Recent Advances in the Development of Phytoestrogens and Derivatives: An Update of the Promising Perspectives in the Prevention of Postmenopausal Diseases

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Abstract: Phytoestrogens constitute a promising alternative in the treatment of diseases associated with menopause. Nevertheless, the lack of data concerning their pharmacology and their toxicology requires use precautions. After reminding the pharmacology of estrogen receptors, this review outlines the estrogenicity and the therapeutic potentialities of phytoestrogens according to their structure.

KeyWords: Phytoestrogens, estrogen receptor, menopause, pharmacology, therapeutic, structure, SAR.

INTRODUCTION

Estrogen receptors (ERs) are members of the superfamily of intranuclear receptors such as glucocorticoid receptors, androgen receptors, progesterone receptors, thyroid hormone receptors, retinoid receptors or vitamin D receptors [1, 2]. They are involved in the growth and in the development of a wide range of tissues. In women, ERs play a critical role in the maintenance of primary and secondary sexual characters as well as in the control of the reproductive system. In physiological conditions, the best known endogenous ligand for ERs, 17β-estradiol (E_2) (Fig. (**1**)) acts not only on sexual functions but also on non-reproductive organs such as bones [3], the cardiovascular system [4, 5], or the central nervous system (CNS) and principally in the maintenance of cognitive functions [6].

In postmenopausal women, E_2 loss is often accompanied by some disorders of the hormonal metabolism and of the urogenital sphere, leading not only to the stop of the menstrual cycle, but also to the disruption of non reproductive functions, particularly by decreasing the calcium/phosphate complexes and the protein matrix of bones, leading to osteoporosis and fractures of wrist, spine, proximal femur, distal forearm or hip [5], heart diseases [4, 5], autoimmune disorders [7, 8, 9], mode disorders, or cognitive deficiencies [6].

To fight these disagreements associated with menopause, women ask often to their physician E_2 or combined progestin and estrogen for hormonal replacement therapy (HRT) [8]. Unfortunately, the use of E_2 increases significantly uterine bleeding and the risks of breast cancers or endometrial cancers, genomic alterations being also implied in the cell degeneration process [10]. Hence the growing necessity to develop new derivatives issued from environmental estrogens less active than E_2 [7] and more selective in terms of receptor and tissue.

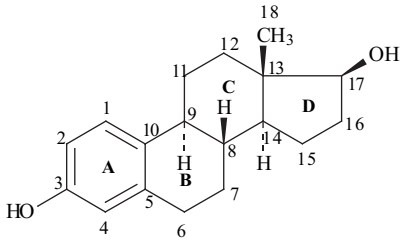

Fig. (1). 17β-estradiol (E_2).

Biochemical mechanisms implying estrogens are very complex, because of their interaction with receptors and associated proteins such as coactivators or corepressors, which are components of the transcriptional machinery [2, 11]. First and foremost, ERs involved in the intranuclear mechanisms implying E_2 exist under different isoforms. These isoforms are called ERα [12], ERβ [11, 13, 14] and ERγ [15, 16], discovered in 1990, 1996 and 2000 respectively. ERs are located not only in the nucleus but also in the cytosol and the cytoplasmic membrane [17]. When E_2 is liganded to the hydrophobic pocket of the receptor, i.e. the ligand binding domain (LBD) [18], a highly conserved 450 $Å^3$ region of the receptor [19], heat shock proteins (HSP-56, HSP-70, HSP-90) leave the receptor by an ATP-dependent mechanism [20, 21] before phosphorylation [1]. Then, dimerization involving helix H8, H10, H11 and the loop between H9 and H10 activates the receptor [18, 21-23]. Structure of the ligand-bounded ER

