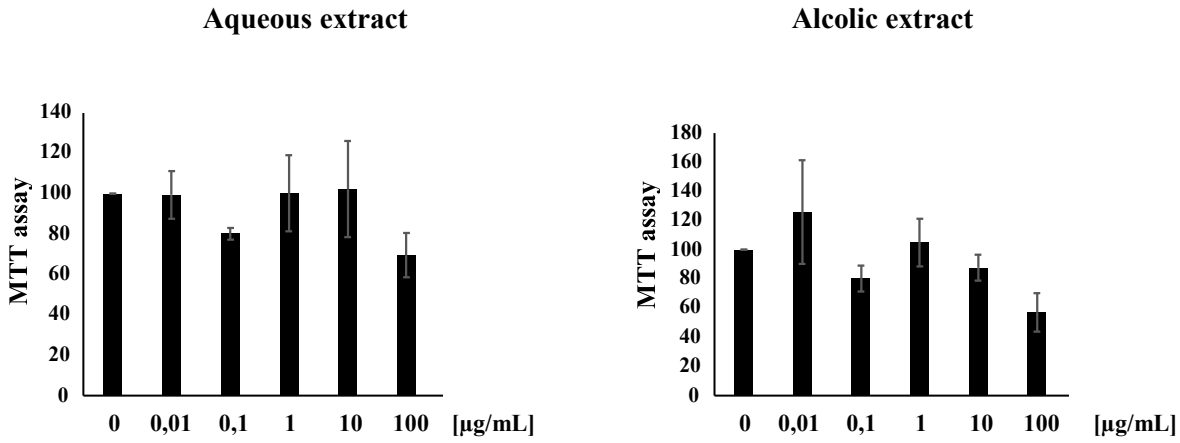
The aqueous and the butanol extracts, and the pure catalposide were initially used in a MTT assay to evaluate their toxicity on HT-29 and HCT-116 cells. For this purpose, increasing concentrations were used for the aqueous and alcoholic extract (0.01, 0.1, 1, 10, 100 µg/mL) and for the pure catalposide (0.01, 0.1, 1, 10 µg/mL). The different concentrations used for pure catalposide are justified by the fact that it represents 10% of the concentration of the

extracts, consequently the concentrations used are 10 times lower. The results have been reported in figure 18a for HT-29 and in figure 18b for HCT-116.

a)



b)



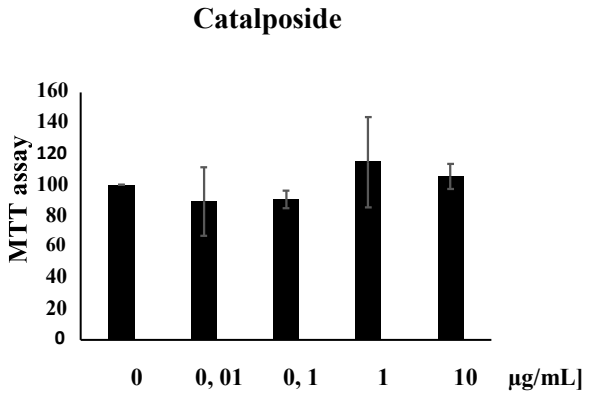


Figure 18. Determination of cellular toxicity of eaC, eohC and pC. (a) HT-29 and **(b)** HCT-116 cells were treated with the aqueous (eaC), butanol (eohC) extract, and pure catalposide (pC) at different concentrations and analyzed by MTT assay. The results were expressed as a percentage of baseline conditions.

As shown in figure 18, for both cell lines the aqueous and butanolic extract of *C. bignonioides* and the catalposide are not toxic, for all the concentrations used, since the cell vitality has not been significantly altered compared to the control conditions.

Having verified the non-toxicity of the aqueous, butanolic and pure catalposide extracts, their activity in modulating the ability of cells to form clones was subsequently studied both in basal and in protumoral conditions of inflammation and induction of cell proliferation induced by IL -1β and EGF. For this purpose, a Clonogenic assay was performed using HT-29 cells. Cells were seeded in 6-well plates at a density of 750 cells/well. After 24h, the cells were first treated for 1h with aqueous or butanol extract, at concentrations of 10 and 100 µg/mL and with catalposide at concentrations of 1 and 10 µg/mL. After that, some cells were treated with IL-1β 10 ng/mL or with EGF 25 ng/mL. They stayed in an incubator for 10 days at 37°C after which they were fixed, stained and counted. As shown in Figure 19, both the aqueous and the alcoholic extract induced a significant reduction in the formation of colonies, demonstrating an inhibitory action on tumor growth. Pure catalposide, at both concentrations used, showed no activity.

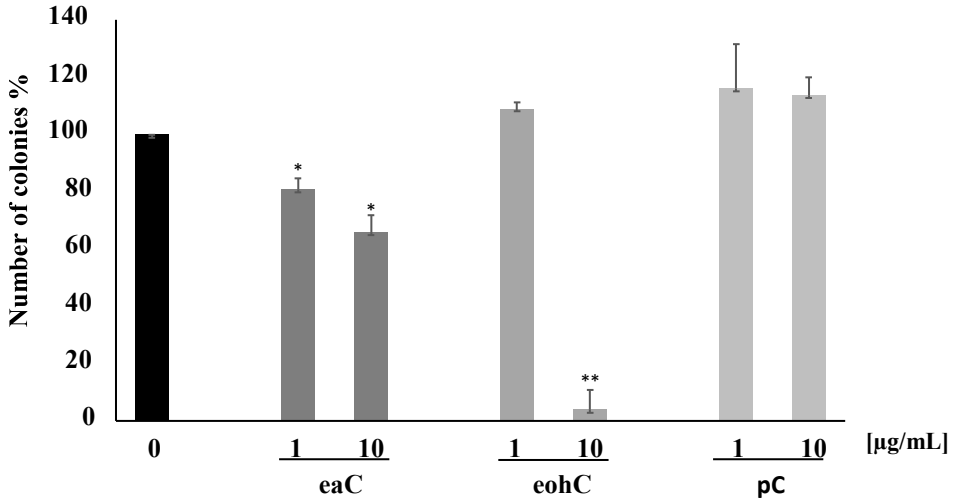


Figure 19. **Evaluation of cell growth of HT-29 cells by Clonogenic assay.** HT-29 cells were treated with the aqueous (eaC) and butanol extract (eohC) at concentrations of 10 and 100 µg/mL and with catalposide (pC) at concentrations of 1 and 10 µg/mL and analyzed by clonogenic assay. The results were expressed as the percentage of colonies formed, compared to the control. The results shown are the mean of three different experiments performed in duplicate and are expressed as mean ± SD (*p<0,05; **p<0,01).

The results obtained following treatment with IL-1β 10 ng/mL and EGF 25 ng/mL are shown, in figure 20 and figure 21 respectively.

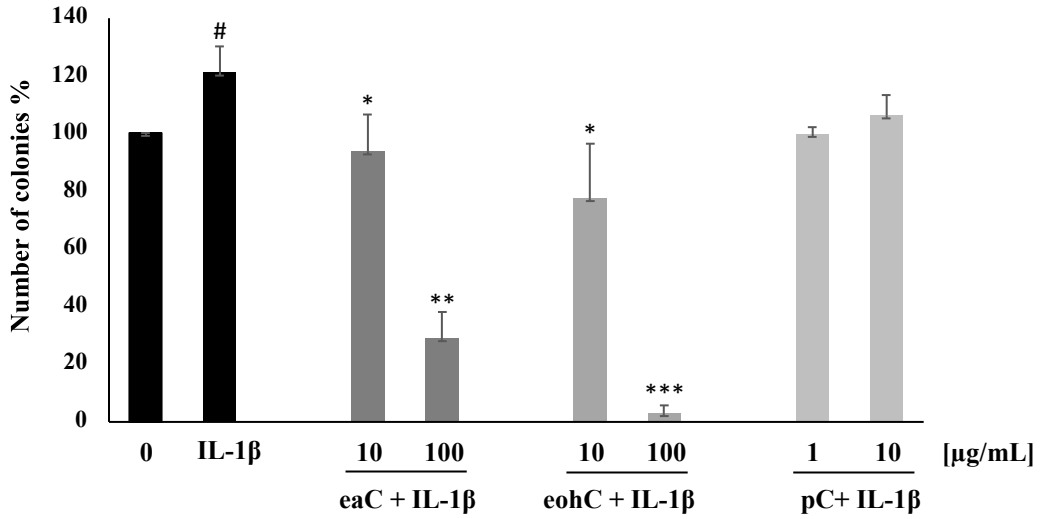


Figure 20. **Evaluation of cell growth of HT-29 cells by Clonogenic assy.** HT-29 cells, pretreated with aqueous (eaC) and butanol extract (eohC) at concentrations of 10 and 100 µg/mL and with catalposide (pC) at concentrations of 1 and 10 µg/mL, were exposed to IL-1β (10 ng/mL) and analyzed by Clonogenic assy. The results were expressed as the percentage of colonies formed, compared to the control. The results shown are the mean of two different experiments performed in duplicate and are expressed as mean ± SD (#p<0,05; *p<0,05; **p<0,01; ***p<0,001).

