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REVIEW

ISOFLAVONES – BIOCHEMISTRY, PHARMACOLOGY AND THERAPEUTIC USE

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There has been a veritable explosion of interest in phytoestrogens, a family of plant-derived compounds with weak estrogenic and antiestrogenic proprieties, and in particular in the potential health-benefits that a rich diet in these compounds might confer on many hormone-related diseases. Phytoestrogens compounds include isoflavones, other phenolic structures, lignans, coumestans and resorcylic acid lactones. Much of the current interest in the pharmacology and physiology of these bioactive phytochemicals has focused on the class of isoflavones. These phenolic compounds possess in addition to estrogenic activity, a myriad of biological properties that can impact on many biochemical and physiological processes. This review focuses on recent studies pertinent to this field and includes the chemistry, dietary sources, pharmacology, and potential therapeutic use of isoflavones class and also the risks and safety of isoflavones consumption.

INTRODUCTION

Phytoestrogens are a family of plant-derived compounds with weak estrogenic and antiestrogenic properties. In the past several years, phytoestrogens have gained considerable attention, from both health practitioners and researchers. There are many types of phytoestrogens: lignans, coumestans, isoflavones, resorcylic acid lactones and some flavonoids.

Epidemiological data indicate that people from Asian cultures have lower rates of certain cancers, and soy usually found in their daily diet, could be a contributing factor. Also, numerous human, animals, and *in vitro* studies have demonstrated that soy isoflavones are effective chemoprotection agents for certain types of cancer and as a result, soy became a promising nutraceutical with potentially significant health benefits.[35] Certain cells in the body have estrogen receptors, special sites that allow estrogen to attach. When estrogen attaches to a cell's estrogen receptor, estrogenic effects occur. Isoflavones latch on to estrogen receptors too, but produce weaker estrogenic effects. This leads to an interesting two-part action. When there is not enough estrogen in the body, isoflavones can stimulate cells with estrogen receptors and partly make up for the deficit. However, when there is plenty of estrogen, isoflavones tend to block real estrogen from attaching to estrogen receptors, thereby reducing the net estrogenic effect. This may reduce some of the risks of excess estrogen (*e.g.*, breast and uterine cancer) while still providing some of estrogen's benefits (preventing osteoporosis).

The benefits of plant extracts containing phytoestrogens as alternatives to conventional hormone replacement therapy (HRT) have been debated in the last years. Red clover and soy extracts contain isoflavones which have a high affinity to estrogen receptors, progesterone receptor and androgen receptor. Furthermore, it is indicated that they have protective effects on osteoporosis and the cardiovascular system.[16] The isoflavones are now in the attention of the whole scientific world: about 600 papers on isoflavones are published annually.

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BACKGROUND ON ISOFLAVONES

Early evidence that plants produced estrogen-like compounds occurred from the observation of infertility among sheep that ate large amounts of clover in Australia. Similarly, Californian Quails were thought to feed on high isoflavone legume seeds during periods of food shortage to reduce their fertility.

Isoflavonoids are a subclass of the more ubiquitous flavonoids, which are naturally occurring polyphenols. Isoflavonoids differ from other classes of flavonoids by their greater structural variability, their frequent presence in plants in their free form, rather than as a glycoside, and by the greater frequency of isoprenoid substitution. They are divided into subclasses depending on the oxidation level of the central pyran ring. Isoflavones are the most abundant of the subclasses of isoflavonoids but, they have a very limited distribution in nature, and soybeans, red clover, peas, nuts, grain products and certain herbs, are the main natural dietary source of these compounds.

Until now it was found twelve different soybean isoflavone isomers. The primary isoflavones in soybeans are the glucosides, named genistin, daidzin, glycitin, 6"-O-malonylgenistin and 6"-O-acetyldaidzin and their respective aglycones, genistein, daidzein and glycitein. Genistein, one of the best known and studied isoflavones, has been isolated from soybeans and has been focus of scientific research since 1966. Daidzein is also classified as a phytoestrogen since it is a plant-derived nonsteroidal compound that possesses estrogen-like biological activity, respectively weak estrogenic and weak anti-estrogenic effects. Genistein glycosides are the most abundant and daidzein glycosides are the second most abundant isoflavones in soybeans and soy foods; nonfermented soy foods, such as tofu, contain daidzein, mainly in its glycoside forms. Fermented soy foods, such as tempeh and miso, contain significant levels of the aglycone.[72]

CHEMISTRY OF ISOFLAVONES

1. Structure of isoflavones

Genistein, daidzein and glycitein are commonly present in plants and foodstuffs as glucose conjugates which are referred to as glucosides or glycosides. The glucosyl group is conjugated at the 7-isoflavone position and the sugar is often esterified with acetyl or malonyl groups at the 6”-position (Fig. 1). 7-glucosylgenistein is known as genistin, 7-glucosyldaidzein is known as daidzin and 7- glucosylglycitein is known as glycitin. When the sugar bears acetyl or malonyl groups these terms are prefixed with 6”-acetyl or 6”-malonyl. Red clover also contains two other isoflavones: biochanin A (which can be turned into genistein) and formononetin (which can be turned into daidzein). Biochanin A and formononetin are methylated derivatives of genistein and daidzein. Biochanin A and formononetin can also occur as glucosides, which are known as ononin and sissotrin, respectively. Little information is available on the prevalence of these particular isoflavones in plants and foodstuffs.

Dietary isoflavones can be divided into four categories:

Aglycones: daidzein, genistein, glycitein, formononetin and biochanin A.

Glucosides or glucones: daidzin, genistin, glycitin, ononin and sissotrin.

Acetylglucosides or acetylglucones: 6”-acetyldaidzin, 6”-acetylgenistin and 6”-acetylglycitin.

Malonylglucosides or malonylglucones: 6”-malonyldaidzin, 6”- malonylgenistin and

6”-malonylglycitin.

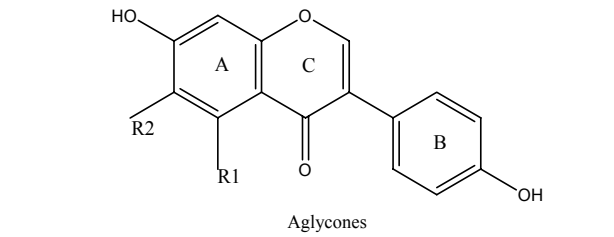
The structure of major isoflavones, aglycones and glycosides are represented in the Fig. 1.

