

Abstract

Between one-third to one-half of all breast cancers are steroid sensitive. Steroid-pathway enzymes (sulfatase, 17 β -hydroxysteroid dehydrogenases, aromatase and sulfotransferases) are thus prime candidates for therapeutic approaches based on the control of intracrine activity. Some phytoestrogens, ubiquitous in our diet, are inhibitors of these enzymes. Such a therapeutic potential has stimulated research and progress has been achieved during the last years. Complementary to previous reviews on

phytoestrogens, this contribution covers the estrogen pathway inhibition effects of these compounds and special attention will be given to isoflavonoids, flavonoids and lignans. Furthermore, the research on structurally-related compounds as therapeutic agents will be discussed briefly.

Key words

Phytoestrogens · aromatase · hydroxysteroid dehydrogenases · sulfatase · sulfotransferases · endocrine disrupters

Introduction

Over the past 15 years, there has been a tremendous increase in the number of papers published on the biological activities of phytoestrogens. The estrogen receptor binding is the best-documented biological action of phytoestrogens and, as estrogen agonists and antagonists, they can be classified as selective estrogen receptor modulators (SERMs) [1]. These compounds also have a diverse range of other biological effects including the potential to alter the biosynthesis of endogenous hormones through a number of pathways.

The high incidence of breast cancer in post-menopausal women and the lack of any correlation between the estrogen levels in plasma and the growth of breast cancer suggest that local estrogen synthesis plays an important role in the pathogenesis of estrogen-dependent breast cancer [2]. Two principal pathways are involved in the last steps of formation of 17 β -estradiol: (i) the

“aromatase pathway” which respectively transforms androstenedione (AD) to estrone (E₁) and testosterone (T) to estradiol (E₂) and (ii) the “sulfatase pathway” which converts estrone sulfate (E₁S) into estrone (E₁); estrone is then transformed into estradiol (E₂) by 17 β -hydroxysteroid dehydrogenase type 1. By analogy with a selected estrogen receptor modulator (SERM), the concept of a selective estrogen enzyme modulator (SEEM) as a therapeutic agent has recently emerged [3], [4]. The SEEM can control the enzymatic mechanisms involved in the formation and transformation of estrogens (Fig. 1).

This contribution covers the inhibition of estrogen pathways by phytoestrogens. In this way, previous reviews will be complemented [5], [6], [7].

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Bibliography

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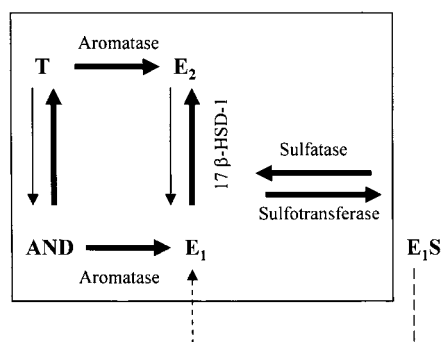


Fig. 1 Enzymatic steps involved in the synthesis of estrogenic steroids: the concept of selective estrogen enzyme modulator (SEEM; from Refs. [3] and [4]). 17β -HSD-1 = 17β -hydroxysteroid dehydrogenase type 1. **SEEM-I** inhibits the estrone sulfatase; **SEEM-II** inhibits the 17β -HSD-1; **SEEM-III** inhibits the aromatase activities; and **SEEM-IV** stimulates the estrone sulfotransferase activity. T: testosterone; AND: androstenedione; E_1 : estrone; E_2 : estradiol; E_1S : estrone sulfate.

bits the 17β -HSD-1; **SEEM-III** inhibits the aromatase activities; and **SEEM-IV** stimulates the estrone sulfotransferase activity. T: testosterone; AND: androstenedione; E_1 : estrone; E_2 : estradiol; E_1S : estrone sulfate.

Phytoestrogens: General Aspects

Dietary phytoestrogens are defined as natural chemicals present in our diet that can mimic or modulate the action of endogenous estrogens, usually by binding to estrogen receptors [6], [7]. Based on their chemical structure, phytoestrogens can be classified into four main groups, i.e., isoflavonoids, flavonoids, stilbenes and lignans (Fig. 2). To be complete, as mentioned recently by Cos [7], some terpenoids and saponins have been reported to exert similar effects.