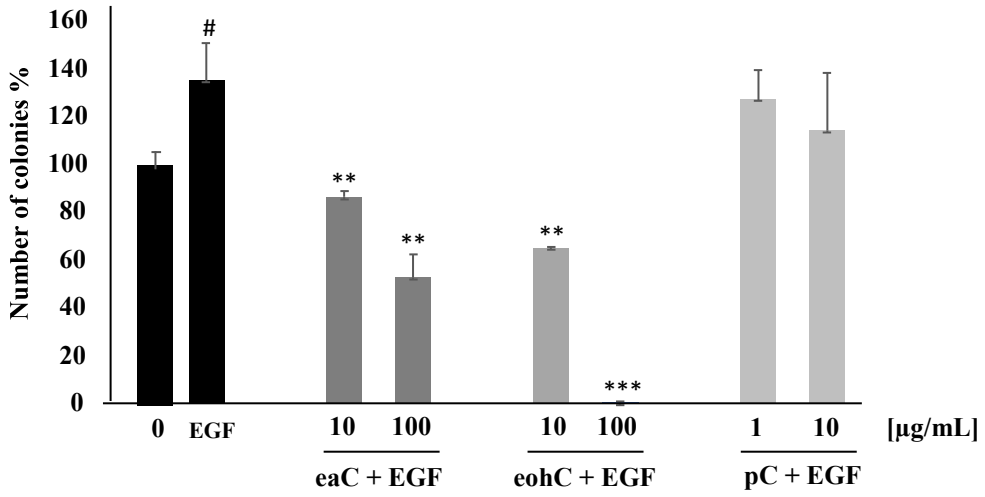


Figure 21. **Evaluation of cell growth of HT-29 cells by Clonogenic assay.** HT-29 cells, pretreated with aqueous (eaC) and butanol extract (eohC) at concentrations of 10 and 100 µg/mL and with catalposide (pC) at concentrations of 1 and 10 µg/mL, were exposed to EGF (25 ng/mL) and analyzed by Clonogenic assay. The results were expressed as the percentage of colonies formed, compared to the control. The results shown are the mean of two different experiments performed in duplicate and are expressed as mean ± SD (#p<0,05; **p<0,01; ***p<0,001).

The data reported in figure 20 and in figure 21 indicate that in the experimental conditions used, both the aqueous and the butanol extracts are able to inhibit the colony formation induced by IL-1β and EGF although with different entities. Pure catalposide, at both concentrations, does not inhibit colony formation.

The results therefore suggest an inhibitory activity of the aqueous and butanol extracts on IL-1β and EGF-induced tumor growth, suggesting a possible anti-inflammatory and antiproliferative activity of the mixtures under study.

On the basis of the data obtained and in order to further investigate the activity of the Catalpa extracts and of the catalposide, further experiment have been performed to evaluate the effect on the modulation of the expression of COX-2, the main enzyme involved in inflammation, and on the modulation of the phosphorylation of ERK 1/2, a signal pathway that plays a fundamental role in the regulation of cell proliferation and the expression of pro-inflammatory enzymes, induced by IL-1β and EGF.

For this purpose, HT-29 cells were plated in 6-well multiplates and after 24h they were first treated for 1h with the extracts at a concentration of 100 µg/mL and with pure catalposide at a concentration of 10 µg/mL. Where indicated, cells were treated with 10 ng/mL IL-1β or 25 ng/mL EGF for 48h, depending on the type of signal to be studied. The cell lysates obtained were subsequently analyzed by Western blotting.

As shown in figures 22 and 23, both the aqueous and the butanol extracts are able to significantly reduce the expression of COX-2 following stimulation with IL-1β and EGF. Different results were observed for pure catalposide which exerts a significant action in the reduction of COX-2 expression induced by IL-1β (Fig. 22) but not by EGF (Fig. 23).

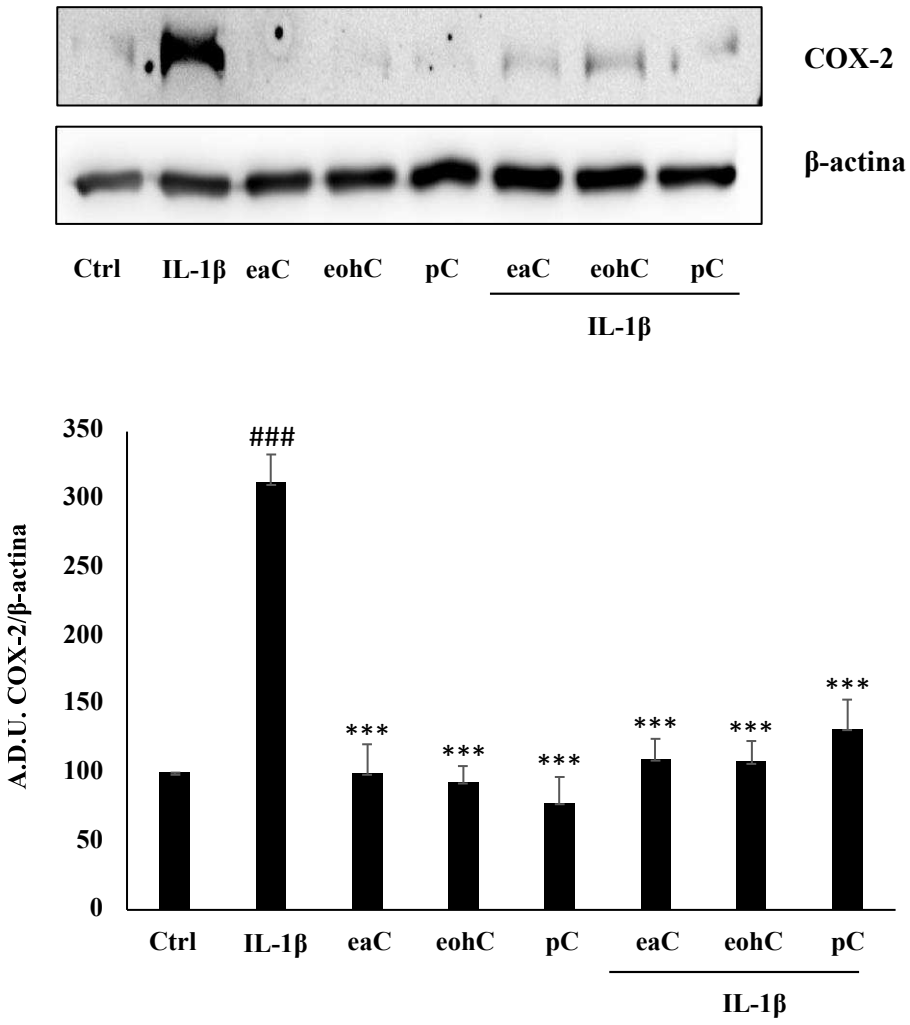


Figure 22. Evaluation of the effect of eaC, eohC and pC on COX-2 expression in the presence of IL-1β. HT-29 cells were treated with eaC and eohC at a concentration of 100 µg/mL and with pC at a concentration of 10 µg/mL. Where indicated the cells were stimulated with IL-1β (10 ng/mL). The image is representative of three experiments. The intensity of the immunoreactive bands was determined using the ImageJ program and expressed as A.D.U. (arbitrary densiometric unit) compared to the control. The data are expressed as a percentage of the control, refer to the mean obtained from three experiments and are expressed as mean ± SD (###p<0,001; ***p<0,001).