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orientates the C-terminal helix H12 of the LBD - implying conformational changes of the receptor - to an appropriate direction [18, 23-25] for transcriptional machinery activation in the case of agonist ligands. The ligand-dependent transcriptional autonomous function AF-2 located in the C-terminal LBD binds to specific DNA sequences (while the autonomous function AF-1, constitutively active, is located in the N-terminal LBD) [2, 26]. This interaction with DNA is carried out by a specific domain of the receptor called DNA binding domain (DBD) [27-29]. The DBD is structurally well defined and contains two tetrahedral zinc ions, which form a complex with four cysteine residues per ion [1, 28, 29]. Then, coactivators such as TFIIs (transcriptional factors) or TBP (TATA binding protein) interact with the dimerized ERs [1, 29]. TBP, required by RNA polymerase, leads to the interaction of the activated ER with the proximal promoter TATA box - a specific region of DNA principally constituted by thymine and adenine - required before transcription [1]. At the same time, a wide range of coactivators such as the CBP/P300 protein complex or the SRC-1/TIF2 protein complex interact with ERs through complex biochemical cascades [30]. Then, the activated ERs interact in a high selectivity with a DNA site called estrogen response element (ERE) [28, 31]. At last, the cellular response is characterized by cell proliferation, the transcription of prooncogens such as *c-fos* or *c-jun*, and the production of coactivators or growth factors involved in the cell proliferation. It is interesting to outline that the functionality of the ER is regulated by different endogenous ways, as recently shown [32-35].

The particularity of ERs is their ability to accept a wide variety of derivatives that mimic E_2. It is the case of some environmental compounds, such as pollutants, industrial chemicals or pesticides, able to act on the hormonal metabolism of different vertebrates and invertebrates [36]. Among these derivatives, plant-derived estrogen-like compounds called phytoestrogens exist in diet and in numerous plants and fruits [37, 38]. Among them, we can cite nuts, spices, soybean, peas, beans, spinach, clovers, alfalfa, oat, barley, rye, wheat, hops, cabbage, liquorice, or coffee [39-41]. These phytoestrogens, particularly their aglycones [42], are often 100 to 1000 fold less active than E_2 [7, 43]. They exercise their action through different pathways, the direct estrogenic mechanism through ER being the most interesting [42]. Moreover, in an ageing society, women are tendency to survive longer in an age exposed to osteoporosis and cardiovascular diseases (CVD). For this reason, phytoestrogens are of a growing interest because of their physiological activity against the disagreements associated with menopause in long-term treatment and their chemopreventive activity. Moreover, they do not exhibit a growing risk of endometrial cancers or breast cancers [8] because of their low uterotropic activities [44]. It is probably one of the the best convincing argument to develop phytoestrogen derivatives. This review focuses on the therapeutical potency of phytoestrogens by presenting the different structures developed during these last years and by presenting their binding affinity and their hormonal activities according to their structure. At last, the most important features required for phytoestrogen binding to ER would be detailed.

THERAPEUTIC POTENTIALITIES AND PHARMACOLOGY OF PHYTOESTROGENS

(A) Phytoestrogens and Bone

Bone is a living tissue for which the loss of E_2 associated with menopause induces a net fragility, conducting to multiple fractures. This postmenopausal pathology is called osteoporosis, a major public health problem. Even if the pharmacology of estrogens in bone has not been yet totally elucidated, it is known that among the low level of ERs in bone, the most frequent isoform is the receptor β located in osteoblast-like cells [3]. Moreover, it has been shown that phytoestrogens present not only moderate bone trophic properties [8, 45] but also significant anabolic effects on bones and a reduction of bone resorption [46, 47] as it has been demonstrated with E_2 [46, 48]. More precisely, phytoestrogens are active on bones by two different mechanisms involving a stimulatory effect on osteoblasts and an inhibitory effect on osteoclasts. Moreover, it is interesting to outline that phytoestrogens are not only present in bones but also exhibit a significant preference for hERβ, with an affinity 7 to 30 times more important than that for hERα, probably because of the structural differences of the LBD (ie, Leu 384 for hERβ instead Val 408 for hERα, amino acids alignment). From these differences, the positioning of the helix H-12 when ligand binds to the receptor may be a decisive factor for the estrogenic activity [1, 11, 23, 40]. These structural differences are accompanied by significant changes of the total volume of the LBD of the two isoforms, 450 $Å^3$ for hERα *vs.* 390 $Å^3$ for hERβ [11, 18]. This observation could explain why substitutive estrogen therapy presents potent preventive effects on osteoporosis progression by an osteoblastic stimulatory activity. Moreover, a recent study from Gao *et al.* [49] has shown that the partial agonist genistein possesses an inhibitory activity at 10 μM. This leader compound of the isoflavonic phytoestrogens, may be mediated *in vivo* by a calcium-dependent mechanism on osteoclasts, inducing by this way an apoptosis process, which could explain the antiestrogenic activity of some phytoestrogens. This mechanism seems to be dose-dependent and time-dependent.