Isoflavones, found in Leguminosae (soybeans in particular) constitute the largest group of natural isoflavonoids. In this subclass, the most thoroughly investigated and interesting compounds are genistein (**1**), daidzein (**2**), biochanin A (**3**) and formononetin (**4**). Coumestans represent a fully oxidized version of pterocarpan and are included in the isoflavonoids classification. The most representative, coumestrol (**10**), has been isolated from a few fodder and pasture plants (e.g., clover, alfalfa) belonging to the Leguminosae. Estrogenic activities of isoflavonoids have been widely studied [8], [9], [10], [11] and coumestrol is the phytoestrogen that has the higher binding affinity for the estrogen receptors [9], [11].

Over 4000 flavonoids have been identified in plant sources. They are usually subdivided into flavonols (found throughout the plant foods), flavanols (catechins, epicatechins, galocatechins, epigallocatechins), anthocyanidins, chalcones, flavanones and flavones. Chalcones, flavanones (citrus fruits) and flavones (spices and herbs) are described as minor flavonoids in spite of sometimes being present in food at a dietary significant concentration. Recent studies on the estrogenic activity of flavonoids *in vitro* revealed that the estrogenic potency of flavonoids is 10^{-2} to 10^{-5} -fold less than that of estradiol. However, 8-prenylnaringenin (**20**), a recently discovered flavanone from hops and beer, demonstrated a higher estrogenic activity than coumestrol or isoflavones [12], [13].

Stilbenes are 1,2-diarylethenes detected in relatively few plant families where they contribute to the resistance to microbial degradation and act as phytoalexins. The major dietary sources of stilbenes are grapes, grape juices, wine and peanuts. Among monomeric stilbenes, resveratrol (**24**) (3,5,4'-trihydroxystil-

bene), produced by plants as a defence mechanism against *Botrytis cinerea* has been identified as the major active compound and most of the studies about estrogenic/antiestrogenic activity have focused on it [7], [14].

Current research is focusing on a number of lignans recently identified in humans and several animals. Lignans are widely distributed throughout the plant kingdom, where they are involved in plant defences, and the major sources are the outer layer of cereals, mainly rye and flaxseed. They are converted into active phytoestrogens called "mammalian enterolignans" by the proximal colon microflora [15]. Both *trans*-2,3-bis(3-hydroxybenzyl)- γ -butyrolactone and 2,3-bis(3-hydroxybenzyl)-butane-1,4-diol, known respectively as enterodiol (**25**) and enterolactone (**26**) have been described as the major lignans. Lignans are closely related to phenolic estrogens and may function as weak estrogens or estrogen antagonists [16].

SEEM 1: Sulfatase Inhibitors

Sulfatases are a group of hydrolytic enzymes that catalyse the conversion of various sulfated compounds to their corresponding unconjugated derivatives [17]. Desulfation of estrone sulfate by estrone sulfatase (**SEEM 1**) represents an important step in the transformation of inactive steroids to estrogenic hormones. Quantitative determinations in breast cancer tissues indicate that the "estrone sulfatase pathway" is about 130 – 200-fold more important than the aromatase pathway [3]. Inhibition by isoflavone metabolites has been recently reported [18]. The most potent inhibitor was daidzein 7,4'-bisulfate (**7**) which exhibited an IC_{50} value of $0.8 \mu M$. Daidzein 7-O-sulfate (**5**), daidzein 4'-O-sulfate (**6**) and genistein 7-O-sulfate (**8**) inhibited sulfatase activity with IC_{50} values $< 20 \mu M$. These results are quite similar to values previously reported by Wong and Keung [19]. It seems that, since less than 0.1% of the conjugation products of isoflavones in humans are bisulfates [20], concentrations of daidzein 4',7-bisulfate, even in those consuming high soy/isoflavone-supplemented diets, would not be sufficient to influence sulfatase activity *in vivo* [18].

None of the unconjugated flavonoids and isoflavonoids tested by Harris were found to have any effect on sulfatase ($IC_{50} > 25 \mu M$) at concentrations likely to be achieved from the diet. This conclusion is the opposite to Huang's paper where it was reported that kaempferol (**11**), quercetin (**12**) and naringenin (**16**) act significantly as estrone sulfatase inhibitors [21].