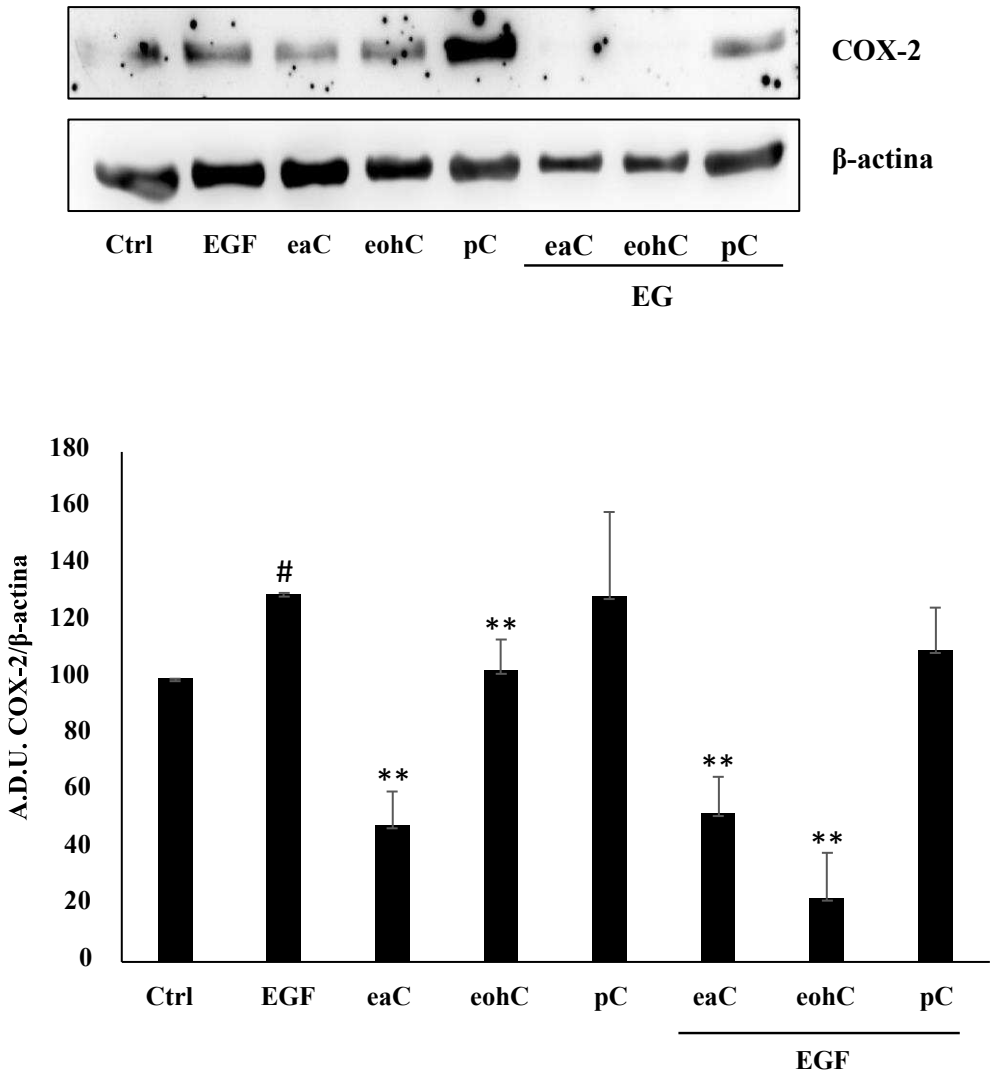


Figure 23. **Evaluation of the effect of eaC, eohC and pC on COX-2 expression in the presence of EGF.** HT-29 cells were treated with eaC and eohC at a concentration of 100 µg/mL and with pC at a concentration of 10 µg/mL. Where indicated they were stimulated with EGF (25 ng/mL). The image is representative of three experiments. The intensity of the immunoreactive bands was determined using the ImageJ program and expressed as A.D.U. (arbitrary densiometric unit) compared to the control. The data are expressed as a percentage of the control, refer to the mean obtained from two experiments and are expressed as mean ± SD (#p<0,05; **p<0,01).

Based on the previous data, the activity of the different samples on ERK1/2 activation was also investigated. The results shown in Figures 24 and 25 indicate that all the samples under investigation inhibit ERK1/2 phosphorylation induced by both IL-1β and EGF.

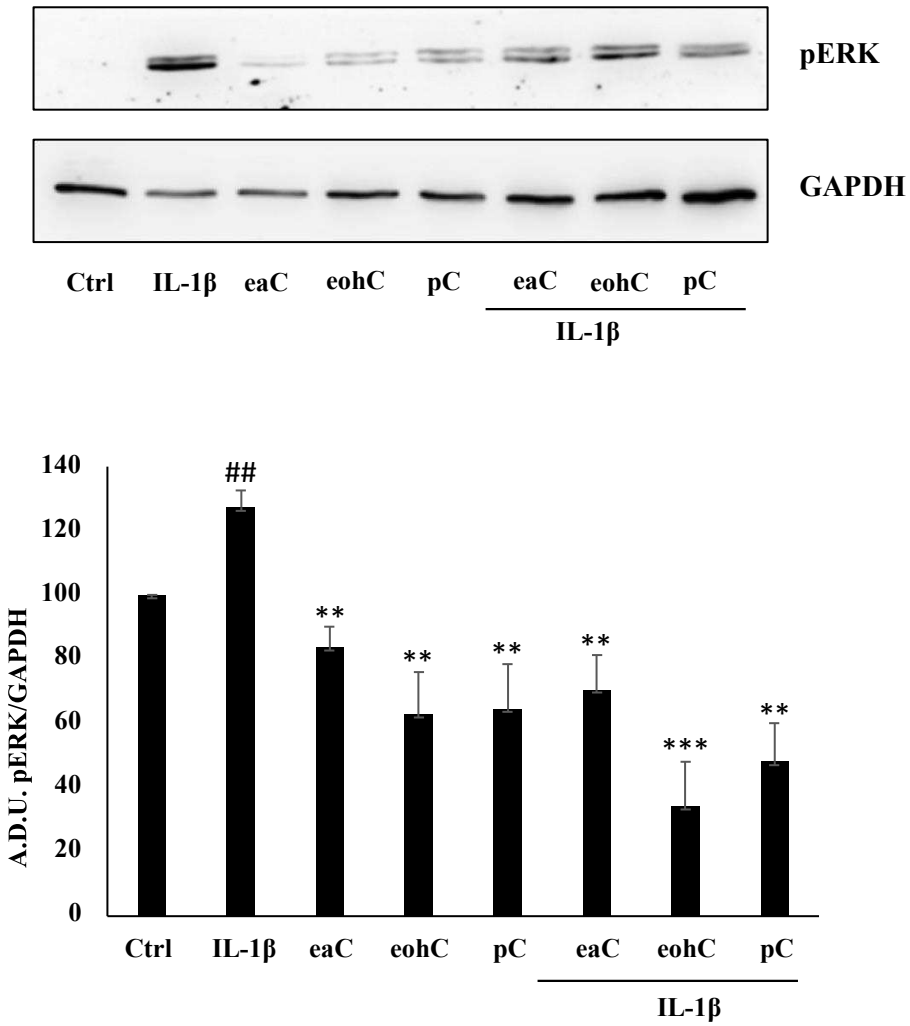


Figure 24. Evaluation of the effect of eaC, eohC and pC on IL-1β-induced ERK 1/2 phosphorylation. HT-29 cells were treated with eaC and eohC (100 µg/mL) and with pC (10 µg/mL) for 1 h and, where indicated, they were stimulated with IL-1β (10 ng/mL) for 15min. The image is representative of two experiments. The intensity of the immunoreactive bands was determined using the ImageJ program and expressed as A.D.U. (arbitrary densiometric unit) compared to the control. The data are expressed as a percentage of the control, refer to the mean obtained from three experiments and are expressed as mean ± SD (##p<0,01; **p<0,01; ***p<0,001).

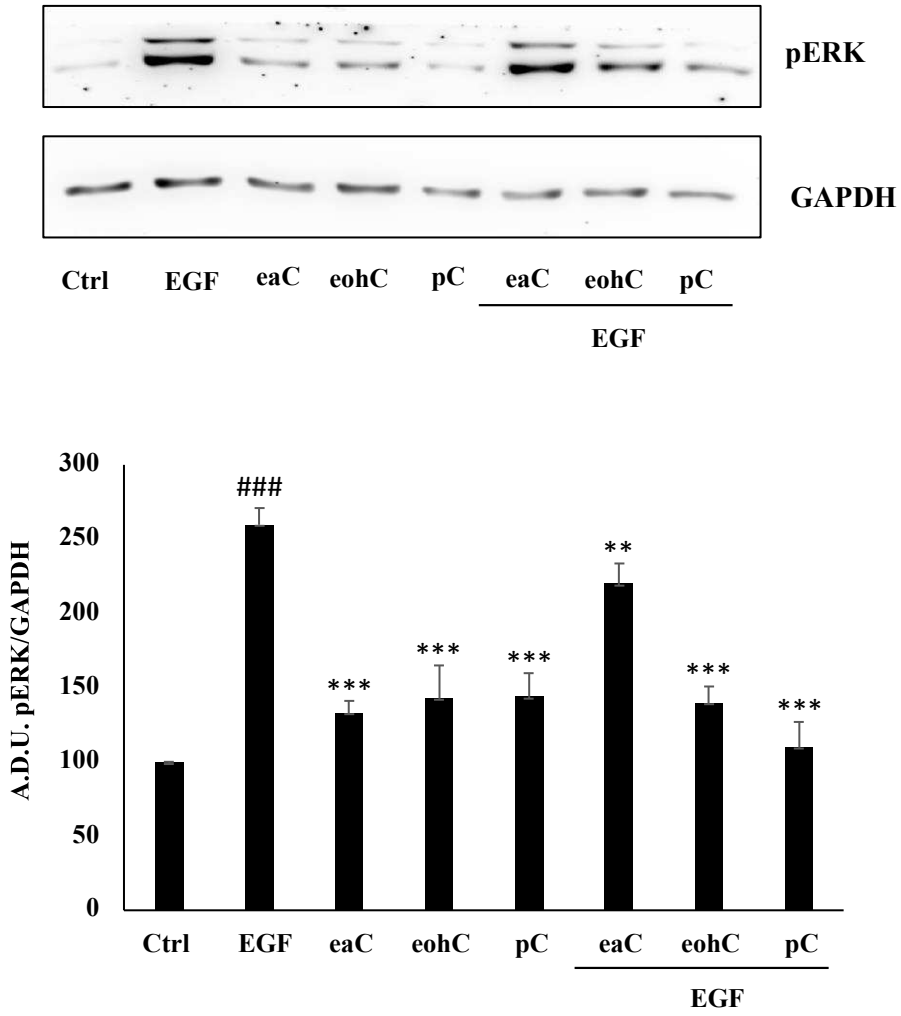


Figure 25. **Evaluation of the effect of eaC, eohC and pC on the expression of ERK 1/2 in the presence of EGF.** HT-29 cells were treated with eaC and eohC (100 µg/mL) and with pC (10 µg/mL) for 1 h and, where indicated, were stimulated with EGF (25 ng/mL). The image is representative of three experiments. The intensity of the immunoreactive bands was determined using the ImageJ program and expressed as A.D.U. (arbitrary densiometric unit) compared to the control. The data are expressed as a percentage of the control, refer to the mean obtained from two experiments and are expressed as mean ± SD (###p<0,001; **p<0,01; ***p<0,001).

