Chapter 12

New Insights Regarding the Potential Health Benefits of Isoflavones

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Abstract

Isoflavones are a class of plant secondary metabolites, with an estrogen-like structure presenting a plethora of biological activities. The chapter discusses important facts about this class of phytoestrogens, from biosynthesis to the latest research about their health benefits. The following major points discussed are: biosynthesis, regulation, isolation, metabolism and bioavailability, isoflavones in diet and intake, and new insights regarding the therapeutic effect including cancer chemoprevention. The chapter ends with a mini review of own research of the anti-inflammatory and chemopreventive activity of isoflavonoid genistein alone and incorporated in modern pharmaceutical formulations. The chapter updates the interested researchers in the field with the latest progress regarding potential health benefits of isoflavones.

Keywords: isoflavones, biosynthesis, regulation, isolation, metabolism, bioavailability, therapeutic effect

1. Introduction

Isoflavonoids, a class of secondary metabolites including over 1000 structures [1], are polyphenolic derivatives of 1,2-diphenylpropane, as opposed to the larger group of flavonoids having a 1,3-diphenylpropane skeleton. They encompass several subgroups, the most prominent being the isoflavones, the rotenoids, the pterocarpans and the coumestans (**Figure 1**). The multiplicity of isoflavonoid structures is accounted by different oxidation levels of the backbone, the presence of additional heterocyclic rings and the diversity of substituents. These substances occur

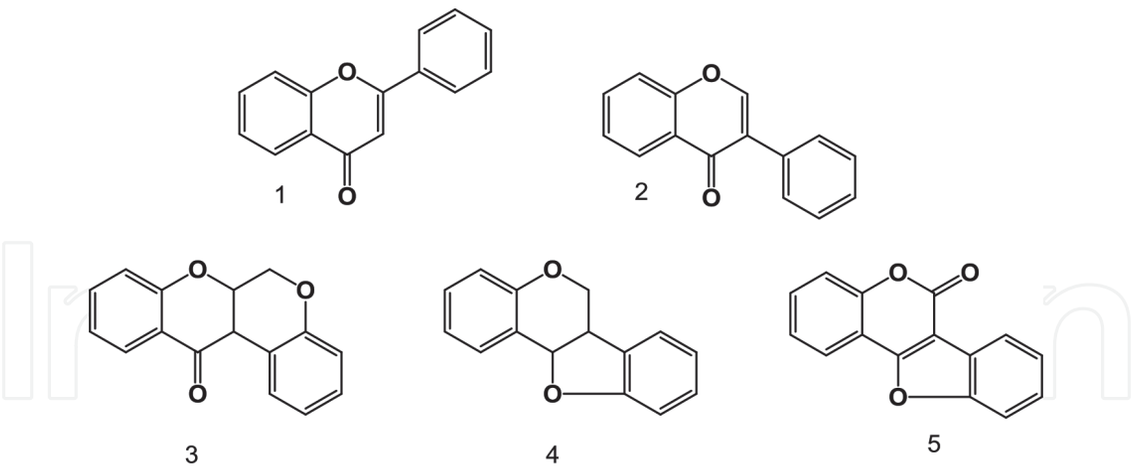


Figure 1. Flavonoid and isoflavonoid backbones. 1: Flavones, 2: Isoflavones, 3: Rotenoids, 4: Pterocarpans and 5: Coumestans.

mostly in free forms as aglycones (**Figure 2**); glycosides are formed with glucose, rhamnose or apiose as the sugar moiety and are mainly O-glycosides.

Isoflavonoids are a major biochemical marker of the Fabaceae, especially of the subfamily Faboideae [2]. As much as 95% of the isoflavonoid aglycones and about 90% of isoflavones were reported in this family [3]. In recent years, the distribution of isoflavonoids has reliably been proven in a variety of nonlegumes, including bryophytes, gymnosperms and angiosperms. In flowering plants, they occur in over 50 families, and their distribution pattern is unrelated with the degree of phylogenetic closeness. Such families include among monocots such as the Iridaceae, Liliaceae, Asphodelaceae, Poaceae, Zingiberaceae and Cyperaceae as well as a large number of dicots such as Asteraceae, Apiaceae, Malvaceae, Rosaceae, Rutaceae or Solanaceae [1, 4]. Among plant organs, isoflavonoids are mainly present in underground parts, wood and bark as compared to flowers and leaves [1]. *In planta*, they act as anti-microbial compounds synthesized in response to the attack of pathogens, representing the first identified type of phytoalexins. Isoflavonoids may either be preexistent in plant tissues before microbial attacks or be produced only upon exposure to infection or environmental stressors. The prominent distribution of these secondary metabolites

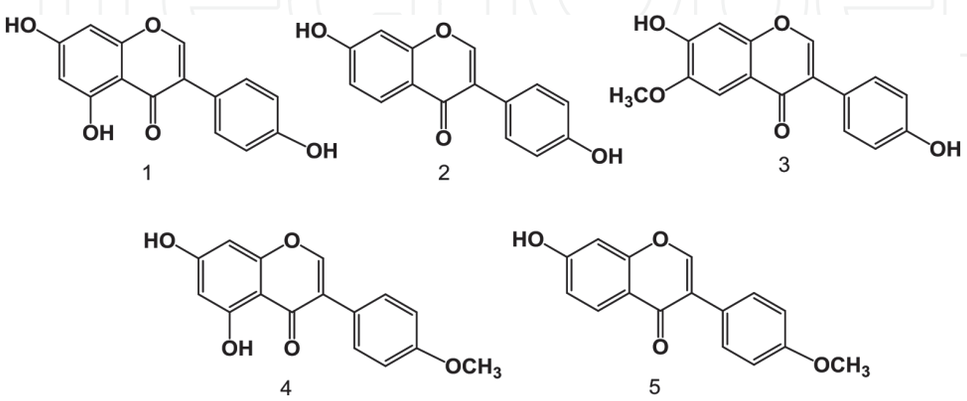


Figure 2. Main isoflavone aglycones: 1: Genistein, 2: Daidzein, 3: Glycitein, 4: Biochanin A and 5: Formononetin.

in legumes is related to their physiological role in nodulation. The isoflavonoids present in root exudates are associated with the attraction of symbiotic *Rhizobium* bacteria and promotion of their growth within root nodules [5], leading to an improved nitrogen uptake.