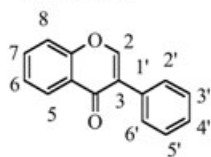
SEEM 2: 17β -Hydroxysteroid Dehydrogenase

The 17β -hydroxysteroid dehydrogenases (**SEEM 2**), key enzymes acting at the last step of androgen and estrogen formation, are nicotinamide adenine dinucleotide [NAD(H)]- and/or its phosphate form [NADP(H)]-dependent enzymes that catalyse the oxidation and reduction of 17β -hydroxy and 17 -ketosteroids in a positional and stereospecific manner [22]. 17β -HSD1 is expressed in steroidogenic tissues including estrogen target tissues such as normal and malignant breast tissues [23] and is a prognostic marker in breast cancer [24]. It catalyses predominantly the conversion of estrone to estradiol and can be considered to be pri-

Fig. 2 Chemical structures of phytoestrogens discussed in this review.

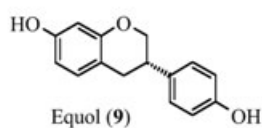
Isoflavonoids

Isoflavones



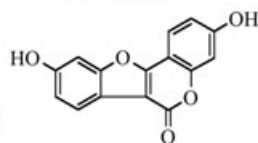
	5	7	4'
Genistein (1)	OH	OH	OH
Daidzein (2)	H	OH	OH
Biochanin A (3)	OH	OH	OMe
Formononetin (4)	H	OH	OMe
Daidzein-7-O-sulfate (5)	H	OSO ₃ ⁻	OH
Daidzein-4'-O-sulfate (6)	H	OH	OSO ₃ ⁻
Daidzein-4',7-di-O-sulfate (7)	H	OSO ₃ ⁻	OSO ₃ ⁻
Genistein-7-O-sulfate (8)	OH	OSO ₃ ⁻	OH

3-Phenylchromans



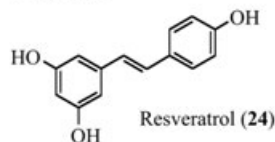
Equol (9)

Coumestans



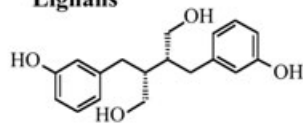
Coumestrol (10)

Stilbenes

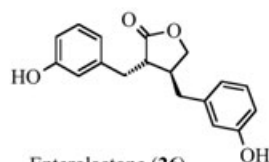


Resveratrol (24)

Lignans



Enterodiol (25)



Enterolactone (26)

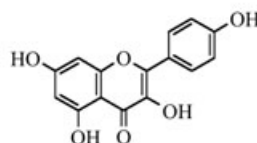
	5	7	8	3'	4'
Naringenin (16)	OH	OH	H	H	OH
Hesperetin (17)	OH	OH	H	OH	OH
Eriodictyol (18)	OH	OH	H	OMe	OH
Naringin (19)	OH	hesp*	H	H	OH
8-prenylnaringenin (20)	OH	OH	CH ₂ -CH=C(CH ₃) ₂	H	OH

* : neohesperidose

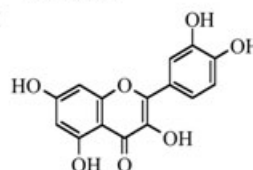
	5	7	3'	4'
Chrysin (21)	OH	OH	H	H
Apigenin (22)	OH	OH	H	OH
Luteolin (23)	OH	OH	OH	OH

Flavonoids

Flavonols

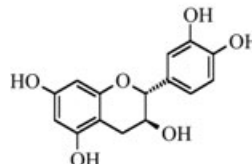


Kaempferol (11)

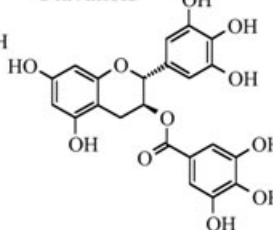


Quercetin (12)

Flavanols

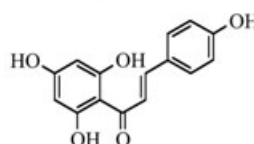


Catechin (13)