Taken together, the results obtained in this work strongly suggest a potential antitumor action exerted by *C. bignonioides* extracts on *in vitro* models of colon cancer.

2.2. *P. vulgaris* var. Venanzio

In the present work, the "Fagiola di Venanzio" (FV), a recently identified Italian variety of *P. vulgaris*, was analyzed for the first time, with the aim of characterizing its chemical composition and potential biological activities. Initially, four different samples of beans grown in four different areas within Murlo, a small town in southern Tuscany, were analysed. The areas of origin of the four samples are quite close to each other, but differ in soil composition and exposure.

2.2.1. Chemical composition of FV extracts

Chemical analysis of aqueous extracts obtained from four different FV samples (Table 6) was focused on the determination of the polyphenolic fraction. To identify the main polyphenolic constituents, a high-performance liquid chromatography-diode array detection (HPLC-DAD) analysis was carried out. FV was found to be rich in polyphenols (Table 6), including phenolic acids and hydroxycinnamic derivatives, in accordance with published papers regarding *P. vulgaris*.

Table 6. Polyphenolic composition of FV extracts.

Components	**Quantification (mg/g)**			
	#1	**#2**	**#3**	**#4**
Total polyphenols	0.142 ± 0.018	0.123 ± 0.011	0.129 ± 0.016	0.120 ± 0.015
Total hydroxycinnamic derivatives	0.054 ± 0.004	0.051 ± 0.003	0.046 ± 0.005	0.052 ± 0.005
Isoflavones	<0.005	<0.005	<0.005	<0.005

HPLC-DAD analysis revealed that two main polyphenols subclasses can be identified in FV extracts, namely simple phenolic acids and hydroxycinnamic derivatives. As a water extraction was performed, the prevalence of hydrophilic compounds in the extract was expected and consistent with a previous work on common beans endemic of Southern Italy. Gallic acid (Figure 26 related to #1 FV extract, Retention Time, RT = 4.41 min) and chlorogenic acid (RT = 7.94 min), resulted the main phenolic and hydroxycinnamic acid, respectively. The other main peaks before gallic acid (RT = 3.88–4.18 min) could be assigned to phenolic acids by monitoring UV spectra for their typical λmax at 270–280 nm. Other hydroxycinnamic derivatives, different from caffeic acid, were recognized by UV spectra (λmax at 270–280 nm and 320–330 nm) and linked with the peak at 6.47 min. Flavonoids were found only in small amounts. At RT = 11.33, 11.71 and 12.40 min, the zone of the chromatogram where isoflavones are recorded, two constituents with UV spectrum similar to genistein and daidzein (λmax at 250–255 nm), but with different RTs, were found. Other flavonoids referable to the standards

and their derivatives were not detected as present in concentrations below the detection limits of the method. Table 7 shows the quantification of gallic and chlorogenic acid identified in FV extracts and total phenolic and hydroxycinnamic derivatives, expressed as gallic and chlorogenic acid, respectively.

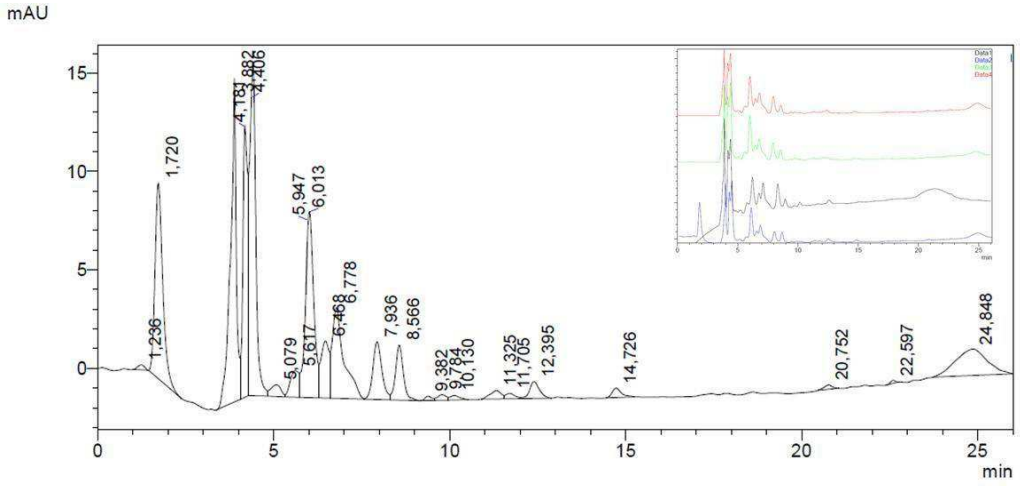


Figure 26. HPLC-DAD profile of polyphenols in FV extract. Chromatogram of sample #1. In the top box, chromatograms of all samples are reported: blue = sample #1, grey = sample #2, green = sample #3 and red = sample #4.

Table 7. Chemical composition of #1 FV extract.

Composition	Quantification
Total polyphenols	0.131 ± 0.016 mg/g
Total hydroxycinnamic derivatives	0.046 ± 0.004 mg/g
Gallic acid	0.052 ± 0.005 mg/g
Chlorogenic acid	0.011 ± 0.002 mg/g
Isoflavones	<0.005 mg/g
Total soluble carbohydrates	10.032 ± 0.820 mg/g
Total proteins	15.190 ± 2.020 mg/g

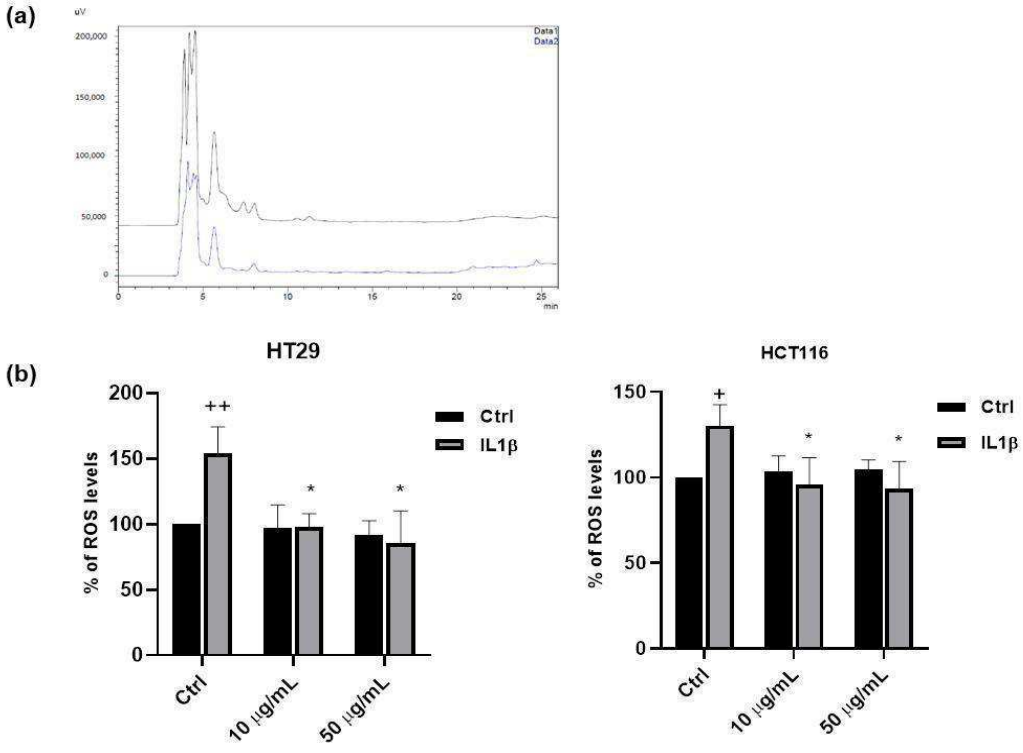
Due to the minimum variance in polyphenols content and a strong similarity in chromatogram, profiles of the four different samples (see Table 6 and Figure 26), only the extract obtained from sample #1, available in the highest amount, was further analyzed and investigated in chemical and biological tests. Table 7 summarizes the chemical composition of the selected FV extract. In accordance with known nutritional data and literature on *P. vulgaris* (nutritiondata.com), the concentration of soluble carbohydrates in the FV extract was shown to be 10.032 mg/g. The concentration of total proteins was 15.190 mg/g.