2. Biosynthesis and regulation

The biosynthesis of isoflavonoids occurs as a branch of the phenylpropanoid pathway, involved in the biologic obtainment of all flavonoids [6]. The departing point is represented by the amino acid phenylalanine, sequentially converted to cinnamate, p-coumarate and p-coumaroyl-CoA by the relevant enzymes of each step (phenylalanine ammonia-lyase, cinnamic acid-4-hydroxylase, and 4-coumarate-CoA ligase, respectively). Subsequently, chalcone synthase is involved in the creation of the 15-carbon flavonoid backbone from p-coumaroyl-CoA; several derivatives may be produced via different branch pathways. The crucial enzyme for isoflavone biosynthesis is isoflavone synthase, a cytochrome P450 monooxygenase, which catalyzes the migration of the aryl moiety and transforms flavanones to isoflavones [7]. The reaction involves keto-enol tautomerism of flavanones and epoxidation, followed by dehydration [8].

Relevant metabolic pathways for the biosynthesis of the major isoflavonoids genistein (precursor naringenin) and daidzein (precursor liquiritigenin) have been well-studied in soybean (*Glycine max* (L.) Merr.). Their understanding opened possibilities of metabolic engineering, leading to the development of soybean lines with an increased content of isoflavones in comparison to wild-type seeds. On the other hand, the health benefits of isoflavones prompted strategies to induce the synthesis of these compounds by non-legume plants (broccoli and tomatoes). This approach could be achieved in plants with an active phenylpropanoid pathway [9]. In fact, the flavanone naringenin is not only the substrate of isoflavone synthase, but as well that of flavanone-3-hydroxylase, involved in the biosynthesis of flavonols, anthocyanins and condensed tannins. The introduction of the isoflavone synthase gene in plants that do not express this enzyme was able to trigger genistein production in corn [7] and the model cruciferous *Arabidopsis thaliana* [10]. The disadvantage was represented by low isoflavone content, related to the competition between the flavonoid and the isoflavone pathways. Blocking the alternative flavonoid/anthocyanin branch of the phenylpropanoid pathway while upregulating the synthesis of isoflavones by introducing foreign transcription factors yielded very favorable results. Yu et al. could increase up to fourfold the level of isoflavones in soy by introducing the transcription factors C1 and R from corn in soybean (involved in the regulation of the phenylpropanoid pathway genes), and by co-suppressing flavanone-3-hydroxylase, hence diverting the whole substrate to the isoflavonoid pathway [11]. An additional regulation of isoflavone biosynthesis involves the enzymes catalyzing the glycosylation of aglycones, as the majority of isoflavonoids accumulate in conjugated form. In soybean, the malonylglycosides are predominant, followed by glucosides. These conjugates are obtained upon catalysis by malonyl transferase and glycosyl transferase, respectively, in specific positions. The engineered obtainment of isoflavonoids in nonlegumes is crucially related to the presence of isoflavonoid-specific malonyl transferases and glycosyl transferases [12]. In soy,

the content and composition of isoflavonoids are subjected to polygenic regulation and highly variable in response to drought, temperature, fertilization, carbon dioxide content and genetic factors [13]. The level of isoflavonoids is higher in wild-growing populations than in cultivated soybean; this situation is thought to be a consequence of domestication [14].

3. Isolation

The isoflavonoid aglycones such as genistein and daidzein are compounds with a low polarity and hence practically insoluble in water. The polarity is lowered by methylation, as in formononetin and biochanin A. After glycosylation, the water solubility increases; glucosides have a higher solubility in water than their malonylated and acetylated derivatives. The glycosidic bond may be hydrolyzed under acidic or basic conditions [15]. Early extraction of isoflavones was performed by refluxing alcohol, but had the disadvantage of converting malonyl- and acetyl-glucosides into glucosides and aglycones [16]. A mixture of methanol 80% was as well proposed [17]. An optimized extraction method was developed by Griffith and Collison, using acetonitrile/water, without the addition of an acid [18]. Acetonitrile is considered to yield higher extraction ratios than solvents such as acetone, ethanol and methanol, during the analysis of 12 main soy isoflavones from foods; the organic solvent (53%) is mixed with water [19]. The preparative isolation of isoflavones could be achieved by high-speed countercurrent chromatography (HSCCC). In one setting of HSCCC, acid-free solvents were employed; the isolation of malonylglucosides was performed with the aid of a solvent *tert*-butyl-methyl ether/*n*-butanol/acetonitrile/water in a ratio of 1/3/1/5 [20]. Monoglucosylated and acetylated isoflavones were obtained more recently by HSCCC after a cleaning-up step on Amberlite XAD-7 material [21].

Quantification of isoflavones is usually performed by HPLC-DAD, using reversed-phase columns and eluents containing 95% acetonitrile with 0.1% trifluoroacetic acid. Validated methods with good peak resolution are available [18]. Detection may be performed at 262 nm. For the quantification in biologic samples (urine, saliva and blood), HPLC-MS/MS spectrometry is employed after solid-phase extraction (SPE) of isoflavones; the glycosides are hydrolyzed enzymatically prior to SPE [22].

4. Bioavailability and metabolism

Bioavailability of isoflavones is based on data from absorbtion, metabolism, distribution and excretion studies. After the intake of pure compounds, isoflavone-rich extracts or foods containing high levels of isoflavones, the parent compound and their metabolite can be found in plasma and urine of human volunteers. Following ingestion, soy isoflavones attain maximal plasma concentration within 4–8 h and then eliminate from the body through the bile and kidneys with an elimination half-life that is on average 8 h [23, 24]. Aglycones are well absorbed due to their low water solubility and small molecular weight [25]. After ingestion, isoflavone glycosides are hydrolyzed by intestinal glucosidases, which partially release the aglycones daidzein,

genistein and glycitein [24]. These may be absorbed or converted to a number of metabolites including equol and p-ethyl phenol [24].

It was proved that intestinal microflora plays an important role in the metabolism and bioavailability of isoflavones [26]. It is considered that about 50% of Asians and 25% of non-Asians host the intestinal bacteria that convert daidzein into the isoflavonoid equol [27]. Variation in individual metabolism of phytoestrogens due to differences in gut microflora might influence the serum concentration of phytoestrogens. It was found that the capacity to produce equol is higher among Japanese and Korean men than among American men [28]. After an intake of 50 mg isoflavone, the urinary excretion was 42% for daidzein and 16% for genistein [29]. Fermented soy products, or supplements in which the soy extract has been hydrolyzed, contain mostly the aglycone forms of isoflavones; however, following ingestion, the plasma profile of isoflavone metabolites is the same, no matter the form ingested [30]. Free aglycones, released after hydrolysis, are absorbed by passive diffusion across the intestin [31].