Hence, phytoestrogens may be assimilated in part to SERMs since they present both estrogenicity and antiestrogenicity. Moreover, their specificity of action relies on tissue selectivity without increasing the risk of endometrial or breast degeneration conducting undoubtedly to cancers [47, 50, 51].

(B) Phytoestrogens and Breast

Phytoestrogens may exert both agonistic and antagonistic properties on both normal and cancer breast cells, probably by different modes of action. More significative results about a potential benefit of phytoestrogens were recorded in studies concerning the prevention of breast cancer [52, 54]. Thus, eating soy phytoestrogens has been reported to be associated with a decrease in the apparition of estrogenic molecules that may cause mutations (i.e., forms with free radicals, some quinolones) and non estrogenic molecules

(nitrites, nitrates, food additives, etc.) potentially leading to cancer. Phytoestrogens may also affect communication pathways between cells, prevent formation of blood vessels to tumors and alter processes involved in DNA processing for cell multiplication [55, 56].

Soy phytoestrogens could also modify breast cancer risk by changing production and/or elimination of reproductive hormones, especially estrogens. Even results of such studies aimed to examine hormone changes among women eating phytoestrogens are not very consistent, recent data suggest a small decrease in the levels of estrogens. (One of the mechanisms of enhancing breast cancer risk is the ability of the estrogens to increase growth of milk ducts from which most breast cancers arise). On the other hand, several but not all studies examining the effect of soy phytoestrogens on breast growth suggested that a high proportion of these compounds have a weak estrogen-like effect. Hence, more studies are needed to evaluate the possible effects of soy phytoestrogens on hormone levels and breast growth [55, 56, 57].

While significative data concerning the potential estrogenic effect of phytoestrogens on breast cells is still lacking, data relevant to their uterotropic activity are already available. It seems likely that some of these data may also concern breast cells. In this regard, it has been demonstrated that direct interaction of phytoestrogens with the nuclear ER is not an absolute requirement for the induction of specific estrogen-like effects. Estrogen-like effects could be induced *via* an indirect action on the pituitary gland or a non-genomic activation of signal transduction cascades, depending upon an increase of intracellular calcium levels, or an activation of MAP-kinases requiring or not ERα or β [52, 58, 59].

In fact, phytoestrogens as well as pesticides, phtalates, and a wide range of diverse phenolic derivatives could be subdivided into distinct categories according to their biological properties [54]. The first group comprises substances, which possess some uterotropic activity and mimic the action of E_2; this group of compounds can be classified as weak estrogens. The second group is characterised by a relatively strong estrogenic activity with a different gene expression fingerprint compared to that of ethinylestradiol. Such compounds are able to induce estrogen-regulated genes in the uterus. However, analysis of gene expression revealed a very specific profile of molecular action in response to compounds, which cannot be detected by judging the uterotropic response alone [54]. The third class of compounds shows a low estrogenic activity able to modulate uterine gene expression by molecular mechanisms differing from that of natural estrogens. It is possible that action of these compounds involves a crosstalk with other signal transduction pathways, binding to other steroid hormone receptors or direct action on the pituitary gland [52].

Treatment of MCF-7 breast cancer cells with a panel of phytoestrogens may generate distinct effects on ER levels (ERα isoform). For example, it has been reported that the administration of coumestrol reduces the receptor content as well as its mRNA, as found after exposure to E_2. Hence, coumestrol exerts molecular properties, which are very similar to E_2 and can be classified as a pure agonist in this test system. In contrast, genistein shows no effect on mRNA ER but decreases ER level as found with the partial antiestrogen raloxifene. Therefore, genistein could be classified as a partial antiestrogen [54, 55, 57]. The effective down regulation of the ERα content of treated cells could serve as a mechanistic clue for the growth inhibitory action of these phytochemicals. However, reported non-ER inhibitory actions must also be considered. Indeed, it is now established that flavonoids at high concentrations exert antiproliferative activity through ER-independent mechanisms [58]. For example, genistein at 1 μM stimulated growth in MCF-7 cells, while at 10 μM it was shown to arrest the growth of six cell lines (ER^+ and ER^-) with some variations according to cell type and/or medium requirements [53]. As events that may contribute to the growth inhibitory effect of the compound, other investigations suggests induction of apoptosis, G_2 cell cycle arrest and inhibition of *c-fos* expression, AP-1 transactivation or ERK phosphorilation [52, 58, 59]. Also, genistein decreases tyrosine phosphorilation induced upon treatment with transforming growth factor-α [52, 53, 55]. Hence, consumption of genistein may reduce breast cancer onset. However, studies are still required to assess whether or not genistein as well as other phytoestrogens act in a similar way in the complex environment of an organism.