2.2.2. Antioxidant properties of FV extract

In is known that the beneficial effects of polyphenols on human health are expressed mainly through the reduction of oxidative stress. It has also been reported that extracts of common beans obtained from different Italian varieties of *P. vulgaris* possess antioxidant and antiradicalic properties. According to that, the antioxidant activity of the FV extract was initially evaluated by DPPH test.

The outcomes of this experiment indicated that the FV extract possesses antiradicalic activity, monitored by DPPH reduction. The comparison between the HPLC-DAD chromatograms obtained before and after adding DPPH to FV extract showed that differences in peak areas related to polyphenols occur (Figure 27a), thus demonstrating that some constituents of FV extract were able to react with DPPH and underwent oxidative degradation.

Chlorogenic acid was the most degraded molecule, more than 75.0% after DPPH reaction, meaning that this hydroxycinnamic derivative primarily contributed to the antiradicalic activity of FV extract. The other main hydroxycinnamic derivative displayed a minor degradation (-39.4%); among phenolic acids, only gallic acid seemed to participate in DPPH reaction and its recorded degradation was 37.6%. Other polyphenolic constituents of FV extract did not show a significant degradation after DPPH reaction.



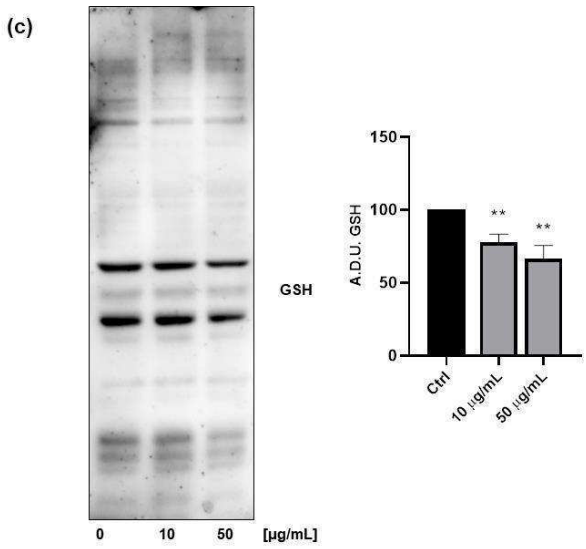


Figure 27. Antioxidant activity of FV extract. (a) HPLC-DAD-DPPH test. Chromatograms showed the differences in peak areas between the chromatograms obtained before (black) and after (blue) mixing DPPH and FV extract. **(b)** ROS measurement in HT-29 and HCT-116 cells after 18h of exposure with the different concentrations of FV extract (10–50 µM) followed by a 24h incubation with IL-1β (10 ng/mL). Data are expressed as relative fluorescence units (++ $p < 0.01$ vs. Ctrl; + $p < 0.05$ *vs*. Ctrl; * $p < 0.05$ vs. IL-1β). **(c)** Evaluation of the levels of glutathionylated proteins. HT-29 cells were treated with different concentrations of FV extract (10–50 µM) for 36h and then analyzed by western blot under non reducing conditions using an anti-GSH primary antibody. The gels are representative of three independent experiments (A.D.U.: arbitrary densitometry units). ** $p < 0.01$ vs. Ctrl.

To further analyze the antioxidant properties of FV extract, *in vitro* experiments were performed on cellular models. Due to the high nutraceutical impact and to the findings that common bean consumption is associated with *in vivo* chemoprotective effects at the early stages of colon cancer and with pro-apoptotic and anti-proliferative activities *in vitro*, two different cell models were selected of colorectal adenocarcinoma, HT-29 and HCT-116 cells. To mimic a pro-oxidant and pro-inflammatory milieu, colon cancer cells were stimulated with interleukin 1β (IL-1β, 10 ng/mL, 48h) in the presence and in the absence of different concentrations of FV extract (10, 50 µg/mL), and ROS levels were measured using the $DCFH_2$-DA assay. As reported in Figure 27b, the ability of IL-1β to promote ROS production was inhibited by the FV extract, at both concentrations and in both cell lines. It is well known that, in addition to triggering traditional post-translational protein modifications (including phosphorylation, acetylation, ubiquitination, etc.), ROS can directly modify cellular proteins, adding another layer of protein regulation to the proteome classified as oxidative post-translational modifications (OPTMs). In particular, ROS may cause various types of chemical modifications of proteins, including glutathionylation. To explore the possibility that FV extract could affect these mechanisms, colon cancer cells were treated with the FV extract for 36 h and the levels of glutathion–protein complexes were measured by western blotting.

Figure 27c shows that the FV extract was able to significantly reduce the levels of total glutathionylated proteins, indicating the antioxidant activity of the FV extract.

2.2.3. Anti-Inflammatory properties of FV extract

To further explore the biological properties of the FV extract, its ability to reduce the inflammation related to cancer was investigated. It is well known that inflammation is a key component in colon cancer onset and progression and that the COX-2 pathways play a major role in modulating cell growth, apoptosis and EMT. Recent reports indicate that a direct interplay exists between inflammation and carcinogenesis. In fact, the risk of developing colon cancer is increased by chronic inflammatory diseases (such as inflammatory bowel disease), chronic infections or inflammations caused by environmental exposures. In addition, administration of COX inhibitors, such as aspirin and other non-steroidal, anti-inflammatory drugs (NSAIDs), relates to a lower risk of developing colon cancer and its recurrence.

IL-1β, a pro-inflammatory cytokine, has shown to induce COX-2 expression in colorectal cells and that COX-2 drives colon cancer progression. In this context, the activity of FV extract on COX-2 expression induced by IL-1β was evaluated. HT-29 cells were stimulated with IL-1β (10 ng/mL, 48h), in presence of 10 and 50 µg/mL of FV extract and it was observed that at the higher concentration FV extract was able to strongly inhibit IL-1β-induced COX-2 expression (Figure 28a). These data clearly indicate that FV extract could reduce the inflammation related to cancer.

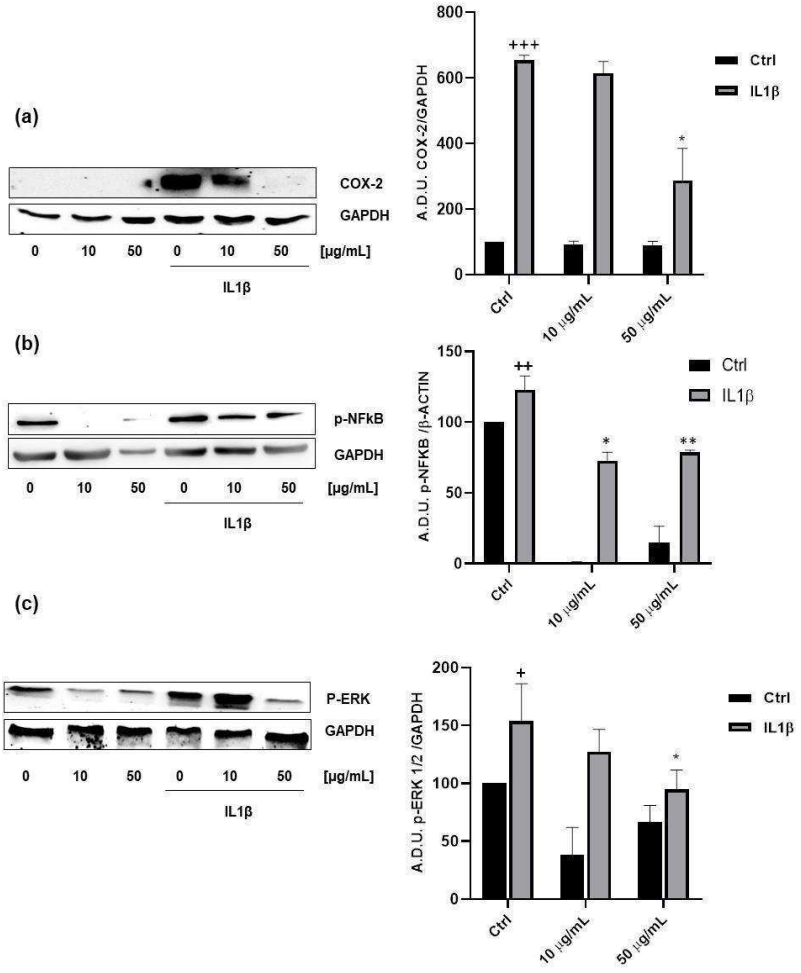


Figure 28. Anti-inflammatory activity of the FV extract. (a) Western blot analysis and quantification of COX-2 expression in HT-29 cells after 18h of exposure with different concentrations of FV extract (10–50 µM) followed by a 24h incubation with IL-1β (10 ng/mL). +++ $p < 0.001$ vs. Ctrl, * $p < 0.05$ vs. IL-1β. (b) Western blot analysis and quantification of NFκB phosphorylation in HT-29 cells after exposure with FV extract (10–50 µM) followed by incubation with IL-1β (10 ng/mL). ++ $p < 0.01$ vs. Ctrl, **$p < 0.01$ vs. IL-1β, * $p < 0.05$ vs. IL-1β (c) Western blot analysis and quantification of ERK 1/2 phosphorylation in HT-29 cells. (A.D.U.: arbitrary densitometry units). + $p < 0.05$ vs. Ctrl, * $p < 0.05$ vs. IL-1β. The gels showed in the figure are representative of four independent experiments.