As can be seen, genistein has three hydroxy groups, while daidzein has only two. Due to the fact that the 5-hydroxy group on the genistein binds to the 4-ketonic oxygen, genistein is a more hydrophobic molecule than daidzein. This confers genistein some of its unique therapeutic effects.[35] The biological and biochemical properties of isoflavonoids vary considerably with only minor modifications in structure. The number of phenolic hydroxyl groups, the nature of substitutions and the specific position in the ring structures influence the functions as imodulators of enzyme activity or as antioxidant, cytotoxic or antimutagenic agents *in vitro* and *in vivo*.

2. Physico-chemical properties

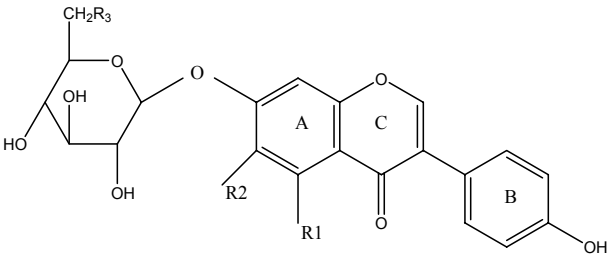
Genistein also known as 5, 7-dihydroxy - 3 - (4-hydroxyphenyl) - 4H - 1- benzopyran - 4- one or 4', 5, 7-trihydroxyisoflavone, is the aglycone of genistin. This isoflavone is found naturally as 7-β-glucoside of genistein, glycosides 6"-O-malonylgenistin and 6"-O-acetylgenistin. Genistein is a yellow crystalline solid substance that is

practically insoluble in water, but is soluble in DMSO and ethanol. Genistin has greater water solubility than genistein. Molecular formula of genistein is $C_{15}H_{10}O_5$, molecular weight 270.24 daltons and melting point 200-202^{0}C.[30] The chemical structure is represented in Fig. 1.



Aglycones

R_1	R_2	Chemical name
H	H	daidzein
OH	H	genistein
H	OCH_3	glycitein



Glycosides

R_1	R_2	R_3	Chemical name
H	H	H	daidzin
OH	H	H	genistin
H	OCH_3	H	glycitin
H	H	$COCH_3$	acetyldaidzin
OH	H	$COCH_3$	acetylgenistin
H	OCH_3	$COCH_3$	acetylglycitin
H	H	$COCH_2COOH$	malonildaidzin
OH	H	$COCH_2COOH$	malonilgenistin
H	OCH_3	$COCH_2COOH$	malonilglycitin

Fig. 1 – Structures of major isoflavones: aglycones and glycosides.

Daidzein, also known as 7-hydroxy-3- (4-hydroxyphenyl)-4*H* -1-benzopyran-4-one or 4', 7-dihydroxyisoflavone, is the 7-β-glucoside of daidzein. The structural formula of daidzein is represented in Fig. 1. Daidzein is a solid substance, insoluble in water with molecular formula $C_{15}H_{10}O_4$, and molecular weight 254.24 daltons.

Glycitein (Fig. 1) is found naturally as the glucoside glycitin and as the glycosides 6"-0-malonylglycitin and 6"-0-acetylglycitin. Soybeans, soy foods and chickpeas are the major dietary sources of these substances. Glycitein also known as 7-hydroxy-6-methoxy-3-(4-hydroxyphenyl) -4*H*-1-benzopyran-4-one or 4', 7-dihydroxy-6-methoxyisoflavone is a solid substance, insoluble in water.

The isoflavone aglucones are stable under physiological conditions. However, the acetyl- and malonyl-glucose ester bonds are labile at elevated temperatures and under acidic or basic conditions. The glucose-isoflavone ether bonds are stronger but can break under acidic conditions and/or high temperatures.

The low aqueous solubilities of the isoflavone aglucones are due to the acidic nature of the phenolic groups and are pH dependent. Conjugation to glucose residues increases the solubility, while acetylation or malonylation of the glucones reduces solubility.[89] The methylated derivatives, biochanin A and formononetin are less soluble than genistein and daidzein, respectively.

One of the most important chemical properties of isoflavones is the ability to participate in redox processes. Isoflavones act as free radical scavengers (chain-breaking antioxidants) when the phenoxilic head group encounters a free radical. The antioxidant activity, free radical-scavenging properties and selected cellular effects of the isoflavone metabolites (equol, 8-hydroxydaidzein, O-desmethylangiolensin, and 1,3,5 trihydroxybenzene) were investigated in comparison with their parent aglycones, genistein and daidzein. Electron spin resonance spectroscopy indicated that 8-hydroxydaidzein was the most potent scavenger of hydroxyl and superoxide anion radicals. Isoflavone metabolites also exhibited higher antioxidant activity than parent compounds in standard antioxidant (FRAP and TEAC) assays. However, for the suppression of nitric oxide production by activated macrophages, genistein showed the highest potency, followed by equol and daidzein. The metabolism of isoflavones affects their free radical scavenging and antioxidant properties, and their cellular activity, but the effects are complex.[105]

Isoflavones have antioxidant activities *in vivo*, but are effective only at a relatively higher concentration, and for a longer time period. Although isoflavones had *in vitro* antioxidant activities, the *in vivo* enzyme activities increased. Isoflavones might have their *in vivo* antioxidant

effects by either inducing the expression level or modifying the enzyme activity rather than exerting scavenging effect directly.[83]

3. Biosynthesis of isoflavones

Isoflavones are produced from a branch of the general phenylpropanoid pathway that produces all flavonoid compounds in higher plants. Soybeans are the most common source of isoflavones and the major isoflavones in soybean are genistein and daidzein. The phenylpropanoid pathway begins from the amino acid phenylalanine, and an intermediate of the pathway, naringenin, is sequentially converted into the isoflavone genistein by two legume-specific enzymes isoflavone syntethase and a dehydratase. Similarly, another intermediate naringenin chalcone is converted to the isoflavone daidzein by sequential action of three legume-specific enzymes chalcone reductase, type II chalcone isomerase and isoflavone synthase. Plants use isoflavones and their derivatives as phytoalexin compounds to ward off disease causing pathogenic fungi and other microbes. In addition, soybean uses isoflavones to stimulate soil-microbe rhizobium to form nitrogen fixing root nodules.[38]