(C) Phytoestrogens and Lipid Metabolism

Estrogens exert significant effects on lipids and lipoproteins [60]. The activity of phytoestrogens on lipid metabolism is in close contact with their activity on the cardiovascular function [8, 61]. Even if some contradictory results have been reported [62], phytoestrogens seem to decrease levels of LDL cholesterol, total cholesterol, lipoprotein (a), triglycerides and to increase apolipoprotein A1 and slightly HDL cholesterol [8, 46, 47, 60, 61, 63, 64]. This tendency might lower the risk of CVD by 25% [47]. Nevertheless, their activity on lipid metabolism is less important than their cardiovascular protective effect. We can note that phytoestrogens exert their antioxidant effects on lipid metabolism by reducing LDL oxidation [65, 66] and by inhibiting in part the glucose mediated LDL oxidation as shown by genistein or its metabolite equol [67, 68].

(D) Phytoestrogens and Heart

It is evidence that phytoestrogens as well as estrogens present a promising alternative to protect postmenopausal women against CVD [5, 8, 61, 69] particularly against coronary heart disease such as smooth muscle cells proliferation-dependent coronary atherosclerosis, the main cause of death among women after sixty years old [65, 69, 70]. This heart protective activity, is due in part to an endothelium-dependent relaxation [71] and to the nitric oxide (NO) release mediated through the stimulatory effects of phytoestrogens on the nitric oxide synthase (NO synthase) [72]. The decrease of thromboxane A_2 or endotheline on the one hand [47], and the L-type calcium channels blocking activity in cardiac myocytes on the other hand [73, 74] result

from the cardioprotective activity of phytoestrogens. Nevertheless, some contradictions exist between *in vitro* and *in vivo* studies. Actually, Mishra *et al.* [71] have shown that daidzein and genistein, two phytoestrogens of reference, exhibit a significant endothelium-dependent relaxation in rats with EC_{50} at 5.7 μM and 3.7 μM respectively, whereas Simons *et al.* [75] have clearly shown no activity of these two phytoestrogens on the endothelial function in postmenopausal women. Such contradictory results may be due to the high and unclear complexity of the cardiovascular protective effects of estrogens [6].

(E) Phytoestrogens and Vasomotricity

Despite contradictory results, the activity of phytoestrogens on the vasomotor response seems to decrease weakly hot flushes [8]. In this context phytoestrogens act as calcium channel blockers on vascular smooth muscle, rich in ERα [61, 72, 74] and as stimulating NO synthase derivatives. Actually genistein inhibits L-type calcium channel on isolated rabbit coronary arteries at 2 μM, pharmacological mechanisms of estrogens on the vasomotricity being similar to those of estrogens on the cardiovascular system [72].

(F) Other Activities of Phytoestrogens

Phytoestrogens are active on others non reproductive organs. It is the case of the CNS on which they exert a neuroprotection. Phytoestrogens act on brain by a mechanism regulating the hypothalamic calcium-binding proteins level, a peptide involved in some calcium-dependent mechanisms such as apoptosis associated with neurodegenerative diseases [76]. Nevertheless, the mechanisms by which phytoestrogens act on CNS are still unclear.

Phytoestrogens act not only through ERs, but also by other mechanisms which could explain their antiproliferative potential [77].