It has been described that natural compounds, and in particular dietary polyphenols, exhibit a relevant anti-inflammatory activity linked to the inhibition of NFκB, MAPK and iNOS signalling. In this light, the activity of the FV extract on NFkB and MAPK activation was studied. By analysing the phosphorylation levels of NFkB and ERK1/2, it was demonstrated that the FV extract was able to reduce both p-NFkB and p-ERK1/2 levels in IL-1β-stimulated colon cancer cells (Figure 28 b,c), indicating once again an anti-inflammatory activity of FV components.

It has been measured the ROS levels and COX-2 expression in non-cancer cells. To this aim, HUVECs were used as a model in which it is well known the activity of IL-1β in inducing ROS production and COX-2 expression. As reported in Figure 28, while IL-1β treatment induced

both ROS production and COX-2 expression, FV extract was shown to be inactive (Figure 29 a,b), indicating a potential selective role of FV extract in cancer cells.

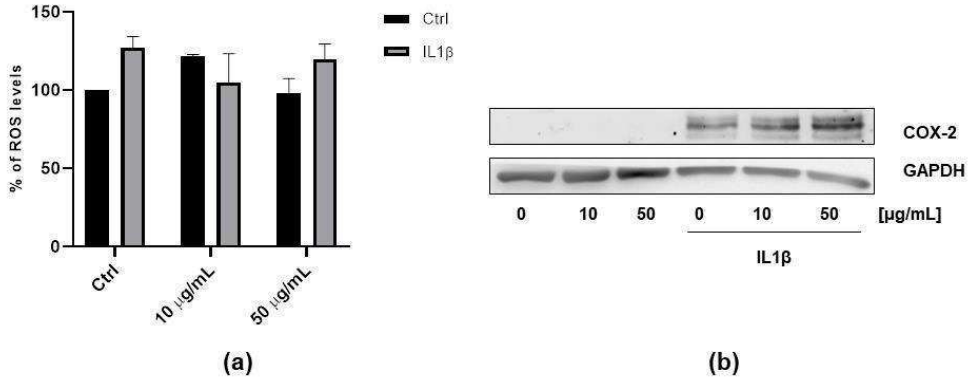


Figure 29. FV extract activity in endothelial cells. (a) ROS measurement in HUVEC cells after 18h of exposure with the different concentrations of FV extract (10–50 µM) followed by a 24h incubation with IL-1β (10 ng/mL). Data are expressed as relative fluorescence units. **(b)** Western blot analysis of COX-2 expression in HUVEC cells after 18h of exposure with different concentrations of FV extract (10–50 µM) followed by a 24 h incubation with IL-1β (10 ng/mL).

2.2.4. Anti-proliferative activity of F.V extract

To evaluate whether the FV extract could modulate tumor progression, the proliferation of colon cancer cells was studied by MTT test. HT-29 and HCT-116 were treated with 10 ng/mL IL-1β for 48h in the presence of increasing concentrations of the FV extract (from 5 to 100 µg/mL). According to previous studies conducted on white beans, the FV extract did not modify cancer cells' growth in basal conditions. However, when the cells were exposed to an inflammatory milieu (IL-1β), FV extract was able to reduce cell growth in a concentration-dependent manner (Figure 30 a,b). Taken together, these data strongly support a potential biological activity of the FV extract, especially in inflammatory conditions.

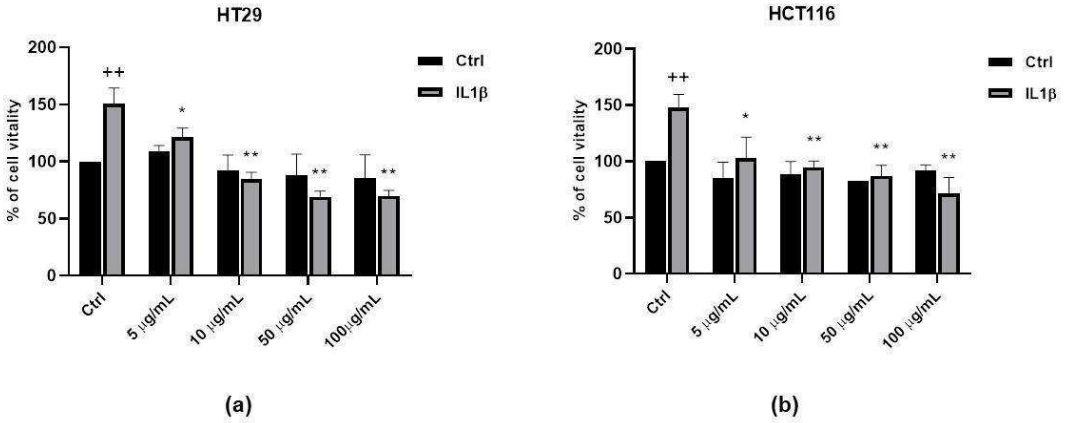


Figure 30. FV extract inhibits colon cancer cell growth. Cell proliferation induced by IL-1β (10 ng/mL) in the presence or absence of FV extract (5, 10, 50 and 100 µg/mL) was measured by MTT assay. HT-29 **(a)** and HCT-

116 **(b)** cells were exposed to IL-1β for 48h. Data are reported as % of cell viability and are the means of 4 experiments run in triplicate. ++ $p < 0.01$ vs. Ctrl, * $p < 0.05$ and ** $p < 0.01$ vs. IL-1β.

One of the main limitations of *in vitro* studies on the biological activity of foods or food extracts is due to the fact that the common extraction methods differ considerably from the transformations that the food undergoes in the digestive tract following the ingestion. In this work, a protocol was developed that simulates the traditional bean cooking procedures and their digestion by the gastrointestinal tract. As described in the Materials and Methods section, the soaking water (AA), the cooking water (AC) and the digested fraction (Dig) were prepared with this procedure. The activity of the three FV fractions against two colorectal cancer cell lines, HT-29 and HCT-116, and a human fibroblast (FU) cell line was subsequently evaluated. The three FV samples were initially used in an MTT assay to evaluate the effects on cell viability of HT-29 and HCT-116 cells. Three different concentrations of each sample were used for this purpose: 1, 10 and 100 µg/ml. The results are reported in Figure 31 (HT-29 cells) and in Figure 32 (HCT-116 cells).

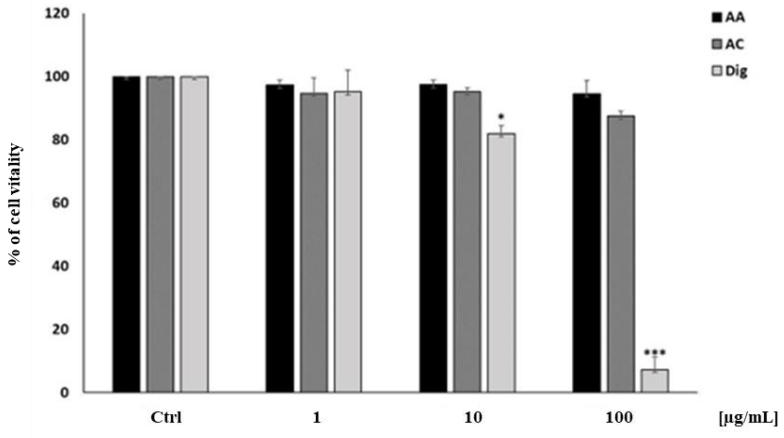


Figure 31. **Determination of cell viability by MTT-HT-29 cell assay.** HT-29 cells were treated with the three FV samples: soaking water (AA), cooking water (AC), digested fraction (Dig) and analyzed by MTT assay. The results were expressed as a percentage of cell viability, compared to baseline conditions. The results shown are the mean of two different experiments and are expressed as mean ± SD (* p< 0.001).

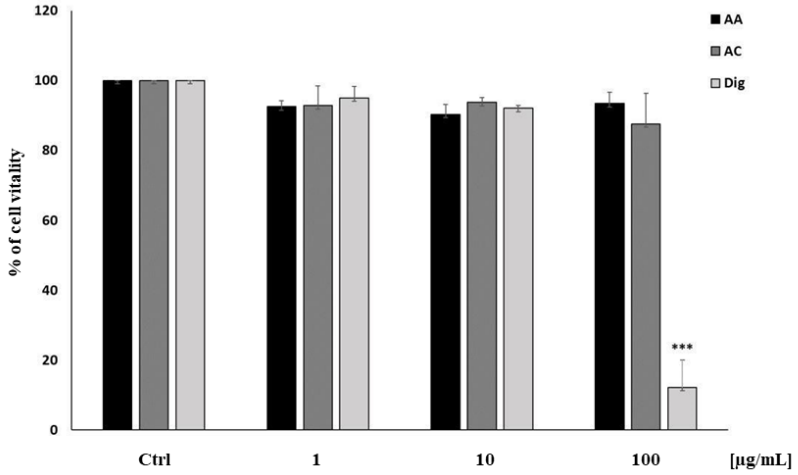


Figure 32. **Determination of cell viability by MTT-HCT-116 cell assay.** HCT-116 cells were treated with the three FV samples: soaking water (AA), cooking water (AC), digested fraction (Dig) and analyzed by MTT assay. The results were expressed as a percentage of cell viability, compared to baseline conditions. The results shown are the mean of two different experiments and are expressed as mean ±SD (***$p < 0.001$).