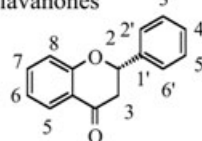
Gallicocatechin gallate (14)

Chalcones



Naringenin chalcone (15)

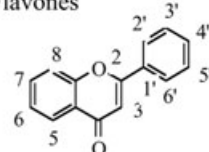
Flavanones



	5	7	8	3'	4'
Naringenin (16)	OH	OH	H	H	OH
Hesperetin (17)	OH	OH	H	OH	OH
Eriodictyol (18)	OH	OH	H	OMe	OH
Naringin (19)	OH	hesp*	H	H	OH
8-prenylnaringenin (20)	OH	OH	CH ₂ -CH=C(CH ₃) ₂	H	OH

* : neohesperidose

Flavones



marily responsible for estradiol biosynthesis in gonads and in peripheral tissues. Several phytoestrogens, i.e., genistein (1) (IC₅₀ ~ 1.2 μM), daidzein (2) (IC₅₀ ~ 1.2 μM), coumestrol (10) (IC₅₀ ~ 0.12 μM), naringenin (16) (IC₅₀ ~ 1.2 μM) and apigenin (22) (IC₅₀ ~ 0.12 μM) were found to inhibit significantly estrone reduction catalysed by purified 17β-HSD1 or cultured T-47 D cells [25]. Le Bail [26], [27], [28] found almost similar results although these authors used human placental microsomes instead of purified enzyme for inhibition studies. On the contrary, Vinh [29] found no inhibitory activity for genistein even at 100 μM.

Recent works were focused on type 5 17β-HSD inhibition. This isoform is expressed in reproductive and hormone target tissues including breast tissues [24] and catalyses the conversion of androstenedione to testosterone [30]. Phytoestrogens, i.e., biocha-

nin A (3), coumestrol (10) and quercetin (12), are potent inhibitors in the micromolar range [31], [32].

SEEM 3: Aromatase

Aromatase converts the Δ⁴-3-one ring of C₁₉ androgenic steroids to the phenolic A ring of estrogen. The gene expressing cytochrome P-450arom, referred to as CYP 19, is part of the cytochrome P-450 superfamily. This enzyme is expressed in numerous tissues such as placenta, ovarian granulosa cells, testicular Leydig cells, adipose tissue, liver and brain [33] and the aromatase activity in breast cancer tissues has been demonstrated to be higher than in normal tissue [34]. The biochemical mechanism of aromatase has been studied extensively and consider-

able progress has been made in understanding the reactions catalysed by this enzyme. Hypotheses have been advanced for the mechanism of the third oxidation step in androstenedione conversion, the Akhtar peroxy intermediate model remaining the most accepted hypothesis [35]. Several main groups of phytoestrogens, e.g., flavones, flavanones and lignans modulate aromatase activity *in vitro*. An early study made by Kellis and Vickery [36] reported inhibition by chrysin (**21**) ($IC_{50} = 0.5 \mu M$) and apigenin (**22**) ($IC_{50} = 1.2 \mu M$). Numerous studies had confirmed the potential of flavones as aromatase inhibitors [26], [28], [37], [38], [39], [40], [41]. Potent inhibition of aromatase occurred with flavanones such as naringenin (**16**), hesperetin (**17**), erodictyol (**18**) and naringin (**19**) or flavanone precursors, i.e., naringenin chalcone (**15**), in the 1 – 10 μM range [26], [28], [39], [40], [42], [43]. However, natural flavones are consistently more potent inhibitors than flavanones [41] and all studies were made with racemic mixtures of flavanones. Nevertheless, a computer modelling [42] revealed that only the 2S-configuration isomer of naringenin could bind to the active site and the values for the natural S-configuration isomer may be lower than those reported. Other flavonoids such as flavanols [catechin (**13**), gallic catechin gallate (**14**)] [44], [45] or flavonols [kaempferol (**11**), quercetin (**12**)] are inactive or weak inhibitors [37], [38], [40].