The first type of non ER-dependent targets is charactedrized by the 3β-hydroxysteroid dehydrogenase Δ_5/Δ_4 isomerase (3β-HSD) and the 17β-hydroxysteroid dehydrogenase (17β-HSD) both involved in the metabolism of steroids. As evocated by Le-Bail *et al.* [78], the antiproliferative activity of phytoestrogens is in favour of an antiproliferative activity mediated by their anti-3β-HSD and their 17β-HSD activities (particularly 17β-HSD type 5 [79]) two microsomial enzymes involved in the biosynthesis and in the metabolism of E_2. Actually, most of phytoestrogens present an IC_{50} able to inhibit 17β-HSD type 5. Zearalenone, coumestrol, quercetin or biochanin A present an IC_{50} between 2 μM and 14 μM for the most active compounds, with a weak difference between the inhibition of 17β-HSD type 5 involved in the reduction of androstenedione to testosterone, and the 17β-HSD type 5 involved in the oxidation of androstenediol to androsterone [79]. Nevertheless, 17β-HSD is less inhibited by phytoestrogens than aromatase which is significantly inhibited by phytoestrogens. The most potent compounds are flavonoids with a phenyl ring located at the position 2, i.e. flavones (Fig. (**2**)), as shown by chrysin and apigenin which exhibit IC_{50} of 0.7 μM and 2.9 μM respectively, brain aromatase being not concerned [78, 80].

An other target of phytoestrogens is tyrosine kinase. Actually, phytoestrogens represent competitive inhibitors at the ATP-binding site of the protein tyrosine kinase (PTK) activity of the epidermal growth factor (EGF) receptor, a key enzyme involved in the cell cycle and indirectly in the steroidogenesis [42, 81]. Actually, genistein and quercetin exhibit an inhibition of the protein tyrosine kinase activity of EGF receptor derived from A431 cells of 63% and 30% respectively at 150 μM, genistein exhibiting such activity at 2.6 μM [72, 82]. By this way, the antioxidant activity of phytoestrogens could be attributed in part to the tyrosine kinase-regulating properties of some phytoestrogens and the arrest of mitosis. It is interesting to note that these antioxidant activities protect DNA against damages induced by oxidative stress [68] and proliferative activity [66]. It is why genistein has revealed promising results in the treatment of leukemia [83].

At last, topoisomerase II, an enzyme involved in cell proliferation, constitutes also a potential target for phytoestrogens such as flavonoids and coumestans [84, 85]. Breithofer *et al.* [86] and Cotroneo *et al.* [87] have recently shown that phytoestrogens could exhibit their antiproliferative activity by regulating the expression of ERs. Nevertheless, the implication of ERs in their antiproliferative activity seems marginal as shown by Singh *et al.* [88]. This observation is especially true that the hydrophilicity of flavonoids increases.

CHEMISTRY OF PHYTOESTROGENS

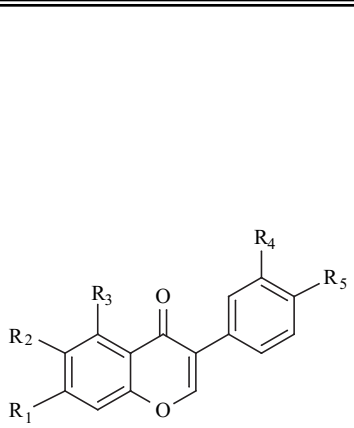
Phytoestrogens are natural plant or fungi based phenolic compounds divided into five chemical categories: the first, constituted by the flavanones, flavones and isoflavones, the second constituted by coumestans, the third, constituted by fungi based resorcylic acid lactones, the fourth is represented by chalcones, and finely, the class of lignans [50, 70, 73, 77, 81, 83, 89, 90].

(A) Isoflavones, Flavones and Flavanones

Flavonoids constitute the most diversified family of non-steroidal estrogen-like compounds. Such compounds being present in a wide variety of plants, many of them have been extensively evaluated [81].

Isoflavones are characterized by the presence of the phenyl ring at the position 3 (table **1**), the best required position for estrogenic activity. The presence of phenolic hydroxyls at the positions 3', 4', 5 and 7, R_4, R_5, R_3 and R_1 respectively in table **1**, are required to optimise their estrogenic activity [81]. Nevertheless, the 3'-methoxy derivative (biochanin A, estrogenicity 95%) or the 4'-hydroxy derivative (genistein, estrogenicity 100%) act both indiscriminately *in vivo* probably because of their