4. Method of determination

The diverse structures and chemical properties of phytoestrogens and their metabolites, in addition to the range of matrices of which they are constituents, make phytoestrogen isolation and analysis particularly challenging. Initially, phytoestrogens were analysed using imprecise and insensitive techniques such as thin-layer and paper chromatography. However, with the development of increasingly sensitive and accurate analytical technologies the ability of analysts to isolate, detect and quantify phytoestrogens has advanced considerably. As a result, the information available on phytoestrogen levels in foodstuffs and biological matrices has increased significantly in the last five years. HPLC-UV is a relatively rapid method for phytoestrogen analysis. Phytoestrogens, once isolated from the matrix, can be directly resolved by HPLC and quantified by UV spectrometry. This allows simultaneous purification and quantification of complex mixtures of phytoestrogens. Quantification is achieved by creating calibration curves with reference standards. The limits of detection for HPLC-UV methods are generally in the range of 1-2 ng/mL.[71]

Concentrations of daidzein, genistein, and glycitein in soy products could be determined using a high-performance liquid chromatography method modified from Franke et al.[28] after acid hydrolysis of the endogenous 12 isoflavones to their aglycon forms (daidzein, genistein, and glycitein), which were summed to obtain total isoflavone concentrations. The aglycon weight corresponded to approximately 55% of the weight in the naturally occurring glycosylated forms. The standard aglycons of isoflavone need to be used. For example, finely ground soybean seed was weighed in duplicate samples of 0.5000 g each and dispersed in 10 mL of ethanol plus 2 mL of concentrated HCl. The resulting solutions were hydrolyzed by heating to 125 °C for 2 h in a sand bath. After the samples were cooled, they were centrifuged at 3000 rpm for 10 min. The clear aliquot was filtered through a filter. Individual hydrolyzed daidzein, genistein, and glycitein were separated on a HPLC equipped with a photodiode array (PDA) detector (200-300 nm) using C18 column, mobile phases, solvent A was 4% aqueous acetic acid and solvent B was 100 % pure methanol. Recovery was monitored by the addition of a recovery standard, flavone, to the sample prior to hydrolysis.[99]

GC-MS is widely used in phytoestrogen analysis and is sufficiently sensitive to measure levels of phytoestrogens in the ng/mL range.[90] Therefore, GC-MS is often used to measure the concentrations of phytoestrogens present in biological samples such as plasma and urine, as well as foods.[1] GC resolves the different components of compound mixtures. In GC-MS analyse, phytoestrogens must be chemically treated prior to analysis.[86] Chemical or enzymatic hydrolysis is used to remove conjugated groups and release the aglycones. Enzymatic methods of hydrolysis are preferable for isoflavones as they can be unstable under acid hydrolysis conditions.[57] Lignan glucosides are resistant to enzymatic hydrolysis and require strong acid to release the aglucones. Once the phytoestrogens are hydrolysed, the aglycones are derivatised with a silylating agent to prevent the analytes binding irreversibly to the GC column. This treatment prerequisite for GC-MS analysis means that it is not possible to analyse the chemical forms of the analytes as present in the sample matrix. As consequance, analytical results are expressed as "total phytoestrogen".[66]

DIETARY SOURCES OF ISOFLAVONES

1. Soybeans and soy products

Soybeans contain various proteins, vitamins, and minerals, as well as significant amounts of isoflavones (Table 1) and are a good source of fiber. Soy (Glycine Max) also known as soybean, soya, and Glycine soja is the source of many foods like: raw soybeans, low-fat soy flour, roasted soybeans, dry-roasted soy beans, soy milk, tofu, and soy protein isolate. Soy is a bushy, coarse annual herb originating in China where it is a long-established cultivated plant, as well as in others East Asian countries. Soybean plants were cultivated between 17th and 11th century BC in the eastern half of China where they were cultivated into a food crop. From about the first century AC to the Age of Discovery (15-16th century), soybeans were introduced into several countries such as Japan, Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal and India. The spread of the soybean was due to the establishment of sea and land trade routes. Soybeans are grown primarily for their protein content, and secondarily for their oil. But that is only the beginning of the story, because processing of soybeans yields a number of other products as well. Since 1945 it has become the most important world protein and oil crop. One of the most important physiological characteristics of soybeans is that it can take nitrogen from the air and "fix" it to be used by the soybean plant.

The main constituents with biological activity in soybeans are: isoflavones, phospholipids (phosphatidylcholine, lecithin, linoleic acid, oleic acid), proteins, carbohydrates, protease inhibitors, lignans, phytosterols, coumestans, saponins, phytates. Fukutake et al.[29] analyzed soy products for genistein and genistin content. In general, they found that fermented soy products contain more genistein than soybeans, soymilk and tofu. The results are outlined in Table 1.

Table 1

The content of Genistein and Genistin in soy foods[35]

Analyzed food	Genistein	Genistin
Soybeans, soy powder	4.6 -18.2 mcg/g*	200.6 – 968.1 mcg/g
Soy milk and tofu	9 – 13.9 mcg/g	94.8 – 137.7 mcg/g
Fermented soy (misso)	38.5 – 229.1 mcg/g	71.7 – 492.8 mcg/g
Calculated daily dietary intake by the Japanese people	5 – 4.1 mg/day/person	6.3 – 8.3 mg/day/person

*mcg/g of food

The most abundant of the soy isoflavones in soybeans are the genistein glycosides (about 50%), followed by the daidzein glycosides (about 40%). The least abundant of the soy isoflavones in soybeans are the glycitein glycosides (about 5 to 10%). Soy protein derived from soybeans contains about 2 mg of genistin and daidzin per gram of protein. In soy germ, the order is different. Glycitein glycosides comprise about 40% of soy germ, daidzein glycosides about 50% and genistein glycosides about 10%[35].