The aromatase inhibition effect of isoflavonoids has been examined and these compounds were reported to be inactive in several studies [26], [27], [38], [39], [42], [46]. But, recently Almstrup [43] found that biochanin A (3) and formononetin (4) are aromatase inhibitors at low concentrations; moreover, except for genistein, all the studied isoflavonoids were both aromatase inhibitors at low concentrations (< 1 μM) and estrogenic at higher concentrations (> 1 μM) resulting in U-shaped dose-response curves. To be complete, Wang [38] and Adlercreutz [47] studied the inhibition of human aromatase by mammalian lignans. Lignans were shown to be weak inhibitors, e.g., enterolactone (**26**) ($IC_{50} = 14 \mu M$ [47], $K_i = 14.4 \mu M$ [38]) or inactive, e.g., enterodiol (**25**) [38], [47].

Computer modelling and site-directed mutagenesis have revealed the structural features for flavonoids to inhibit aromatase [42]. Flavones bind to the active site in an orientation in which rings A and C of flavones mimic rings C and D of androstenedione. By doing so, the *p*-phenyl substituent (ring B) of flavones is oriented in a similar position to that of the substrate's ring A. The C-4 keto group of flavones points towards the haeme prosthetic group indicating that this group is essential for the inhibition [36], [48]. Isoflavones have the 4'-hydroxyphenyl group at position C-3, greatly reducing the ability to bind and inhibit aromatase. This computer modelling revealed that the 3-hydroxy group of flavonols significantly changes the orientation of ring B, resulting in a large decrease in the ability to inhibit aromatase.

Recently, Saarinen evaluated the *in vivo* effect on aromatase of selected flavonoids using uterotrophic tests in immature rats [49]. Phytoestrogens such as naringenin (**16**), apigenin (**22**) or luteolin (**23**) neither induced uterine growth nor reduced estrogen- or androgen-induced uterine growth. According to these authors, the inability of these flavonoids to inhibit aromatase may be due to their relative poor absorption and/or bioavailability.

SEEM 4: Sulfotransferases

The concept that steroid-modifying enzymes such as estrogen sulfotransferase (EST) have critical physiological roles in modifying steroid hormone action at target cells has been emerging based upon multiple studies in different systems [50]. Estrogen sulfotransferase (SULT 1E1) belongs to the family of cytosolic sulfotransferases [51] and catalyses the transfer of a sulfonate radical to the 3-hydroxy group of estrogens using 3'-phosphoadenosine 5'-phosphosulfate as a donor for the sulfonate group; it is well accepted that estrogen sulfonation diminishes the estrogen receptor binding activity (SEEM 4). Otake reported that estrogen sulfotransferase (SULT 1E), from both human recombinant sources and human mammary epithelial cells, was inhibited competitively by quercetin [12] and resveratrol [24], with IC_{50} values of 0.61 μM and 0.36 μM , respectively, using recombinant estrogen sulfotransferase [52]. Phenol sulfotransferases (SULT 1A1/2, SULT 1A3, SULT 2A1) [53], [54] also sulfate estrogens at physiologically relevant concentrations and may represent a route for estrogen sulfation in some mammary tumour cells lines that lack estrogen sulfotransferase. SULT 1A1 was reported to sulfate phytoestrogens, e.g., isoflavonoids (**1**, **2**, **4**, **9**) and flavonoids (**11**, **12**, **13**, **16**, **17**, **21**, **22**, **23**) with $IC_{50} \ll 0.5 \mu M$ [18], [55], [56], [57] and genistein (**1**), daidzein (**2**), quercetin (**8**) and luteolin (**23**) inhibited SULT activity against a physiological concentration of estradiol with IC_{50} values in the low micromolar range [57].

Dietary Phytoestrogens: Potential Selective Estrogen Enzyme Modulators?