The extensive metabolization of the aglycones is evident in the extremely low content of the free form in body fluids, of less than 1% in human plasma and urine [32]. The metabolization of genistein has intensively been studied and thoroughly reviewed [25]. Following the faith of most xenobiotics, it undergoes detoxification, being conjugated to glucuronides and sulfates. Among them, monoglucuronides are present in the highest proportion (62.5%), followed by diglucuronides, sulfoglucuronides, disulfates and monosulfates in human urine after dietary intake of soy products [33]. The percentage of sulfates was reported to be slightly higher in blood (20%) than in urine (13%) [32]. In humans, the plasma level of genistein (all forms) is situated in the micromolar range, while the level of free genistein is in the higher nanomolar range. The rate of metabolization after oral ingestion is less than half an hour, with conjugations occurring in the intestine, the liver but as well in the kidneys, heart and lungs. The tissue distribution of genistein is highest in the gastro-intestinal tract and liver, consistent with its enterohepatic recycling [34]. While the differences between individuals are very high, the oral bioavailability of genistein is low due to extensive metabolization and high expression level of efflux transporters (such as breast cancer resistance protein (BCRP)) [25].

5. Dietary intake and sources of isoflavones

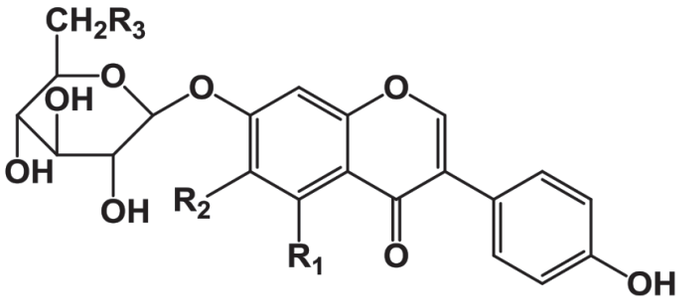
As already mentioned above, isoflavones are flavonoid compounds that are biogenetically produced by plants belonging mainly to the Fabaceae family. Main sources of dietary isoflavones are soybeans (*Glycine max*) [35, 36] and red clover (*Trifolium pratense*) [37]. Other plants with a high content of isoflavones are: mung beans (*Vigna radiata*) [38], kudzu (*Pueraria lobata*), lupine (*Lupinus spp*.), fava bean (*Vicia faba*), psoralea (*Psoralea corylifolia*) [39], poinciana (*Caesalpinia pulcherrima*) [40] and alfalfa (*Medicago sativa*) [41].

Soybeans are widely employed for the preparation of food and dietary supplements. They contain both isoflavone aglycones: genistein, daidzein, glycitein, and glucosides: 7-*O*-glucosides: genistin, daidzin, glycitin, and three 6″-*O*-acetyl glucosides: 6″-*O*-acetyl-genistin, 6″-*O*-acetyldaidzin, and 6″-*O*-acetyl-glycitin, and three 6″-*O*-malonyl-glucosides: 6″-*O*-malo-

nyl-genistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylglycitin (**Figure 3**) [42, 43]. The highest percentage of soy isoflavones in soybeans is represented by genistein glucosides, approximately 50%. Daidzein glucosides are about 40% and glycitein glucosides 5–10% [15]. More exactly, the concentration of daidzein and daidzin in soy extract is 10.4 and, respectively, 244.5 mg/g soy extract corresponding to 1.7 and 39.6%. Concerning the concentration of genistein and genistin, it is 5.3 mg/g soy extract and, respectively, 319.6 mg/g soy extract representing 0.9 and 51.8% [44].

Comparing the isoflavones from soybeans with those extracted from red clover, it is remarkable that there are four main isoflavones (daidzein, genistein, formononetin and biochanin A) from red clover and only three in soybeans [15].

In China, the first reference to soybeans dates from 2853 BC [45]. In contrast, Western cultures have adopted these products only lately. A variety of soy foods is currently available throughout the world, produced with modern processing techniques or using the traditional methods. They can be classified into fermented (tofu, okara, yuba, fresh greed soybeans, whole dry soybeans, soy nuts, whole-fat soy flour, soy sprouts, soymilk and soymilk products) and nonfermented (tempeh, natto, miso, fermented tofu and soymilk products and soy sauces) products [46]. Epidemiological studies among the Japanese population suggest that fermented soybean products have better effects than nonfermented soy products, probably because of a higher bioavailability of isoflavone aglycones [47].



Name	R_1	R_2	R_3
Daidzin	H	H	H
Acetydaidzin	H	H	$COCH_3$
Malonyldaidzin	H	H	$COCH_2COOH$
Genistin	OH	H	H
Acetylgenistin	OH	H	$COCH_3$
Malonylgenistin	OH	H	$COCH_2COOH$
Glycitin	H	OCH_3	H
Acetylglycitin	H	OCH_3	$COCH_3$
Malonylglycitin	H	OCH_3	$COCH_2COOH$

Figure 3. Structure of main isoflavone glycosides.

During the processing of raw soybeans, the composition of isoflavones is altered. The loss of isoflavones in the water used to soak raw soybeans, whey and the okara, was 4, 18 and 31%, respectively [42]. The isoflavone loss during coagulation in tofu processing was 44% and during alkaline extraction in soy protein isolate production was 53% [48]. The recoveries of isoflavones in tofu and in soy beverage comparing to their initial concentration in raw soybeans were found to be 36 and 54%, respectively [42].

Several reviews are available on the content of isoflavones in soy foods, including Japanese foods [49], foods used in the US [50, 51] and Europe [52]. A comparison of the most frequently used foods containing soy is performed in **Table 1**.