Table 1. Estrogenic Activity of Isoflavones

Entry	Compound	Trivial name	R_1	R_2	R_3	R_4	R_5	Pharmacological properties	Cell line	Ref.
1		Genistein	OH	H	OH	H	OH	Proliferation*: 62.8% (at 10 nM) luciferase* : 4.9% EC_{50} = 0.60 μM IC_{50} = 15.1 μM IC_{50} = 0.48 μM CAT*** = 988±296	MCF-7 MCF-7 MCF-7 MCF-7 T47D HeLa	[78] [78] [78] [82] [108] [90]
2			OH	H	OH	H	OCH_3	CAT = 331±77	HeLa	[90]
3		Biochanin A	OH	H	OH	OCH_3	H	Proliferation*: 47.6% (at 10 nM) luciferase* : 0.1% Estrogenicity**: 95% IC_{50} = 0.5 μM CAT = 331±77	MCF-7 MCF-7 BT-474 Yeast HeLa	[78] [78] [84] [43] [90]
4		Daidzein	OH	H	H	H	OH	Proliferation*: 9.6% (at 10 nM) Estrogenicity** : 55% CAT*** = 469±74	MCF-7 BT-474 HeLa	[78] [84] [90]
5		Formononetin	OH	H	H	H	OCH_3	Proliferation.*: 3.6% (at 10 nM) Luciferase* : 0.9% CAT*** = 287±96	MCF-7 MCF-7 HeLa	[78] [78] [90]
6		Prunetin	OCH_3	H	OH	H	OH	Proliferation.*: 32.5% (at 10 nM) Luciferase* : 1.0%	MCF-7 MCF-7	[78] [78]
7			OH	H	H	OH	OH	Proliferation.*: 1.6% (at 10 nM) Luciferase* : 0.3% CAT*** = 86	MCF-7 MCF-7 HeLa	[78] [78] [90]
8			OH	OH	H	H	OH	CAT*** = 63	HeLa	[90]

*Proliferation and transcriptional activity compared to E_2 = 100% (EC_{50} = 1 nM).

**Estrogenicity compared to genistein (100%) by dosing the estrogen-regulated protein *p*S2.

*** CAT: The estrogenicity is obtained using HeLa cells transfected by the plasmid pERE-TK-CAT (chloramphenicol acetyl transferase). These assays are expressed in terms of pmoles/min – mg protein (ethanol vehicle: 82).

biotransformation involving dimethylating enzymes of the human colon [91].

The benefit involved by the presence of the phenol at the position 3 of the isoflavone core structure can be elucidated by its superposition with E_2. Actually, Pike *et al.* [11] have shown by crystallographic investigations that the 4' phenolic hydroxyl at position 3 of the benzopyranic ring core structure of isoflavones is analogous to the phenolic hydroxyl of E_2 (A ring) involved not only in the binding of E_2 but also in the affinity of the ligand for ER. Moreover, such comparison led us to understand why the lack of

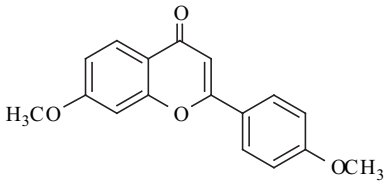
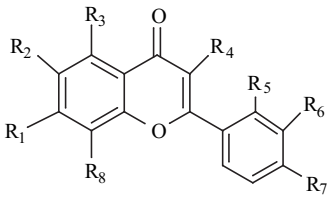


Fig. (2). Structure of 2-(4'-methoxyphenyl)-7-methoxy-4*H*-1-benzopyran-4-one.

hydroxyl at the positions 5 and 7 (R_3 and R_1, table **2**) first, and at the positions 3' and 4' (R_6 and R_7 respectively in table **2**) secondly, decreases dramatically the estrogenicity. By analogy with E_2, the 7 phenolic hydroxyl of genistein lies hERα (β) by the residues His-524 (475)[1] of the helix H11 and Glu-419 (371) of the loop 6-7, the 4' phenolic hydroxyl of genistein lying the receptor by the residues Glu-353 (305), Glu-394 (346) of the helix H3 and a buried water molecule as shown in figure **3**. Nevertheless, the 7 phenolic hydroxyl should be the most important feature for the binding affinity of isoflavones on ERs. Actually, this 7 phenolic hydroxyl is analogous to the 17β-hydroxyl of E_2 (D ring), which constitutes an absolute requirement for binding on ER [11, 25, 92, 93]. Besides, Le Bail *et al.* [58] have shown that pinostrobin, a flavanone without hydroxyl at the position 4' but with a methoxy at the position 7 instead of a hydroxyl is absolutely devoid of estrogenic activity or binding affinity for ERs. Nevertheless, methoxyflavone 2-(4'-methoxyphenyl)-7-methoxy-4*H*-1-

[1] Numbers in brackets represent corresponding residues for hERβ.