The soaking water (AA) and the cooking water (AC) did not significantly reduce cell viability in the two cell lines compared to the control for all concentration conditions used, while the digested fraction (Dig) at the concentration of 100 µg/ml reduced the viability by 93% compared to the control conditions. To exclude the possibility that the reduction in viability observed for Dig at the highest concentration was due to the experimental conditions used and linked in particular to the presence of digestive enzymes in the sample, HT-29 cells were treated with the mixture of reagents used for the digestion (digestive solution).

As can be seen in Figure 33, the digestive solution, at the highest concentration used, reduces cell viability by only 20%. Therefore, the effect observed in Figure 31 for HT-29 cells (about 70% of the reduction in viability) can be mainly attributed to the presence of specific molecules generated following the digestive process.

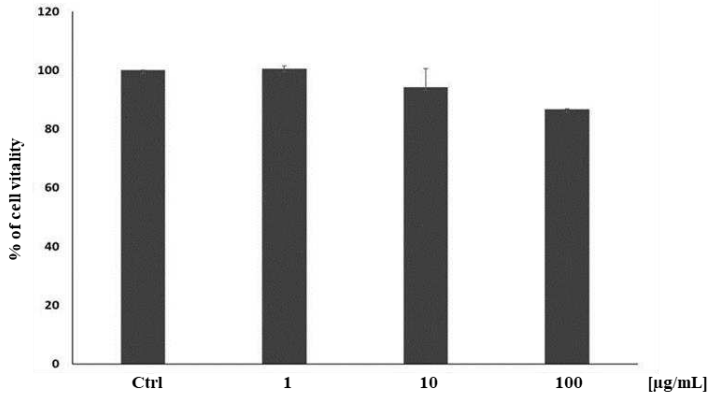


Figure 33. **Determination of cell viability by MTT-HT-29 cells assay.** HT-29 cells were treated with the digestive solution and analyzed by MTT assay. The results were expressed as a percentage of cell viability,

compared to baseline conditions. The results shown are the mean of two different experiments and are expressed as mean ± SD.

Furthermore, to verify whether the activity on cell viability of bean fractions was specific for tumor cells, untransformed human fibroblasts were used as a control cell line to perform an MTT assay with the same samples and under the same conditions used for the two tumor lines. As shown in Figure 34, the three FV samples, for all three concentrations used, did not produce significant changes in cell viability in human fibroblasts. These data indicate a selective toxicity towards tumor cells by the digested fraction, not possessed by the soaking water and the cooking water.

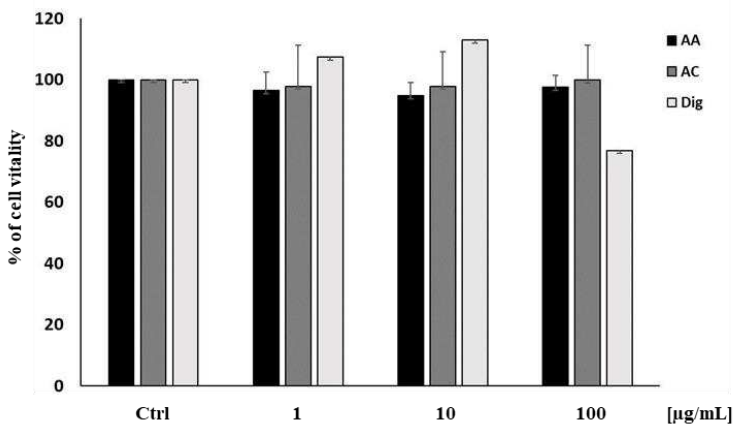


Figure 34. **Determination of cell viability by MTT-FU cells assay.** FU cells were treated with the three FV samples: soaing water (AA), cooking water (AC), digested fraction (Dig) and analyzed by MTT assay. The results were expressed as a percentage of cell viability, compared to baseline conditions. The results shown are the mean of two different experiments and are expressed as mean ± SD.

Taking note of these experiments and considering the possible inhibitory activity of Dig on the growth of tumor cells, its possible cytotoxicity was subsequently evaluated using the Trypan Blue assay. This test allows to discriminate the quantity of live and dead cells thanks to the characteristic of Trypan Blue to penetrate inside the dead cells, making them colored and clearly visible under the microscope. For this purpose, a suspension of HT-29 cells (5.0×10^5 cells/mL) was placed in microtubes and incubated with Dig, or with the digestive solution as control, at a concentration of 100 µg/mL. 48h after the treatment, the dye was added to the cells and live and dead cells were counted. The results were expressed as a percentage of dead cells, compared to the total number of cells. The data shown in Figure 35 indicate a low toxicity of Dig (about 20% of dead cells), while with the digestive solution about 10% of dead cells are obtained.

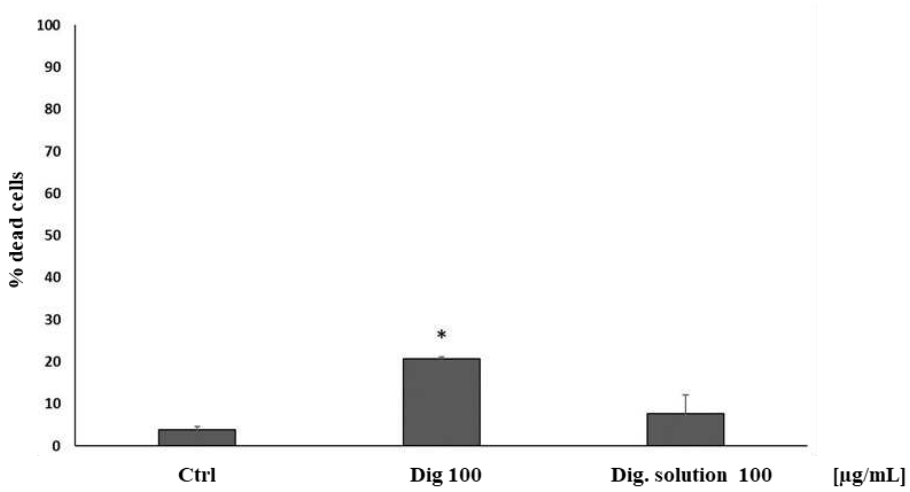


Figure 35. **Determination of the cytotoxicity of Dig in HT-29 cells.** HT-29 cells were treated with Dig and digestive solution and analyzed by Trypan Blue assay. Data were reported as the percentage of dead cells versus control conditions. The reported results are the mean of two different experiments and are expressed as mean ±SD (*$p < 0.05$).

HT-29 cells were used to perform a Clonogenic assay that evaluates the ability of a given substance to induce or interfere with the cells' ability to form clones. Cells were seeded in 24-multiwell plates, at a density of 300 cells/well; after 24h cells were treated with the three FV samples, at concentrations of 1, 10, 100 µg/mL and incubated for 10 days at 37°C. The cells were then fixed, stained and counted. The data shown in Figure 36 indicate that under the experimental conditions used, the Dig sample induced a concentration-dependent reduction in the colony-forming ability, suggesting a promising inhibitory activity of the FV digest against tumor cells.

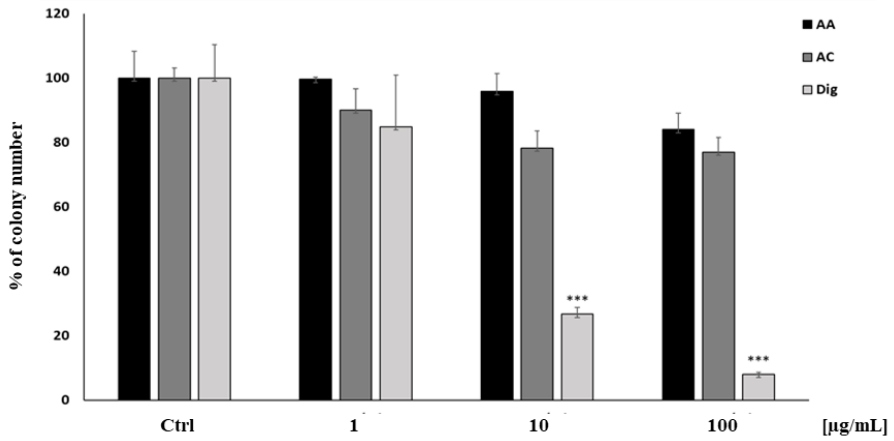
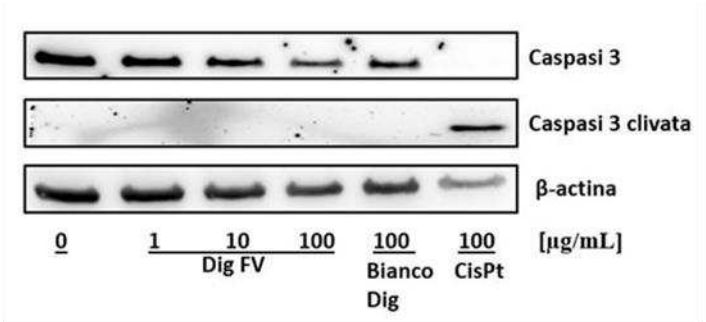


Figure 36. **Evaluation of cell growth of HT-29 cells by Clonogenic assay.** HT-29 cells were treated with different concentrations of the FV, soak water (AA), cook water (AC), and digest (Dig) samples and analyzed by Clonogenic assay. The results were expressed as the percentage of colonies formed, compared to the control. The

results shown are the mean of two different experiments performed in triplicate and are expressed as mean ± SD (***p < 0.001).