Food	**Daidzein**	**Genistein**	**Daidzein**	**Genistein**	**Daidzein**	**Genistein**
Country	**Japanese foods**		**Food in the US**		**Food in Europe**	
	μg/g wet food		**μg/g**		**μg/g**	
Soybean (raw)	1006.3	1437.7	613.3	863.3	580	840
Soybean (boiled)	135.8	472.5	74.1	70.6	150	320
Soybean sprout	49.6	79.3	50	67		
Mung bean sprout	3.4	2.4	0.6	0.8	39	680
Soymilk	78.2	156.6	48.84	60.7	–	–
Okara—bean curd residue	33.1	57.1	36.2	44.7	–	–
Tofu (momen type)	166.2	269.2	–	–	–	–
Tofu (silken type)	130.1	206.4	91.5	84.2	–	–
Tofu (packed type)	168.6	280.7	–	–	–	–
Tofu (baked type)	166.8	291.2	102.6	104.3	–	–
Tofu (dried type)	168.2	556.7	–	–	–	–
Deep fried tofu	–	–	138.0	184.3	–	–
Deep fried tofu (thick type)	148.7	257.4	–	–	–	–
Deep fried tofu (thin type)	84.2	179.1	–	–	–	–
Miso	–	–	164.3	232.4	590	670
Tempeh—fermented soybean from Indonesia	525.9	1326.2	226.6	361.5	–	–
Navy beans (haricot)	–	–	0.137	4.08	0.130	0.110
Broccoli	–	–	0.06	0.08	0	0
Carrots	–	–	0.016	0.017	0	0
Strawberries	–	–	trace	trace	0.046	–
Spinach	–	–	0	0	0	0
Onion	–	–	0	0	0	0

Table 1. Concentrations of daidzein and genistein in Japanese foods [49], food in the US, [50, 51] and food in EU [52].

Soy foods, such as tofu and tempeh, are extremely rich in isoflavones compared to other type of foods [53]. Raw soybeans contain the highest level of genistein and daidzein in the Japanese foods. The soybeans in US have a higher amount of genistein (863.3 μg/g) and daidzein (613.3 μg/g) than in Europe, which have 840 μg/g genistein and 580 μg/g daidzein [51, 52]. The richest sources of isoflavones are tofu, tempeh and miso in Japanese, European and American foods. Small variation from one region to another may occur. The isoflavone level in vegetables, fruits and other types of food are extremely low, sometimes in traces or not detectable. Among these, the navy beans have a higher level of genistein 4.08 μg/g.

One gram of soy protein in soybeans and traditional soy foods contain about 3.5 mg of isoflavones. One serving of a traditional soy food (100 g of tofu or 250 mL soymilk) provides about 25 mg isoflavones. In more refined products, it is possible that 80–90% of the isoflavone content to be lost during processing [27].

5.1. Soy foods in Asia

In Asia, soybeans are used in producing traditional foods such as tofu, soymilk and fermented products, while in Western nations, soybeans are used in the form of refined soy protein ingredients that are further used in food processing [45]. In Japan, the most popular soy food is tofu, served at all meals and in dessert products. Fermented foods such as natto and miso were very popular among Japanese and today are also largely consumed. Japanese adults consume approximately 6–11 g of soy protein and 25–50 mg of isoflavones (expressed as aglycone equivalents) per day. The results were higher than in Hong Kong and Singapore [54]. Concerning genistein and daidzein, the annual report of the national nutrition survey in 1997 in Japan shows that the dietary intake of isoflavones daidzein and genistein was 64.6 and 111.6 μmol/day/capita (16.4 and 30.1 mg/day/capita). The isoflavones intake was mostly attributable to tofu, natto and miso [49].

The mean plasma concentrations of total isoflavones are estimated to be 492.7 nM for genistein and 282.5 nM for daidzein in Japanese men, and 33.2 nM for genistein and 17.9 nM for daidzein in British men [55].

In China, not only tofu, yuba, soymilk and many regional specialities are served, but also soy powder mixes are becoming popular [45]. Isoflavone intake differs very much from a region to another [54]. In Taiwan, the meat substitutes from soy (chicken-like and fish-like products) are highly appreciated and, in Indonesia, tempeh is the most popular soy food [45].

5.2. Soy foods in Europe

Meat and dairy substitutes are the most popular soyfoods in Europe [45]. Traditional soyfoods rich in isoflavones (tofu, tempeh and miso) are rarely eaten in the UK, but soya dairy alternatives (milk, cheese and yogurts) are more commonly eaten. Some commercial products (bread, biscuits and breakfast cereals) contain soy ingredients as food additives which contribute to isoflavone intake [56].

Data from the 1998 UK Total Diet Study shows that daily intake of isoflavone aglycones (daidzein, genistein and glycitein) is approximately 3 mg/day [57].

In a group of 9 omnivores and a group of 10 vegetarians, mean isoflavone intake was measured after a 7-day food diary; mean daily isoflavone intake in the omnivorous and vegetarian groups was 1.2 and 7.4 mg, respectively. Main isoflavones food sources for the omnivorous group were soya yogurts, wholemeal bread and rolls, and for the vegetarian group were soymilk (plain), meat-substitute foods with soy protein isolate, beans, raisins and wholemeal bread and rolls [58].

5.3. Soy foods in the US

The consumption of soy food in the US resembles with the one in Europe. Popular soy foods in the US are tofu, soy sauce, soymilk, miso and tempeh. There can be found some new, adapted soy foods such as tempeh burgers, veggie burgers, tofu hot dogs, tofu ice cream, soymilk yogurt, soymilk cheeses, soy flour pancake mix and myriad [46]. Isoflavone intake is less than 3 mg/day in the US [27].

The Study of Women's Health Across the Nation demonstrated that median intake of daidzein and genistein by white subjects, African American subjects, and Japanese subjects in the US were 6.2 and 3.9 μg/day, 2.7 and 1.7 μg/day, and 4676 and 7151 μg/day, respectively. Women were aged between 42 and 52 years [59].

5.4. Soy foods in Africa

In certain African countries, soy foods gained acceptance due to the high protein level and nutrition quality. In other countries, it is used because of the food aid. In South Africa, modern soy foods are used [45]. The intake of soybeans in South Africa (0.64 g/day) is comparable with the soybeans intake in Germany (0.64 g/day).

As it can be observed in **Table 2**, the soybeans intake in Asian countries is higher than in the Western countries. For example, in 2001, in Taiwan, Japan and China, per capita consumption was approximately 19.15, 7.73 and 7.31 kg/year, respectively. In contrast, in the US, Germany and South Africa, per capita consumption was 0.33, 0.24 and 0.23 kg/year,

Country	Soybeans	
	kg/year	g/day
Taiwan	19.15	52.46
Japan	7.73	21.19
China	7.31	20.03
US	0.33	0.89
Germany	0.24	0.66
South Africa	0.23	0.64
World average	2.39	6.54

Source: Ref. [45].

Table 2. Annual per capita consumption of soybeans (2001).

respectively. Concerning isoflavone intake among Japanese adults, it is ranged from about 30–50 mg/day but is less than 3 mg/day in the US and Europe [27]. In Asian countries, the mean isoflavone consumption is 25–50 mg/day, whereas in Western countries, 1–2 mg/day is typical [60]. However, in the last decade, in Western countries, production and intake of soy foods have increased due to its important health benefits (relief of menopausal symptoms, improvement in bone health and reduced risk of certain types of cancers) [61].