Table 2. Estrogenic Activity of Flavones

Entry	Compound	Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Pharmacologic. properties	Cell line	Ref.
9		Chrysin	OH	H	OH	H	H	H	H	H	Estrog.*: 18% IC_{50} = 10 µM CAT**: 50	BT-474 Yeast HeLa	[84] [43] [90]
10		Apigenin	OH	H	OH	H	H	H	OH	H	Estrog.*: 16% CAT**: 544±157	BT-474 HeLa	[84] [90]
11		Luteolin	OH	H	OH	H	H	OH	OH	H	Estrog.*: 58% CAT** : 168	BT-474 HeLa	[84] [90]
12		Galangin	OH	H	OH	OH	H	H	H	H	CAT** : 72±17	HeLa	[90]
13		Kaempferol	OH	H	OH	OH	H	H	OH	H	CAT** : 858±143	HeLa	[90]
14		Kaempferide	OH	H	OH	OH	H	H	OCH_3	H	CAT** : 130±31	HeLa	[90]
15		Quercetin	OH	H	OH	OH	H	OH	OH	H	Estrog.*: 10% IC_{50} = 24 µM CAT**: 75±28	BT-474 MCF-7 HeLa	[84] [75] [90]
16		Morin	OH	H	OH	OH	OH	H	OH	H	Estrog.*: 15% CAT**: 67±11	BT-474 HeLa	[84] [90]
17			OH	H	H	H	H	H	H	H	Estrog.*: 8% CAT**: 93±22	BT-474 HeLa	[84] [90]
18			OH	H	H	H	H	OH	H	H	CAT** : 162±30	HeLa	[90]
19			OH	H	H	H	H	H	H	OH	Estrog.*: 8% CAT** : 72±19	BT-474 HeLa	[84] [90]
20			OH	H	OH	H	H	H	OH	OH	CAT** : 68	HeLa	[90]
21			OH	H	H	H	H	OH	OH	H	CAT** : 42	HeLa	[90]
22		Fisetin	OH	H	H	OH	H	OH	OH	H	Estrog.*: 12% CAT**: 135±34	BT-474 HeLa	[84] [90]
23			OCH_3	H	H	H	H	H	H	H	Estrog.*: 0%	BT-474	[84]
24			OCH_3	H	H	H	H	H	H	OCH_3	Estrog.*: 0%	BT-474	[84]
25			H	OH	H	H	H	H	H	H	Estrog.*: 15% CAT**: 287	BT-474 HeLa	[84] [90]
26			H	OH	H	H	H	OH	H	H	CAT** : 161	HeLa	[90]
27			H	OH	H	H	H	H	OH	H	CAT** : 427	HeLa	[90]
28			H	OCH_3	H	H	H	H	H	H	Estrog.*: 0%	BT-474	[84]
29			H	CH_3	H	H	H	H	H	H	Estrog.*: 0%	BT-474	[84]
30			H	H	OH	H	H	H	H	H	Estrog.*: 0%	BT-474	[84]
31			H	H	OH	H	H	H	OH	H	CAT** : 389±95	HeLa	[90]
32			H	H	OCH_3	H	H	H	H	H	Estrog.*: 0%	BT-474	[84]
33			H	H	H	H	H	H	OCH_3	H	Estrog.*: 0%	BT-474	[84]
34			H	H	H	H	OCH_3	H	H	H	Estrog.*: 0%	BT-474	[84]
35			H	H	H	OH	H	H	H	H	Estrog.*: 0% CAT**: 92±9	BT-474 HeLa	[84] [90]
36			H	H	H	H	H	H	OH	H	CAT**: 237	HeLa	[90]
37		Flavone	H	H	H	H	H	H	H	H	Estrog.*: 0% IC_{50} = 2 µM CAT**: 52±11	BT-474 Yeast HeLa	[84] [43] [90]

*Estrogenicity compared to genistein (100%) by dosing the estrogen-regulated protein *p*S2.