2. Red clover

Red clover (Trifolium pretense) is a native plant of Europe, central Asia, and Northern Africa and is a member of the legume family, the same class of plants including chickpeas and soybeans. The flower head is the part of the plant used in herbal remedies. The point of difference between isoflavones derived from red clover and those extracted from soybeans is that red clover extracts contain four main isoflavones (genistein, daidzein, biochanin A, formononetin), while soybeans contain only three. Recently, based on reversed phase HPLC, more isoflavone aglycones were separated and identified in acidic hydrolyzed extracts of red clover: pseudobaptigenin, glycitein, calycosin, prunetin, irilone and pratensein.[106]

Red clover is found in many herbal formulas indicated in menopause. Many - but not all - studies show red clover reduces the severity of hot flashes. The treatment with 80 mg red clover isoflavones per day resulted in a significant reduction in hot flushes from baseline. It is difficult to know for sure what role red clover plays in the treatment of menopausal symptoms. It appears that red clover has a weak effect and certainly not as noticeable as estrogen therapy. But, red clover does not have the major safety concerns of estrogen. [40, 76, 79,94,95] Red clover may also improve bone density and reduce blood

pressure.[9, 10] Red clover has been shown in lab studies to reduce the risk for breast and prostate cancer.[11]

3. Kudzu root

Kudzu, native to Asia is a climbing, semi-woody, perennial vine in the pea family. Kudzu includes Pueraria root (Pueraria mirifica from Thailand and Pueraria lobata from Korea) and Pueraria Montana var. lobata. Kudzu root is an important traditional Chinese herb: its crude extract has been used for treatment of hypertension and angina pectoris; roots and flowers have long been used in herbal medicine to help lessen the desire for alcohol.

Pharmacological studies and clinical practice have shown that the active constituents in the methanolic or ethanolic crude extract are isoflavones. Puerarin (daidzein 8-C-glucoside), daidzin and daidzein are the main components and of these, puerarin is present in the greatest amount.[101] The exact way kudzu works is not fully understood. Some studies suggest kudzu may play a role in menopausal symptoms[100], has cholesterol lowering properties and extract of kudzu plant may be a useful aid in reducing alcohol intake in a naturalistic setting. [17,62]

4. Fruits, nuts, cereals and vegetables

The variety of possible beneficial effects of phytoestrogens has stimulated much interest in the investigation of food intake in relation to risk diseases. However, little data exists regarding their levels in foods, except soya. Recently a large range of food chosen because they are common items in the European diet (apples, apricots, avocado, banana, cherries, clementine, cranberries, oranges, mango, kiwi, melon, peaches, nectarines, pears, pineapple, plums, prunes, strawberries, chestnuts, coconuts, peanuts, sesame seeds, sunflower seeds, walnuts, etc.) was selected and analyzed.[59] Eighty examples of fruits and nuts were analyzed for their daidzein and genistein content, of which 36 contained measurable quantities at concentrations ranging from 1 to 2,250 μg of daidzein and genistein combined per kilogram wet weight of food, in comparison, soya beans contain approximately 2 g/kg wet weight. Currants and raisins were the richest, containing 2,250 and 1,840 μg of daidzein and genistein combined per kilogram wet weight of food, respectively.[81]

The levels of isoflavones in a variety of cereals were analysed as well. Of the 78 assayed foods reported in that paper [60], 57 contained measurable quantities of daidzein and/or genistein and three foods contained trace quantities.

When 114 vegetable foods were assayed, 66 were found to contain measurable quantities of daidzein and genistein, in a broad concentration range Foods derived from soybean contained by far the highest concentration, respectively $5x10^5 \div 14x10^5$ μg daidzein and genistein/kg wet weight of food. Cooking by boiling in water generally caused a decrease of isoflavones concentration; however in some vegetables cooking increase these levels, but the increase is relatively small.[58]

The analytical data presented in the more recent papers indicated that there are a number of possible sources of dietary daidzein and genistein, and that many and varied components of the human diet contain phytoestrogens.

PHARMACOLOGY OF ISOFLAVONOIDS

1. Absorption and metabolism of isoflavones

The chemical form in which isoflavones occur is an important consideration because it may influence the biological activity, the bioavailability, and therefore the physiologic effects of these dietary constituents. Almost two decades ago, it was shown that intestinal microflora play a key role in the metabolism and bioavailability of both lignans and isoflavones. [18, 87]

After ingestion, soybean isoflavones are hydrolyzed by intestinal glycosidase[89], which release the aglycones, daidzein, genistein (Fig. 2) and glycitein. These may be absorbed or further metabolized to many specific metabolites including equol and p-ethylphenol.[12,42,48] So, after ingestion of isoflavones-rich foods, the isoflavone glucosides, which are considered biologically inactive, undergo deglycosilation. For many years it was assumed that only the β-glucosidases of gut microflora were responsible for deglycosylation reactions. However, more recent studies have demonstrated that deglycosylation of genistin to genistein already begins in the mouth [4] and then continues in the small intestine.[23] There have been also many discussions if isoflavones conjugates could be absorbed intact or not.

In the recent years, several animal [6,22] and cell culture [102, 103] studies have examined the intestinal absorption of isoflavone glycosides. All of these studies used either intestinal cell culture or

perfusion systems, and although useful data were obtained, the question of whether isoflavone glycosides are absorbed by humans remains to be definitively resolved. The study of absorption of pure genistin in an isolated rat intestine perfusion model showed that small amounts of genistin (1.3% of added amounts) pass into the mucosal cells where hydrolysis take place. It has been shown that genistein but not genistin can be readily absorbed through the wall of the stomach.[78] This may explain the faster absorption rates of aglycones compared to that of glycosides.[45] All these current *in vivo* evidences support the idea that isoflavonoid glycosides are not absorbed intact in humans. Or, the isoflavonoid glycosides are poorly absorbed in the small intestine, due to higher hydrophilicity and greater molecular weight.