6. Isoflavones: new insights regarding the therapeutic effect

An increased consumption of soy products has been remarqued in the last decade of the twentieth century and was associated with the awareness concerning the health benefits of these products, besides their role as a source of proteins [62]. The therapeutic activities of soy isoflavones were also evaluated in numerous studies for their estrogenic [63], lipid-lowering [64], anti-diabetic [65], anti-inflammatory [66], cardioprotective [67, 68] or anticancer effects [69]. *In vitro* and *in vivo* studies were performed in order to evaluate the therapeutic potential and the mechanisms of action of these compounds [70, 71].

Isoflavones are known as phytoestrogens due to their ability to bind the estrogen receptors and to exhibit estrogenic-like properties. Dietary supplements containing soy isoflavones are used to alleviate menopause disturbances as an alternative to hormone therapy [72]. Isoflavones attracted the attention of researchers after the observation of fertility problems in sheep grazing on a clover type rich in isoflavones [62]. The decreased risk of breast, prostate and colon cancer in Asian countries was associated with a higher intake of soy products in these population compared to western countries [70].

The possible benefits of isoflavones thus generated several studies to establish their therapeutic properties. Isoflavones exhibit both estrogenic and anti-estrogenic activity, binding to both α and β subtypes of estrogen receptor (ER) [73]. It was stated that isoflavones act as estrogen antagonists or agonists depending on estrogen concentration. Therefore, they are estrogen antagonists in a high estrogen environment, but when the estrogen quantity is reduced, they act as agonists [74]. The affinity for the estrogen receptors is different; genistein presents a 20–30 times higher affinity for ERβ than for ERα, while daidzein presents a weak affinity for both receptors, still higher for ERβ [70, 75]. Administration of 80 mg/day red clover isoflavones (containing genistein, daidzein, formononetin and biochanin A) for 90 days reduced hot flushes and night sweats, important vasomotor symptoms commonly found in postmenopausal women [76]. Low doses of isoflavones (25 mg/day containing 51.8% genistein, 43.3% daidzein and 4.9% glycitein) can reduce depression and insomnia in postmenopausal women [72].

Soy isoflavones present beneficial effects on lipid metabolism. The lipid-lowering activity of isoflavones has been observed in studies performed in animals. Soy isoflavones reduced the levels of triglycerides (TG) and low-density lipoprotein (LDL) in obese rats, and also exhibited benefits on obesity. The mechanisms involved are the suppression of mechanistic target of rapamycin complex 1 (mTORC1) activity that determines a reduction of

AKT phosphorylation [77]. A decrease of total cholesterol, LDL cholesterol levels and an improvement of ApoA1/ApoB ratio were noticed after the administration of 435 mg isoflavones/day for 2 months in women with type 2 diabetes [64].

The benefits of isoflavones in glucose and lipid metabolism have been previously reported, but the mechanisms of action are not yet fully understood [78]. Potential benefits in obesity were also observed after intake of isoflavones. Dietary modifications in early stages of this condition are important to prevent cardiovascular and metabolic disorders [79]. Some of the mechanisms involved in the anti-diabetic properties of isoflavone genistein are the enhancing of β-cell proliferation and the regulation of insulin secretion [65]. Isoflavones intake might reduce the risk of type 2 diabetes [53]. However, the beneficial role of isoflavones in this disease was not supported by other studies [78, 80]. A decreased risk of type 2 diabetes was noticed for women with high concentrations of genistein in plasma, but this correlation was not observed in men [81]. After the administration of a diet supplemented with 0.02% (w/w) genistein for 8 weeks (10–12 mg genistein/day) in Zucker diabetic fatty (ZDF) rats, there were no beneficial effects on glucose homeostasis or on skeletal muscle oxidative stress [78]. The conflicting results regarding the benefits of isoflavones in diabetes led to the assumption that other soy compounds might be responsible for this activity [65].

Genistein not only reduces weight gain in female obese *ob*/*ob* mice after the administration of 600 mg/kg diet for 4 weeks, but also promotes oxidative stress in the vasculature and inflammation in the perivascular adipose tissue [82]. Daidzin and glycitin (0.06% in diet) decrease blood glucose, insulin and HbA1c levels in mice with obesity and diabetes induced by a high-fat diet [79].

The effects of isoflavones on components of the metabolic syndrome were also evaluated. An extract from the roots of *Pueraria lobata*, administered 0.2% in the diet of female stroke-prone spontaneously hypertensive rats (SP-SHR) improved blood glucose levels, decreased plasma total cholesterol levels and reduced blood pressure, thus indicating beneficial effects on risk factors that led to the development of metabolic syndrome. The major isoflavones in the extract were puerarin (25.3%), daidzin (7.1%) and daidzein (0.8%) [83].

Even though the effects on obesity and diabetes were not sustained by other studies, an increase in bone mass was noticed in obese mice treated with 600 mg genistein/kg for 4 weeks [84]. The bone protective properties were also observed for other compounds. Formononetin, an isoflavone found mainly in the roots of *Astragalus membranaceus* and *Astragalus mongholicus*, improved the mechanical properties of the bones and exerted beneficial effects in rats with osteoporosis induced by ovariectomy after the administration of 10 mg/kg formononetin for 4 weeks [85]. The results of a meta-analysis investigating trials that evaluated the effects of isoflavone in osteoporosis, revealed that the effects of these compounds depend on dose and duration of administration. An increase in mineral density of the bone by 54% and a reduction in the fracture risk were noticed in women included in these studies [86]. A clinical trial regarding the effects of soy isoflavones on bone loss and menopausal symptoms did not reveal benefits in this condition. Women included in the study received tablets containing 200 mg soy isoflavones (genistein and daidzein)/day for 2 years [87].

The cardiovascular diseases are more frequent in women after menopause, due to the modifications in the production of estrogen [88]. Compounds with estrogenic properties, such as the isoflavones found in soy, were evaluated for their potentially cardioprotective activity [89, 90]. The administration of 80 mg/day isoflavones extracted from soybeans for 12 weeks in patients with primary or recurrent ischemic stroke determined a decrease in high sensitivity, C-reactive protein and improved vascular endothelial function [68]. A meta-analysis evaluating the results of nine trials concluded that isoflavone supplementation improves endothelial function in postmenopausal women that presents low flow-mediated dilatation (FMD) levels, but not in ones with high baseline FMD levels [89]. Nevertheless, the benefits of isoflavones regarding the protective effects in ischemic stroke are questionable. Yu et al. associated the high intake of soy isoflavones (53.6 mg isoflavones/day) with an increase in the risk of ischemic stroke in women [91].