Only partial information is available on the quantities of dietary phytoestrogens that are consumed daily throughout the world [58] and the bioavailability is clearly a crucial factor influencing the biological activity of these compounds [58], [59], [60], [61]. After absorption mainly as glycosides and hydrolysis of the sugar moiety, phytoestrogens are re-conjugated predominantly to glucuronic acid and to a lesser degree to sulfuric acid. Only a small portion of the free aglycone has been detected in blood, demonstrating that the rate of conjugation is high. Circulating levels of phytoestrogens (conjugated and unconjugated forms) in adults may reach 4 – 6 μM after ingestion large amounts of fruits, vegetables or soya derivatives [58], [60], a level which seems insufficient to inhibit *in vivo* aromatase and 17 β -hydroxysteroid dehydrogenase. This hypothesis could be contradicted in at least two cases: the ingestion of high dose dietary supplements (>> 50 mg phytoestrogens/day) and the special case of infants. Concentrated isoflavones are available in pill form and are sold extensively in health food stores and on the internet. Recently, the ability of chrysin to inhibit aromatase has led to its marketing as a high-dose dietary supplement for body building and is subject to industrial development [62]. Soy milk is consumed in large quantities by babies or infants who are allergic to cow milk. Isoflavone intake can reach, when expressed relative to body weight, 5 – 10-fold the dose shown to exert a physiological effect on the hormonal regulation of women's menstrual cycles [63].

Sulfotransferases are at least 10 times more sensitive to inhibition by flavonoids or isoflavonoids than aromatase or 17 β -hydroxysteroid dehydrogenase and these enzymes may be inhibited

ited by concentrations that occur *in vivo*. If the effects of phytoestrogens were exerted in breast tissues, phytoestrogen ingestion might contribute to an unfavourable ratio of estrogen/estrogen sulfate in tissues leading to the proliferation of hormone-dependent tumour cells [18]. Hence, as underlined by Harris [18], there is an urgent need for further work to clarify the influence of phytoestrogens on the sulfation/desulfation processes.

Phytoestrogens and, more generally, natural compounds are a mine for drug design and many of the molecules described can be viewed as templates for molecular modifications [15], [64], [65], [66]. Fig. 3 summarizes the recent developments in the research of structurally-related compounds as therapeutic agents. The development of dual inhibitors (sulfatase/aromatase, for example) could constitute a priority approach [68].

Another major question for the future is the biological evaluation of new emerging phytoestrogens such as prenylated flavonoids from hops [77], [78], terpenoids from black cohosh [79], [80]

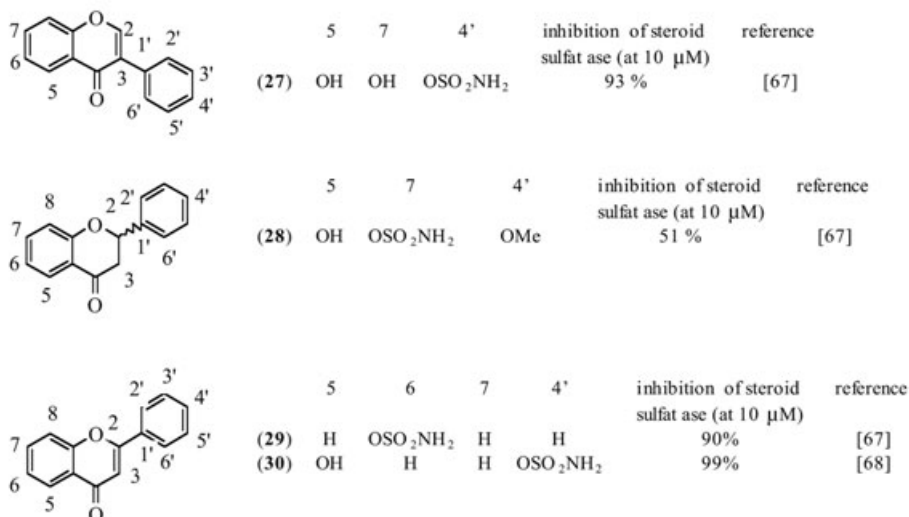
and liquorice [81], [82], saponins from *Tribulus terrestris* [83] and coumarins from Dong Quai [84] (Fig. 4). At this time, only few papers have appeared on steroid biosynthesis inhibition by such compounds [31], [85], [86], [87], [88], [89].

Concluding Remarks

Phytoestrogens are endocrine disrupters according to the definition of the European Commission. In view of the current data, phytoestrogens appear to have steroidal potency not only by acting on steroids receptors but also by modulating steroidogenesis enzymes; there are indications that potential risks may be present, but the magnitude of these risks cannot be determined exactly due to the limited or controversial data available. As concluded recently by Cos in a previous review [7], it is still premature to recommend specific amounts of dietary phytoestrogens.