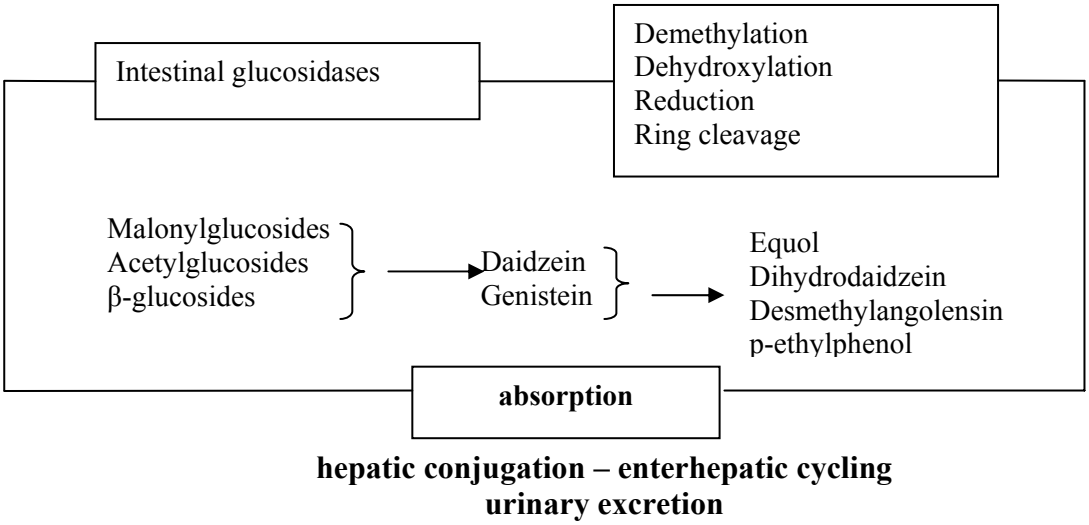


Fig. 2 – Schematic showing the major biotransformations in the metabolism of soybean isoflavones in humans and animals.[89]

The most extensive studied isoflavonoids are daidzein (4',7-dihydroxyisoflavone), genistein (4',5'7-trihydroxyisoflavone), and glycitein (4',7-dihydroxy-6-methoxyisoflavone), the isoflavones of soybean, and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone), the isoflavones of the red clover.

The metabolism of isoflavones has been well characterized in sheep [63] and also in many other species including goats and cows.

Most of the studies on isoflavones metabolism have been concentrated on reductive metabolism (phase I metabolism).

In humans, the reductive metabolism of daidzein and genistein has been studied to a certain extent. Initially, equol [13] [7 – hydroxy -3 -(4'-hydroxyphenyl) - chroman] and O -des-methylangolensin [15]

(O-dma) have been considered as the end products of metabolism of daidzein (Fig. 3).

In 1993, Kelly et al. [48] carried out a comprehensive study on soy isoflavone metabolism. The three main metabolites of daidzein were reported to be equol, dihydrodaidzein and O-dma. Two novel metabolites of genistein, dihydrogenistein and 6'-OH-O-dma were identified for the first time in urine samples after soy supplementation but no metabolites of glycitein were identified in the study of Ioannou et al.[42] The presence of the *cis* isomer of 4-OH-equol, dihydrogenistein and 6'-OH-O-dma in urine samples was demonstrated with authentic reference compounds by Heinonen et al.[36]

The reductive metabolism of the third soy isoflavone, glycitein was for the first time elucidated in humans by Heinonen et al. and three new metabolites of glycitein were identified in urine samples collected after soy supplementation.[37]

So far, the reductive metabolism of the red clover isoflavones formononetin and biochanin A in humans has not been reported.

Isoflavones with methoxyl groups are demehtylated; demethylation extent depends on the position of the methoxyl group: formononetin and biochanin A, which have 4'-methoxyl groups at B-ring are almost completely transformed to the demethylated metabolites daidzein and genistein, while the demethylation of glycitein occurs to a smaller extent. [41]

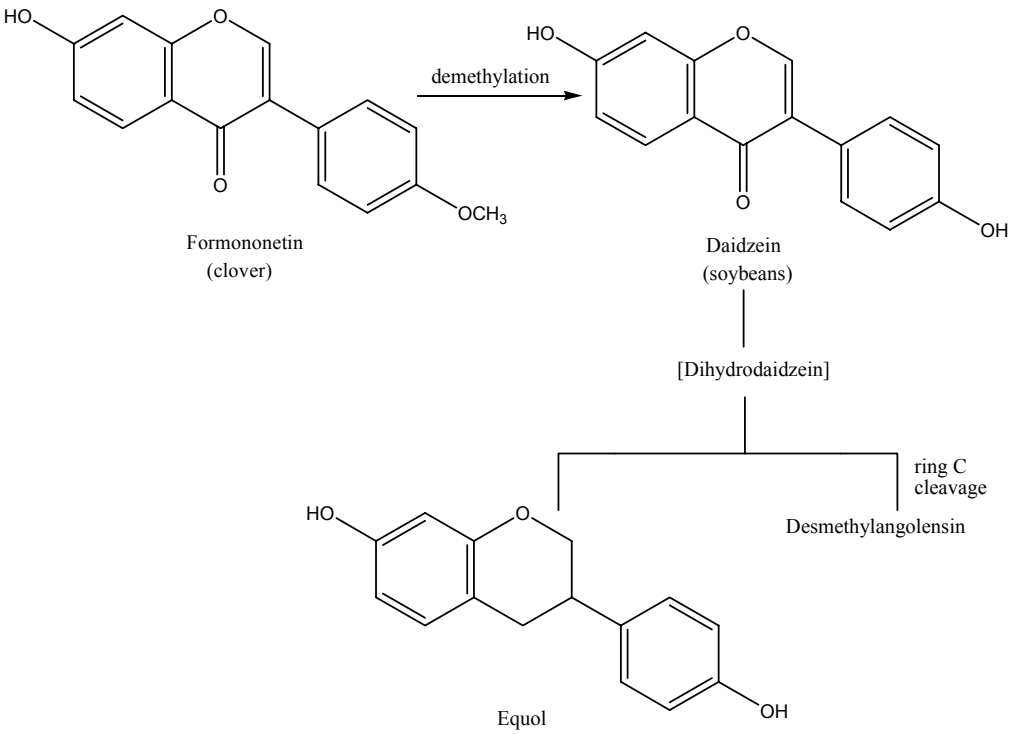


Fig. 3 – Major transformations in the metabolism of formononetin and daidzein. [41]

Roberts-Kirchhoff et al. studied the oxidative metabolism of genistein by rat and human cytochrom P450s and they found out that in rat liver microsomes the metabolism of genistein is NADPH and time-dependent. In incubation samples five oxidized metabolites were found.[84]

Kulling et al. studied further the oxidative metabolism of genistein and daidzein *in vitro* with rat and human microsomes, and they found out that both isoflavones are good substrates for cytocrome P450 enzymes and also extensively metabolized.[52]

Concerning the C-ring fission, as a possible end metabolism of genistein to smaller metabolic products, similar with the metabolism of flavonoids, for many years, was considered as the end metabolite, p-ethylphenol. More recent studies by Coldham et al. [20] show that the main metabolite of genistein after 48 h is 2-(4-hydroxyphenyl) - propionic acid.