The protective effect against the inflammatory vascular disease of isoflavones is due to their anti-inflammatory activity. The inhibition of monocyte adhesion to endothelial cells that involves the activation of PPARγ is related to this effect [66]. The anti-inflammatory properties are due to inhibition of interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) production, NF-κB regulation and their antioxidant activity [92]. Pro-oxidant effects at high doses were also noticed for these compounds. The supplementation with 640 mg/kg daidzein in pigs revealed antioxidant properties in muscle, but were accompanied by pro-oxidant effects in fat and liver tissues [93].

Administration of soy isoflavones (daidzein, genistein and glycetin) in capsules, 20 mg twice daily in female patients improved irritable bowel disease and association with vitamin D can determine a synergistic effect [94]. The estrogen-like effects of isoflavones genistein and daidzein seem to be involved in their beneficial effects on sleep status. A high intake of isoflavones was related to an optimal sleep duration and an increased quality of sleep in Japanese adults [95].

Age-related skin modifications in women emerging with a decline in estrogen production can be reduced by isoflavones. Genistein improves skin appearance and is used to reduce wrinkles and skin dryness in cosmetic preparations. It increases the skin resistance and contributes to skin reparation [96].

Anti-bacterial effects were also reported for isoflavones. For instance, biochanin A, a methylated isoflavone from red clover, inhibits the growth of *Chlamydia trachomatis* and *Chlamydia pneumoniae* [97]. Isoflavones act as anti-viral agents against several types of viruses including herpes simplex virus and human immunodeficiency virus (HIV). Genistein, a tyrosine kinase inhibitor, is one of the compounds most studied for these properties and revealed positive effects in inhibiting HIV-1 infection, especially when applied in entry and early post-entry stages [98].

7. Isoflavonoids: new insights regarding cancer chemoprevention

It is very well known that dietary components are, nowadays, considered important therapeutic agents used to prevent various chronic disorders, especially cancer, cardiovascular pathologies

and inflammation processes [99]. For example, according to the statistics, the incidence of prostate cancer or breast cancer in Asian countries has been lower than in Western countries, mainly because of their high consumption of soy products [100]. Furthermore, the incidence of breast cancer in Asian women who had immigrated to Western countries proved to be similar to that of Western women [101]. In addition, soy isoflavones, with the main representative genistein, proved to be promising phytotherapeutic drugs with chemopreventive effects in chronic disorders caused by exposure to solar UVB radiations, including nonmelanoma skin cancer [102, 103].

Genistein has been extensively studied in different types of cancers cells and cancer animal models [104]. Unfortunately, the low oral bioavailability of genistein [105], due to its high lipophilicity and its extensive metabolism by the phase II enzymes [106], has limited its use in clinical trials [107].

The data regarding the chemopreventive effects of daidzein are limited, more studies being focused on the curative effect of daidzein in cancer and not in its prophylactic properties [108].

Previous *in vitro* evidences have shown that genistein may act not only as an agonist, but also as an antagonist on cancer cells depending on both its concentration and the type of cancer cells on which was tested [109]. In this regard, genistein because of its biphasic effect may be involved not only in preventing, but also in promoting cancer [110].

The *in vitro* chemopreventive effect of genistein might be related to its involvement in epigenetic regulations of gene expression, having a direct effect on histone modifications and DNA methylation [111]. Genistein is also a strong inhibitor of the tyrosine kinase [112] and topoisomerase activities [113]. The *in vitro* apoptotic effect of genistein may be related to the inactivation of NF-kB and Akt signaling pathways [114], although its specific mechanisms of inducing apoptosis have not being fully understood [115]. According to a previous *in vitro* study on pancreatic cancer cells, genistein suppressed the ovarian cancer cell growth and migration through the inhibition of mRNA [116]. The chemopreventive properties of genistein have also been proved on MCF-7 breast cancer cells, in which genistein has inhibited the cell proliferation by inactivating the IGF-1R-PI3 K/Akt pathway and decreasing the Bcl-2/Bax mRNA and protein expressions [115]. Another mechanism responsible for the chemopreventive effects of genistein might consist in the suppression of the microsomal CYP1a1 gene expression in Hepa-1c1c7 liver cancer cells [117].

The association between genistein and daidzein has proved antiproliferative effects on human colon adenocarcinoma grade II cell line (HT-29) [118], the anticancer effect of daidzein being related to copper-dependent pathway and redox cycle [108].

Among the soy isoflavones, GLY has been the most potent activator of extracellular signal-regulated kinase (ERK1/2), exhibiting a significant antiproliferative effect on RWPE-1 nontumorigenic prostate epithelial cells [119]. Thus, according to another study on the same type of cells, GLY has also shown to decrease the expression of cytokeratin 18 and prostate-specific antigen (PSA) [120].

Furthermore, *in vivo* data have shown that oral consumption of genistein during the early prepubertal period decreased the susceptibility of developing breast cancer later in life [121]. Genistein, as a phytoestrogen, may interact with the estrogen receptors. In this regard, a previous

study on HER2 overexpressing mice has shown that genistein mimicked the estradiol effects, in the presence of estrogen receptor alpha [112], while in postmenopausal women, in the absence of estradiol, genistein directly reduced the anticancer activity of cisplatin, a cytostatic drug commonly used in breast cancer [122]. For instance, according to Tonetti et al. *in vitro* and *in vivo* studies, the concomitant administration of tamoxifen with genistein or daidzein might not be safe because this association has produced bigger size tumors than tamoxifen alone [123].

Soy isoflavones exhibited *in vivo* protective effects against skin chronic disorders including cancer by reducing pro-inflammatory cytokines and oxidative stress and through activation of NF-kB [124]. For example, genistein has suppressed UV-induced skin carcinogenesis in mice through its moderate inhibitory effect on ornithine decarboxylase activity [125]. Moreover, 7,3′,4′-trihydroxyisoflavone, a major metabolite of daidzein, has reduced UVB-induced skin cancer in mice through inhibition of cyclooxygenase-2 (COX-2) expression by suppressing NF-kB transcription activity [103]. In this regard, genistein loaded-PLA nanocapsules indicated to be a promising formulation with chemopreventive effects against skin cancer in porcine ear skin not only by increasing the penetration of genistein in skin deeper layers, but also by limiting its degradation in time [107].