Finally, to study the possible molecular mechanisms involved in the functional response of cells exposed to Dig treatment, the effect of this FV fraction on the activation of caspase 3, an important regulator of cellular apoptotic processes, was evaluated.

For this purpose, HT-29 cells were seeded in in 6-well multiplates and after 24h they were treated for 48h with Dig at increasing concentration (1, 10, 100 µg/mL). Cells treated with 100 µg/mL of the digestive solution and with the same amount of cisplatin, a compound with known antitumor activity capable of activating apoptotic processes, were used as negative and positive control respectively. Cell lysates were subsequently analyzed for the expression of the activated form of caspase-3 (cleaved caspase-3) by western blotting. As shown in Figure 37 the Dig fraction does not induce activation of caspase-3 for any of the concentrations used.



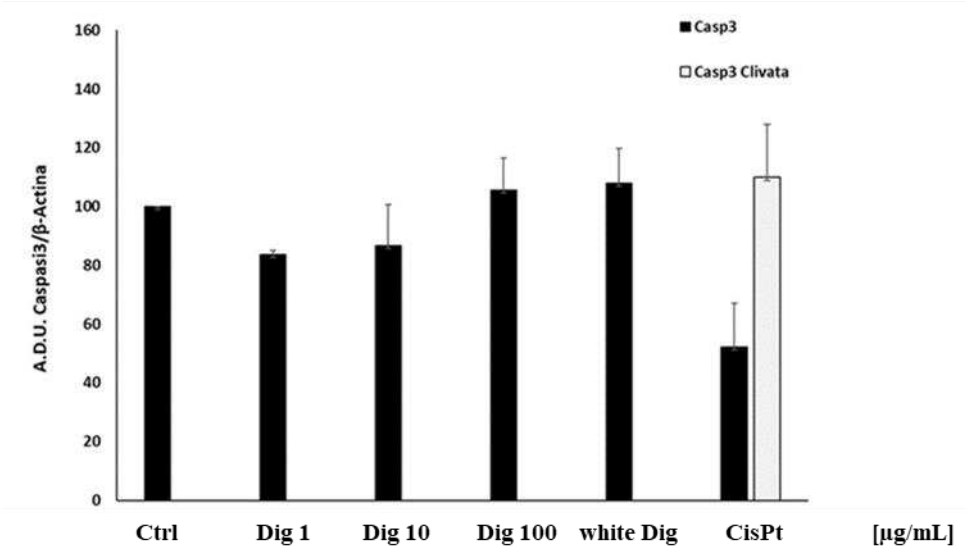


Figure 37. Evaluation of the effect of bean extracts on caspase 3 expression in HT-29 cells. HT-29 cells were stimulated with different concentrations of Dig, and with digestive solution and cisplatin as indicated. The lysates obtained were analyzed by Western Blotting to evaluate the activation of caspase 3. The intensity of the immunoreactive bands was determined using the ImageJ program and expressed as A.D.U. (arbitrary densitometric unit) versus the control. The data were expressed as a percentage of the control and refer to the mean obtained from two experiments ±SD.

IV. Conclusions

Cancer is one of the leading causes of death and a major obstacle to increasing life expectancy in every country in the world. Cancer Research UK reported that in 2021, 14.1 million adults were diagnosed with cancer and 8.2 million people were killed by cancer globally. Among the various types of cancer, colorectal cancer certainly figures as one of the most commonly diagnosed malignant neoplasms; overall, it ranks third in terms of incidence and second in terms of mortality. Therefore, the demand for a cure and the prevention of cancer is extremely high.
The current methods such as chemotherapy and radiotherapy have their limitations due to their toxic effects on non-targeted tissues furthering human health problems.
For this reason, in the last years scientific research has focused on the search for alternative treatments and tried to design and develop alternative, eco-friendly, biocompatible and cost-effective strategies.
Plants represent a rich source of molecules with multiple relevant biological properties often associated with minimal side effects. Many studies have therefore focused on the use of molecules of natural origin in the treatment of various types of cancer, including colorectal cancer, demonstrating their efficacy in tumor treatment and studying the molecular mechanisms underlying the pharmacological activity.
Phytochemicals are present in different parts of the plant, e.g., flowers, pericarp, sprouts, fruits, seeds, roots, rhizomes, stem, leaf, embryo, bark and perform several pharmacological functions. Several plant-derived compounds including alkaloids, flavonoids, lignans, saponins, terpenes, vitamins, minerals, glycosides, and other primary and secondary metabolites have been shown to possess anticancer activities which include antioxidant activity, inhibition of cancer cell growth, induction of apoptosis, target specificity, cancer cell cytotoxicity.
In this thesis, two different plants have been selected on the basis of their content in active ingredients and according to what reported in the literature regarding their antitumor potential. The first one is *Catalpa bignonioides,* available in the Botanical Garden of the Université Paris Cité, Faculté de Pharmacie de Paris, and very interesting for its high content in iridoids.
The chemical composition of three different extracts obtained from *C. bignonioides* fruits was determined by HPLC-DAD-MS analysis and the dosage of catalposide in the three extracts, using an internal standard. From chromatographic analyses, the extracts of *C. bignonioides* fruits consist of different phytochemical classes such as flavonoids, iridoids, phenylpropanoid glicosydes, phenolic acids, oligosaccharides and terpenoid quinones (naphthoquinones). In particular, 17 compounds have been identified and to confirm the presence of these compounds,

several HPTLCs were performed, using three different derivatisation reagents: vanillin, NP/PEG and DPPH that has showed potential antioxidant activities of the three extracts.

Then, the antitumor activity of the extracts was evaluated, together with the pure catalposide, which represents one of the main iridoids present in the fruits of this plant, using *in vitro* models of colorectal cancer (HT-29 e HCT-116 cells). In particular, it has been evaluated the effect of *C. bignonioides* extracts on the modulation of cell growth, the IL-1β and EGF-induced ERK 1/2 activation, and the expression of enzymes related to inflammation in colon cancer cell models (HT-29 and HCT-116). These results showed that the three extracts of *C. bignonioides* fruits and pure catalposide are not toxic for cells, but they are able to inhibit IL-1β and EGF-induced tumor growth, although with different extent.

In addition, the extracts showed to be able to affect COX-2 expression and ERK1/2 activation following stimulation with IL-1β and EGF, suggesting a possible anti-inflammatory and antiproliferative activity.

In the light of the data obtained, it is possible to establish for the first time a potential antitumor action exerted by the extracts of *C. bignonioides* fruits. This activity appears to be linked to the mixture of compounds present in the extracts and not to the pure catalposide. The results obtained in this work are promising and encourage to a more in-depth study in order to evaluate further anticancer properties and possible therapeutic applications of the Catalpa extracts.

The second plant that has been chosen in this work is the Fagiola di Venanzio (FV), an endangered bean grown in a restricted area of the municipality of Murlo (Siena, Tuscany), recently recognized and classified as a novel variety of *P. vulgaris* but never studied before.

The FV has shown, like the other varieties of the species, beneficial properties, including anti-radical activity *in vitro*, the ability to reduce the production of ROS (reactive oxygen species) and inhibition of colon cancer cell growth induced by pro-inflammatory stimuli. It is well known that IL-1β mimics inflammatory conditions that occur in intestinal tract and that may drive the development of such inflammatory chronic diseases as cancer, or such inflammatory bowel diseases (IBD) as ulcerative colitis and Crohn's disease.

The results of this work show that FV extract can reverse the effects of IL-1β on colon cancer cells, strongly suggesting that FV may play an important role in preventing the alteration of molecular processes characterizing the inflammatory microenvironment that leads to cancer and chronic diseases.

To better study the nutraceutical properties of this new bean variety, a protocol was developed that mimicked the traditional bean cooking procedures and their digestion by the gastrointestinal tract. The functional and molecular assays performed with the different FV fractions indicated that the fraction obtained following *in vitro* enzymatic digestion (Dig)

possesses selective activity against tumor cells and can reduce both cell viability and the formation of clones and colonies in models of colon cancer cells. This effect seems to be related to a non-apoptotic mechanism that needs to be further studied. The Dig properties, not possessed by the other fractions (soaking water and cooking water), can likely be attributed to the presence of specific molecules generated following the digestive process and which will be analyzed and identified in a subsequent study.

The results obtained in this work are very promising and indicate an excellent nutraceutical potential of the Fagiola di Venanzio. In general, this study further supports the beneficial effect on human health deriving from the presence of beans in the diet and stimulates the search for the molecules responsible for these health properties.

So, beans, typically known as poor foods and accessible to all, play a key role in nutraceuticals, showing healthy activities and arousing considerable interest in the scientific community.

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