Till now, only a few studies on phase II metabolism of isoflavones in humans have been performed. It has been proposed that isoflavones and their metabolites occur mainly as glucuronide conjugates, but have also been identified as sulfoglucuronides and sulfates.[3] Studies made by Doerge et al.[25] in human tissue microsomes showed that glucuronidation of genistein was more likely to occur in gut than in liver whereas the daidzein glucuronidation occurs most probably in liver.

In 1932, Marrian and Haslewood [65] first isolated and elucidated the chemical structure of equol, found in high levels in pregnant mare's urine. This "contaminant" of the estrus-producing hormone hydroxyestrin was assigned with the chemical composition $C_{15}H_{14}O_3$. It was named equol for its equine origins, and has a structure very similar to the estrogenous hormone estradiol.

In sheep, formononetin and biochanin A are transformed by ruminal bacteria to the demethylated intermediates, daidzein and genistein, and then to the estrogenic isoflavane equol and the inactive metabolite p-ethylphenol, respectively.

Equol is not normally present in the urine of most healthy adults in more than traces unless soy is consumed. Its identification in human urine followed the discovery that the rat, with its large ceccum and abundance of microflora was an "equol producing machine". Sufficient quantities of equol were isolated from urine and its structure was fully elucidated by mass spectrometry and nuclear magnetic resonance spectroscopy.[13] Its formation is exclusively dependent on intestinal microflora; germ-free animals do not excrete equol.[14]

Once formed, equol appears to be metabolically inert, undergoing no further biotransformation, save phase II metabolism. As with genistein and daidzein, the predominant phase

II reaction are glucuronidation and to a minor extent, sulfation.[2] About 30-50% of the adult population does not excrete equol in urine when consuming daily soy foods and the reasons are unclear.[47] Furthermore, even when the pure compounds are administred, many people do not convert daidzein to equol.[88] This phenomenon had led to the terminology of being an "equol-producer" or "nonequol-producer" to describe these two distinct populations. Lampe et al.[53] noted that being an equol converter was a relatively stable phenomenon. Because of excessive use of antibiotics, which wipe out intestinal flora, it is possible to be blocked the formation of equol, but this remains to be established.

2. Molecular mechanism of estrogen/phytoestrogen effect

The isoflavones are very similar in chemical structure to mammalian estrogens. When the structures of the isoflavone metabolite equol and estradiol are overlaid, they can be virtually superimposed. Therefore, on the basis of structure alone it is not surprising that isoflavones bind to estrogen receptors.

The classical mechanism of estrogen action involves interactions with intracellular receptors, members of the superfamily of steroid receptors, and regulation of gene transcription. This genomic effect is characterized by its delayed onset of action and occurs within minutes to hours. In contrast, it is now appreciated that more rapid non-classical pathways of estrogen action also influence cellular function. Typically, these effects occur within seconds to minutes and are mediated by membrane receptors that are coupled to cytosolic signal transduction proteins.

Estrogens mediate their activity by interaction and activation of specific intracellular receptor proteins, the estrogen receptors (ERs). Subsequently the receptor-ligand complex binds to specific DNA sequences located within the regulatory region of the target genes. The steroid receptor complex then, interacts with other cellular components to either activate or suppress transcription of the target gene in a promoter- specific and cell-specific manner.[91]

To date, two isoforms of nuclear ERs have been identified, cloned and characterized from several species: ER-α and ER-β.[70] The ER-α and ER-β proteins bind to 17 β-estradiol with nearly equal affinity and exhibit a very similar binding profile for a large number of natural and synthetic ligands.[51]

Isoflavones phytoestrogens daidzein and genistein are well known ER-β selective compounds.[7] They bind and activate human ER- α and ER-ß with up to 100 fold stronger activation of ER-β.[34] In many cells, the receptors coexist as either monodimers or heterodimers [21] but the distribution of the two receptors does not completely overlap. The tissue distribution and relative ligand binding affinities of the ER- α and ER-β differ, and this finding may help to explain the selective action of estrogens in different tissues. However, ER-β are prominently expressed in ovary, prostate, lung and hypothalamus.[51]

Phytoestrogens are unique among many estrogen-like substances for their preferential binding to ER-ß protein and this may explain some of the reported effects of soy isoflavones in tissues expressing this receptor subtype, such as the bone, brain and vascular endothelium.

Recently (2001) the binding affinity of equol for human ER- α and ER-β was found to be similar to that of genistein, but equol induced transcription more strongly than any other isoflavone, especially with ER-α, thus explaining the actions of isoflavones more those of partial estrogen agonist and antagonist. [68]

Nagel et al. showed that 49,7% of equol circulates in the free or unbound form.[73] This is considerably greater than the proportion of the free daidzein (18,7%). Because it is the unbound fraction that is available for receptor occupancy, this may effectively contribute to enhancing the overall potency of equol. Besides estrogen type effects must be also taken in consideration the nonhormonal secondary mediated action of isoflavones.

3. Potential therapeutic use of isoflavones class

Isoflavones are suggested for having health benefits in a variety of human conditions, including menopause symptoms, endocrine-responsive cancers, coronary heart disease, osteoporosis and cyclic monthly breast pain (mastalgia).