According to Ghaemi et al. study on human papillomavirus (HPV) associated-cervical cancer in mice, genistein has also indicated immunomodulatory effects through increment of interferon-gamma (IFN-gamma) level, lymphocyte proliferation and lactate dehydrogenase (LDH) release [126].

Consequently, the isoflavones from soy products may be considered promising alternative therapies to prevent various types of cancer, more experimental and clinical studies being necessary for establishing the safe dose that can be used especially in patients susceptible to hormone-dependent tumors.

8. Anti-inflammatory and chemopreventive activity of isoflavonoid genistein alone and incorporated in modern pharmaceutical formulations

From the main isoflavones reported above, we have studied genistein. One of the most important lines in our research group on this topic involves the analysis of the chemopreventive effect of the phytoestrogen genistein against malignant melanoma. The isoflavonoid genistein (4′,5,7-trihydroxyisoflavone) is the aglycone of heteroside genistin. It is the most studied compound from the class of isoflavones together with daidzein, glycitein, formononetin, equol and biochanin A. It is the major active compound from soy seeds, *Glycine max* (L.) Merr., family *Fabaceae* [127–129]. Regarding the biological activities, recent papers report that: genistein induces apoptosis, inhibits cell proliferation, modulates cell cycle progression on different cancerous cell lines, inhibits angiogenesis, suppress lymphocyte activation and proliferation, stabilizes mast cells and presents mild anti-inflammatory properties [130, 131]. The phytoestrogen also inhibits the production of reactive oxygen species (ROS) which is directly correlated with DNA modification and tissue damage. Production of ROS, especially

by activated cells of the immune system, has been postulated to play an important role in carcinogenesis, particularly in tumor promotion [132, 133].

In a recent complex study employing, the B164A5 and B16F10 murine melanoma cell lines, we have shown testing a wide range of concentrations (150, 100, 50, 30, 15, 5 and 1 μM) that this phytocompound is an active antiproliferative and pro-apoptotic agent on this two cell lines. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has shown an IC_{50} of 41.1 μM genistein for B164A5 cells and 61.4 μM genistein for B16F10 cells. The carboxyfluorescein diacetate succinimidyl ester (CFSE) assay has shown that after 24 h of incubation, proliferation of B16 cells was decreased when treated with 30 μM genistein. Genistein at 100 μM was able to cause G2/M arrest in the cell cycle of these murine melanoma cell lines [134]. DAPI staining was performed in order to detect first signs of apoptosis. When B16 cells were incubated with 100 μM genistein, this phenomenon could be detected and translated by a reduction of the cell number and increase in nuclear fragmentation compared to the control group [134]. Western blot analysis was furtherer conduced, for four important proteins involved in the process of apoptosis, namely caspase 3, poly(ADP-ribose) polymerase (PARP), Bax and Bcl-2. Incubation with 100 μM genistein conduced for both B16 cell lines to cleaved caspase-3 as well as cleaved PARP as responsible for the mechanism of apoptotic events. In another study using all concentrations previously tested (150, 100, 50, 30, 15, 5 and 1 μM genistein) and after a period of incubation of 72 h, we have shown that the phytoestrogen does not induce caspase-2 activation *in vitro* on B16 melanoma cell lines [135]. In order to get the full picture about apoptosis, namely to detect early and late apoptotic cells, annexin V-FITC/7AAD staining was performed by fluorescence cytometry in parallel for the B16 melanoma cells as well as for the bone marrow-derived dendritic cells (BMDCs). At its highest concentration, genistein induced slightly more apoptotic events in the B16 cells than in the BMDCs [134]. The aim of the above-mentioned study was to find a drug that "kills" the cancerous cells and stimulate the immune activity. On this purpose, the potential immune stimulatory activity of genistein was tested by measuring the anti-tumorigenic cytokine IL-12p70 released by murine primary BMDCs. The phytoestrogen, at the concentration of 5 μM decreased the level of IL-12p70 of the LPS-stimulated DCs. These findings were in line with the observation that also the IL-12p35 mRNA levels were downregulated. T cell activity was further screened by analyzing the concentration of IFN-γ and IL-2 cytokine in the supernatant of spleen cells of OT I mice expressing the ovalbumin-specific transgenic T cell receptors. Results have shown that genistein had no effect on the concentration of IFN-γ and IL-2 cytokine [134].

Besides the chemopreventive activity for murine melanoma, our research group have investigated genistein alone and incorporated in randomly methylated β-cyclodextrin (RAMEB), hydroxypropyl-β-cyclodextrin (HPBCD) and hydroxypropyl-γ-cyclodextrin (HPGCD) in a molar ratio 1:1 for a range of biological activities. This approach was chosen in order to increase the water solubility of this lipophilic compound. CDs are cyclo-oligosaccharides presenting a hydrophilic outside and hydrophobic inner side with the ability to form host-guest inclusion complexes with an increased number of chemical structures [136]. Firstly, quantum chemical calculations were performed analyzing the behavior in gas phase, in water and in dimethyl sulfoxide, the solvent used for the solubilization of active agents for all the mentioned assays. Additionally, it was proofed that incorporation of genistein in the above-mentioned CDs took

place by a series of consecrated techniques such as phase solubility studies, differential scanning calorimetry (DSC), X-ray diffraction and scanning electron microscopy (SEM) assays [136]. Genistein and its inclusion complexes were studied *in vitro* on four types of cancer cells lines such as HeLa (cervical adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (human ovarian carcinoma) and A431 (skin epidermoid carcinoma) cell lines using the following concentrations: 1, 3, 10, 30, 60 and 90 μM and a period of incubation of 72 h. A2780 human ovarian carcinoma cell line proofed to be the most sensitive to genistein, followed by HeLa. Proliferation was not significantly affected for the other two cell lines. After incorporation in the above-mentioned CDs, changes in the antiproliferative action occurred with respect to the tested cell line. Cervical adenocarcinoma HeLa cell line was more sensitive for all three inclusion complexes when compared to pure genistein. The same behavior was found for A2780 cell line, except for the complex with RAMEB. Complexation with RAMEB conduced to an increased IC_{50} also for MCF-7 and A431 cell lines. For this two cell lines, complexation of genistein with HPBCD seemed to be the best option [136]. In the same study, genistein and its CD complexes were analyzed by the agar disk-diffusion method and the dilution method against several bacterial strains: *Bacillus subtilis*, *Enterococcus faecalis, Escherichia coli, Salmonella typhimurium, Shigella sonnei, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Tested compounds at the concentration of 10 mM presented antibacterial activity only for *B. subtilis* [137]. The last line of this study was drawn toward tests for antiangiogenic effects employing the chorioallantoic membrane of the chicken embryo. Pure genistein presented antiangiogenic effects and the HPBCD complex showed superior activity. Also for the other two complexes, namely HPGCD and RAMEB could be described an antiangiogenic effect but a decreased one as evaluated by applying the 0–5 score [136].