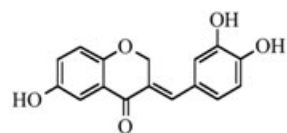
Fig. 3 Structurally related compounds as potent selective estrogen enzyme modifiers (SEEMs).

SEEM 1 : sulfatase inhibitors



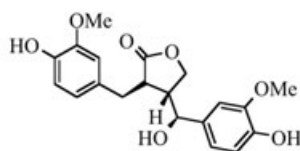
SEEM 2 : 17β-HSD inhibitors

(see also Refs. [69], [70], [71])

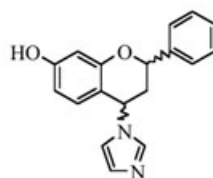


(31) inhibition of 17 β-HSD : 50,2% [72]

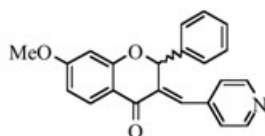
SEEM 3 : Aromatase inhibitors



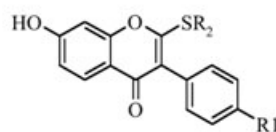
(32) enterolactone precursor [73]



(33) IC₅₀ = 0.041 μM [74]



(34) IC₅₀ = 0.62 μM [75]



(35) R₁ = OMe ; R₂ = (4'-pyridyl)methyl
IC₅₀ = 0.22 μM [76]

(36) R₁ = OH ; R₂ = (4'-pyridyl)methyl
IC₅₀ = 0.28 μM [76]

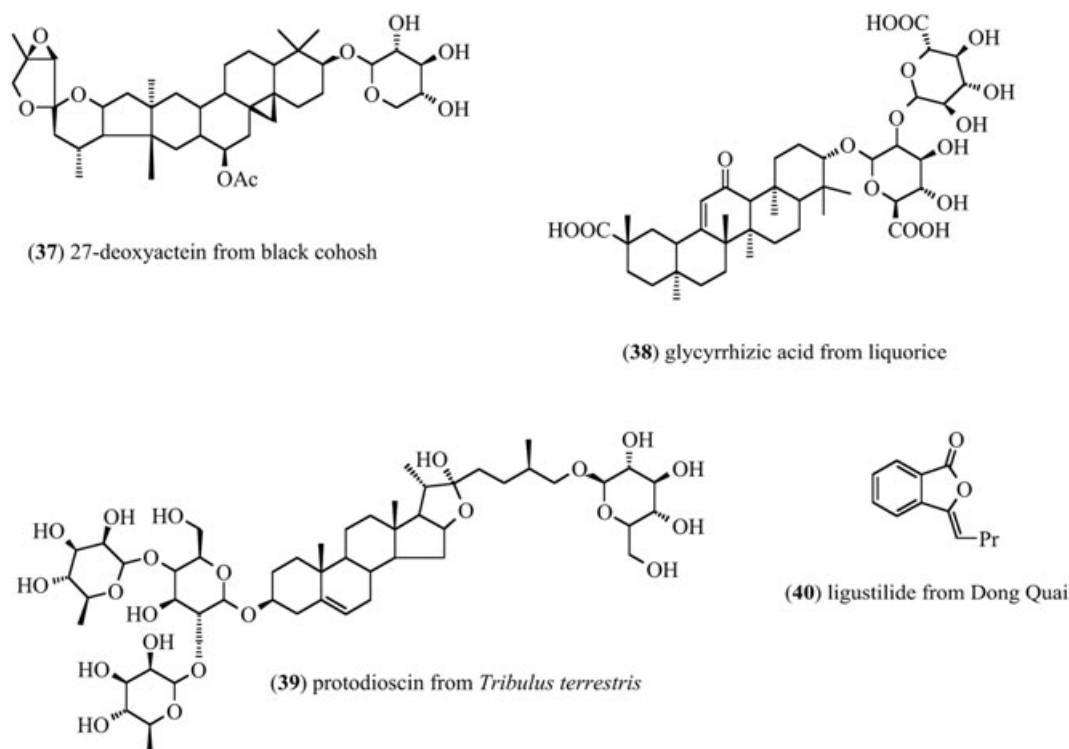


Fig. 4 Examples of emerging phytoestrogens.

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