3.1. Isoflavones in hormone replace therapy

Many isoflavones have estrogen-like properties and, because of a favorable side-effect profile, may be ideal alternatives to HRT (hormone replace therapy). Controlled studies in premenopausal women provide evidence to suggest that diets containing phytoestrogens can produce estrogenic effects. A daily intake of vegetable protein containing 45 mg isoflavones lengthening the

menstrual cycle and consequently reducing the breast cancer risk.[19] In perimenopausal women, the estrogenic effects of soy isoflavones are weak because estrogen is abundant. At menopause, it is believed that this effect increases due to the decrease in endogenous estrogen.[31] When given in adequate doses to postmenopausal women, soy that contains isoflavone improves menopausal symptoms and related quality of life but the results are mixed. Some of these studies indicate beneficial effects when the dosage used appears to be safe, respectively 100 mg isoflavone daily.[50]

Cyclical mastalgia is very common in Western populations and is believed to have a hormonal basis. In a double-blind randomized control trial 40 mg or 80 mg of isoflavones was administrated and the reduction in pain was 13% for placebo, 44% for 40 mg of isoflavone per day and 31% for 80 mg per day. The isoflavones benefit demonstrated in this study adds another therapeutical mean.[44]

3.2. Isoflavones diet in cancer prevention

Epidemiological data indicate that consumption of soy is particularly associated with reduced risk of breast, prostate and lung cancers, as well as leukemia. *In vitro* and animal research has further supported these observations.

People from Asian cultures eating a diet rich in soy foods, such as tofu, demonstrated lower rates of several types of cancers, including types not typically considered to be hormone - or diet-related. Messina et al. reviewed 21 epidemiological studies which evaluated the effect of soy diets on 26 different cancer sites.[67] An evaluation of the effect of non-fermented soy products in these studies found that 10 showed decreased risks for rectal, stomach, breast, prostate, colon, and lung cancers, while 15 showed no significant effect.

An inverse relationship between the risk of both premenopausal and postmenopausal breast cancer and a high intake of phytoestrogens (as measured by the urinary excretion of two classes of phytochemicals-lignans and isoflavonoids) was observed. Also, a significant reduction in breast cancer risk was observed with high excretion of both equol and enterolactone.[43] In contrast to studies showing that conventional HTR increase mammographic breast density, the isoflavone supplement did not.[104] In a case-control study of 500 women from different ethnical groups diagnosed with endometrial cancer and 470 other women from similar backgrounds serving as controls, consumption of phytoestrogens (derived mostly from soybean products) were inversely related to the risk of endometrial cancer. Non-obese postmenopausal women seem to benefit the most.[39]

Epidemiological evidence points to the benefits of soy constituents in the prevention of prostate cancer. Japanese men who consume a low-fat, high soy diet has low mortality rates from prostate cancer. Isoflavones in the plasma of Japanese men were between 7 and 110 times higher than in Western men, with genistein present in the highest concentrations. Mechanisms suggested include genistein-induced prostate cancer cell adhesion, direct growth inhibition, and induction of apoptosis.[35] Genistein and other phytoestrogens inhibit the growth of androgen-dependent and independent human prostate cancer cell lines. Genistein and biochanin A have both shown to inhibit the growth of prostatic cancer cells in vitro, irrespective of whether these were androgen-dependent or independent cell lines.[77] Rather than inhibit etiologic factors, soy protein extracts appear to influence the progression of established tumors. Other proposed mechanisms of prostate cancer prevention include genistein-induced prostate cancer cell adhesion, direct growth inhibition, and induction of apoptosis.[97]

In a large case-control study that involved over 1,600 lung cancer patients and approximately equal number of healthy controls, the consumption of phytoestrogens has been shown to reduce the risk of lung cancer. High intake of soy isoflavones has the most significant protective effect, 72% for men and 44% for women. It is unclear if life style or dietary variations between groups contributed to this reduction in risk. This is the strongest evidence ever presented to support the use of soy as a chemopreventive agent. However, the authors cautioned against overinterpretation of the findings and suggested further large-scale studies to confirm these results.[85]

3.3. Isoflavones effects on cardiovascular disease and bone density

Mortality from cardiovascular disease is similar in man and women, and heart disease is the major cause of death in postmenopausal women. Estrogen deficiency is associated with significant alterations in lipoprotein metabolism, with serum cholesterol concentration increase. The cardio-

protective effects of estrogen replacement therapy are well established and are mediated via effects on lipid metabolism which include a lowering of LDL cholesterol and increases in the level of HDL cholesterol.[54,61] Soy protein containing isoflavones significantly reduced total serum cholesterol, LDL cholesterol, and triacylglycerol and significantly increased HDL cholesterol, but the changes were related to the level and duration of intake and the sex and initial serum lipid concentrations of the subjects.[49] Isoflavones influence vascular function and recent publications demonstrate potent vasodilator effects that resemble to that of estradiol. The most human studies that have been reviewed above show multiple effects on arterial circulations that are clearly beneficial. In particular, improved systemic arterial compliance, reflecting greater distensibility of large arteries, is likely to reduce the risk of systolic hypertension and clinical coronary heart disease. Results from the exposure of vascular system to isoflavones, whether infused into the microcirculation of the human forearm or in pharmacological preparations, suggest a role in avoiding endothelial dysfunction.[5, 26,56, 74,75]

The possibility that phytoestrogens may offer a natural alternative for the prevention of bone loss due to estrogen deficiency during the menopause has led a number of animal and clinical investigations. So, a double-blind, parallel-group study conducted over a 6-month period in 66 postmenopausal women to examine the effect of isoflavones of soy protein (40g/daily) containing varying concentrations, on blood lipids and bone density, was made. Participants presented hypercholesterolemia. Significant increases were observed in bone mineral content and density in the lumbar spine for the group taking the higher isoflavone-containing product as compared with the control group. In sum, both the moderate- and high-concentration isoflavone dosages decreased risk factors for cardiovascular disease, but only the higher concentration demonstrated an ability to protect against spinal bone loss.[80] Daily intake of two glasses of soymilk containing 76 mg isoflavones or a daily dose of 26 mg biochanin A, 16 mg formononetin, 1 mg genistein, and 0.5 mg daidzein (a red clover isoflavone supplement) seems to prevent lumbar spine bone loss in postmenopausal women. Transdermal progesterone had bone-sparing effects but when combined with soymilk a negative interaction between the two treatments occurs resulting in bone-loss to a greater extent than either treatment alone.[8,64]