Another attempt to increase the bioavailability of this lipophilic phytoestrogen was directed toward the synthesis and analysis of a genistein ester derivative with myristic acid and complexed with beta cyclodextrin. The successful synthesis of the new compound as well as the successful inclusion in beta cyclodextrin was determined using consecrated assays such as TLC analysis, HPLC analysis, FTIR spectroscopy, MS spectroscopy, differential scanning calorimetry (DSC) and scanning electron microscopy (SEM). Samples were tested *in vitro*, using the MTT proliferation assay on three human cell lines: HeLa—cervix adenocarcinoma, A2780—ovary carcinoma and A431—skin epidermoid carcinoma. Results have shown that, after a period of incubation of 72 h at the concentrations of 10 and 30 μM, respectively, genistein is an active agent on HeLa (cervix adenocarcinoma) and A2780 (ovary carcinoma) cell lines. The new formulations did not decrease the viability of the cancerous cells. This behavior may be explained by the increased stability of the complex within the *in vitro* environment [138].

A new modern formulation explored by the pharmaceutical industry, but not only, is the polyurethane microstructures (PM). What determined us to focus on these compounds? Depending on the structure of the particle, such approach can offer: the possibility of amending the lipo- or water-solubility of inclusion structures, protection from external agents such as UV radiation, strong acidic or alkaline environments, drug delivery toward a specific receptor or retard activity of the biologically active compound due to the use of transport vehicles with low speed of degradation [139]. Based on these hypotheses, we have synthesized PM with a yield of encapsulation of 68.3% genistein (w/w). The formulation was tested *in vitro* using the

MTT proliferation assay on three human breast cancer cell lines MCF7, MDA-MB-231 and T47D-human breast adenocarcinoma cell lines. Tests were performed also for the antimicrobial and antifungal activity against the following strains: *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella enteritidis*, *B. subtilis*, *Bacillus cereus* and *Candida albicans* employing the dilution method. Results made us to conclude that the PM are a bad in *vitro* carrier partner for genistein [137].

In vivo assays were also performed in order to test the chemopreventive effects of genistein against murine melanoma. During the research in our group, we have observed in a murine model of melanoma, obtained by subcutaneous injection of 0.1 ml of $1*10^5$ B164A5 cells/mouse that, genistein after a period of 15 days at a dose of 15 mg/kg, body weight decreased tumor volume and weigh with about 30% and reduced distance tumors. Noninvasive measurements using the Multiprobe Adapter System (MPA5) from Courage-Khazaka, Germany, Mexameter® MX 18 showed that genistein reduced the quantity of melanin and the degree of erythema directly correlated with the number of days of treatment [140]. Being very well known, the link between inflammation and cancer, we have analyzed the effect of genistein in an animal model of ear inflammation alone and after incorporation in hydroxypropyl-beta-cyclodextrin (HPBCD) and randomly-methylated-beta-cyclodextrin (RAMEB). Cyclodextrins (CDs) are well known agents used to increase the hydro solubility of active lipophilic agents [139]. The study concluded that the phytoestrogen, at a dose of 2 mg can be reconsidered as an active anti-inflammatory natural compound on C57BL/6 J animal model of inflammation. Additionally, complexation of genistein with the above-mentioned CDs was done and led to a stronger anti-inflammatory effect [141]. In a recent study, in order to attempt to increase the bioavailability of this phytoestrogen, we have adopted a new strategy that combines two elements: the formulation and the modality of administration. The formulation was lamellar lyotropic liquid crystal in which genistein was incorporated at the concentration 3% and the formulation was applied local, with or without electroporation (EP), using the Mezoforte Duo Mez 120905-D device on C57BL6J. Results have shown that tumors appeared later when electroporation was applied. During the 21 days of the experiment, genistein incorporated in the new modern formulation, applied topically classic decreased the tumor volume, the degree of erythema and amount of melanin for mice bearing B16 murine melanoma tumors. When the formulation was applied by electroporation, the prognosis was even better. However, the new approach had no effect in terms of serum concentrations of the protein S100B and serum neuron-specific enolase (NSE), or the tissue expression of the platelet-derived growth factor receptor β (PDGFRβ) antibody [142]. Also, soy total extract incorporated in the new modern lamellar lyotropic liquid crystal formulation was tested *in vitro* on the B164A5 mouse melanoma cell line for its pro-apoptotic potential, employing two consecrated assays: 4',6-diamidino-2-phenylindole (DAPI) and annexin-FITC-7AAD double staining. 200 μg/ml of soy extract, respectively 200 μg/ml of soy extract incorporated into the lamellar lyotropic liquid crystalline formulation were incubated for 72 h together with this murine melanoma cell line. Results have shown that soy extract has pro-apoptotic properties and incorporation in the new formulation does not affect in a negative manner this effect thus being a suitable excipient for *in vivo* studies [143]. In a recent study, diffusion and penetration of genistein, respectively, genistein incorporated in lamellar lyotropic liquid crystalline formulation through different membranes (a synthetic membrane *in vitro*, chick chorioallantoic membrane (CAM) *ex ovo*, and excised human epidermis *ex vivo*) were also investigated by conventional treatment without EP, and also with the mediation of EP by the help of a Franz diffusion cell system. *In vivo* ATR-FTIR

and *ex vivo* Raman spectroscopy were applied in order to analyze the effect on mice skin [144]. Results have shown that the new formulation is a suitable carrier for the lipophilic genistein. The formulation with the active agent penetrated the skin, but when electroporation was applied, the transdermal drug transport was more rapid and effective. This observation was validated by both ATR-FTIR and Raman spectroscopy [144].

The research of our group on the phytoestrogen genistein points toward the clear conclusion that this phytocompound is an active chemopreventive agent against malignant melanoma both *in vitro* and *in vivo*. A series of attempts were made in order to increase the bioavailability of this lipophilic compound. We cannot say that we have found the optimal formulation, but we have managed to improve results compared to pure substance. Further studies will be conducted on this matter.

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