3.4. Phytoestrogens as an anti-staphylococcal agent

The bacterial infection therapy of often involves antibiotics. However, due to the increasing prevalence of antibiotic-resistant bacteria, the search for new antibacterial compounds has attained a high priority. Phytoalexins, including isoflavonoids as well, are known to inhibit the growth of pathogenic microorganisms in plants. The antibacterial activity of flavonoids against S. aureus and Staphylococcus epidermidis has been reported recently.[32] In vitro study demonstrated the growth-inhibitory effect of genistein on various bacteria, including methicillin-resistant strains of S. aureus and therefore, isoflavones may represent a new type of anti-staphylococcal agent.[98]

RISK AND SAFETY OF ISOFLAVONES CONSUMPTION

Research indicates that soy and its individual constituents have several potential health benefits. The primary glucoside-isoflavones, genistin, daidzin, glycitin as well as their metabolites, exerts a wide array of effects, which appear to offer protection against cancer, cardiovascular diseases, osteoporosis, and ocular neovascularization. There is dual evidence about the risks and benefits of soy phytoestrogens, research data presenting a contradictory picture. Some reviewers suggest that early exposure to soy may prevent cancer and heart diseases. Against this generally positive view there are an increasing number of recent reports that suggest that in experimental animals, phytoestrogens have adverse effects with respect to carcinogenesis, reproductive function, immunity and hormonal balance. Other experts believe that it is still too early to recommend soy isoflavones for the treatment of menopausal symptoms or for estrogen replacement therapy. They claim the lack of specific information about effective dosage, length of administration, and long-term effects of using phytoestrogens to manage menopausal symptoms. To determine whether their use in these situations is harmful or beneficial further studies are indicated.

Soy is contraindicated in patients hypersensitive to soy products. Have been reported the fallowing adverse reactions: flatulence, allergic reactions. There is an ongoing debate as to whether soy should be contraindicated in those with estrogen-dependent tumors.[55] In some epidemiological

studies the increase in dietary soy was positively associated with elevated bladder cancer risk. The risk was similar between men and women and was not explained by other dietary factors.[92] Long-term treatment (up to 5 years) with soy phytoestrogens was associated with an increased occurrence of endometrial hyperplasia. These findings call into question the long-term safety of phytoestrogens regarding the endometrium.[96]

There are few research data on the effects of consumption of soy phytoestrogens by human neonates. The safety of soy-based infant formulas has been under observation because of the presence of the phytoestrogens and their potential for hormonal actions at critical time of development. For biological effects in infants from exposure to phytoestrogens data are lacking; avoidance of high intake is recommended also no obvious evidence to support negative effects has been found.[89]

A few isoflavones - drug interactions are indicated in the literature: animal studies suggest that genistein, a soy isoflavone, may antagonize the effects of tamoxifen on estrogen-dependent breast cancer (MCF-7). Women with estrogen-dependent breast cancer treted with tamoxifen must limit their isoflavone consumption. Tamoxifen is similar to genistein, both act against tumors containing estrogen receptors, work on estrogen - positive and - negative cells and attach to estrogen-receptor sites. To minimize interference, researchers recommend a restricted soy intake during the treatment with tamoxifen. [46]

Despite, lacking adequate scientific research that quantifies the level of risk in infants, most would argue for a precautionary approach to be taken in situations where there are potential developmental effects from the consumption of pharmacologically active compounds in infancy and childhood.[93] Additionally, a very large percentage (99%) of soy is genetically modified and also it has one of the highest percentage pesticides contaminations from all our foods.

Soybeans are rich in phytic acid, present in the bran or hulls of all seeds. It's a substance that can block the uptake in the intestinal tract of the essential minerals: calcium, magnesium, copper, iron and especially zinc. Phytic acid has been extensively studied; there are literally hundreds of articles on the effects of phytic acid in the current scientific literature. Scientists are in general agreement that grain- and legume-based diets high in phytates contribute to widespread mineral deficiencies in third world countries. Vegetarians consumming tofu and bean soy as a substitute for meat and dairy products risk severe mineral deficiencies. The results of calcium, magnesium and iron deficiency are well known while those of zinc are less known. [33,69,82]

There are abundant evidences that some of the isoflavones found in soy, including genistein and equol demonstrate toxicity in estrogen sensitive tissues and in the thyroid. Additionally, isoflavones are inhibitors of the thyroid peroxidase. Inhibition can be expected to generate thyroid abnormalities, including goiter and autoimmune thyroiditis,[24, 27] that documents the association of soy formula feedings in infancy and autoimmune thyroid disease.

CONCLUSION

Every day, an increasing number of new soy products appear on the stores' shelves. Some remarque soy protein content, while others claim a safety dose of isoflavones. Soybeans have the highest concentration of isoflavones in the Leguminosae family. Isoflavones are phytochemicals and phytoestrogens that can reduce cholesterol, the risk of heart disease, stroke, certain cancers and osteoporosis, relieve menopause symptoms, and resemble human estrogen in chemical structure, but with weaker effects. There is strong evidence that soy isoflavones reduce coronary heart diseases and stroke risk by lowering total and low-density lipoprotein cholesterol levels and by preventing arterial plaque formation, but it seems isoflavones alone do not improve cholesterol profiles. The synergistic effect of isoflavones and soy protein was demonstrated in studies involving both men and pre- or postmenopausal women.

Isoflavones may confer protection against the detrimental effects of human estrogen. Isoflavones can act as anticarcinogenic estrogen-receptor blockers. Their similar chemical structure enables the isoflavones to fit into receptor sites where the endogenous estrogens usually attached. This prevents the more powerful human estrogens, which can promote cancerous tumors, from binding to these receptors and exerting their full effects. Isoflavones from soy protein can lengthen the menstrual cycle, which also reduces estrogen exposure. Reducing estrogen exposure can, over the years, decrease breast cancer risk. There is hypothetical evidence that large amounts of isoflavones may stimulate tumor development, but there is no evidence that eating soy products is a problem for women with breast cancer or those with an increased risk for the disease. These women should be cautious with pure isoflavone supplementation until more is understood.

In conclusion, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several interdependent potential anticarcinogens; improved analytical methods are needed for simultaneous determinations of the content of soy-based materials. More basic research in the absorption, metabolism, physiology, and anticarcinogenic potential of isoflavones in humans should be conducted.

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