**CAT: The estrogenicity is obtained using HeLa cells transfected by the plasmid pERE-TK-CAT (chloramphenicol acetyl transferase). These assays are expressed in terms of pmoles/min – mg protein (ethanol vehicle: 82).

benzopyran-4-one (Fig. (**2**)) is highly antiestrogenic (65%) and possess 31% of the uterotrophicity of E_2 as shown by Ismail *et al.* [94]. At last, the binding mode of genistein on the receptor may involve a change of the configuration of the pocket and a modification of the position of the helix H12 similar to which induced by antagonists, explaining the antagonist behavior of some phytoestrogens [11].

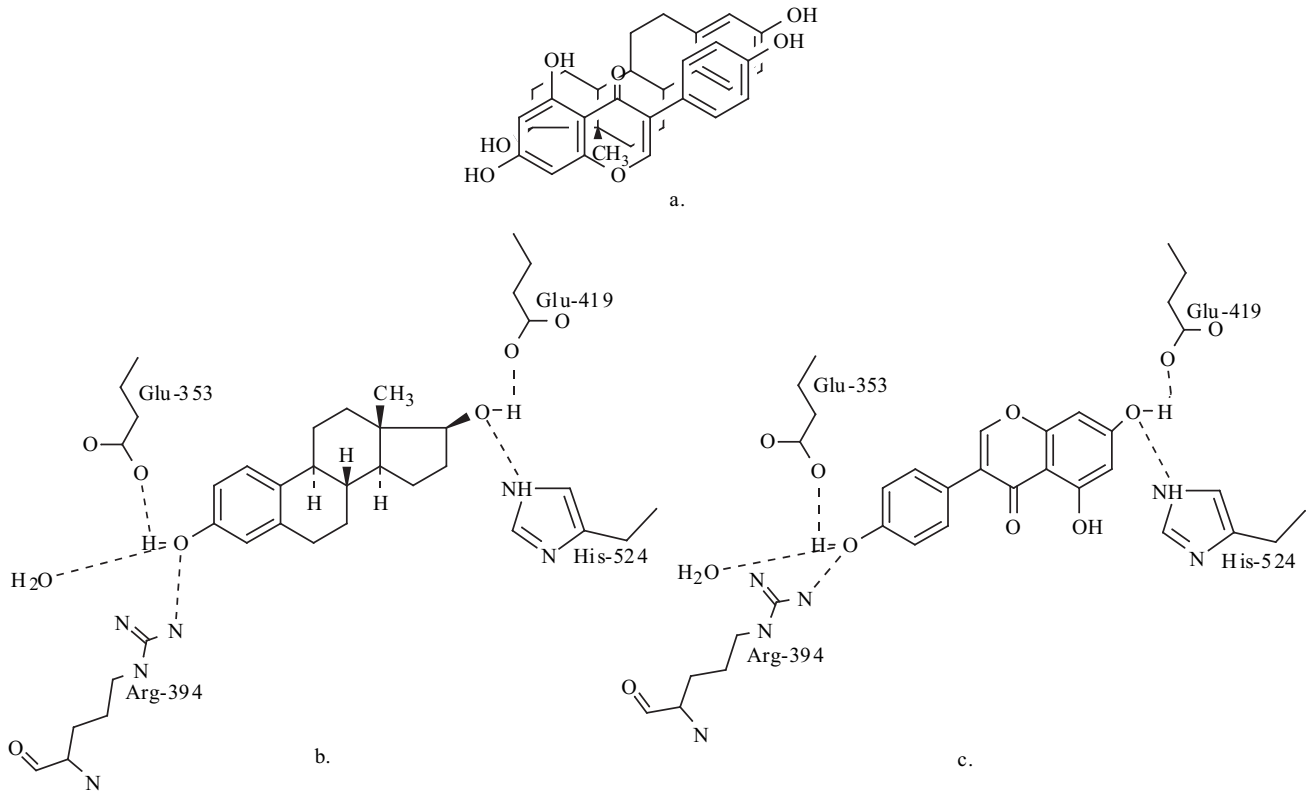


Fig. (3) . Binding mode of genistein on hERα. (a). Superposition of E_2 and genistein. (b). Binding mode of E_2 on hERα. (c). Binding mode of genistein on hERα.

If we compare apigenin to genistein, we can note that the presence of the phenyl ring at the position 2 decreases strongly the estrogenicity (table **1**, table **2**) and increases in a large proportion the progestational activity [84]. Note that a hydroxyl at the position 3 (R_4 in table **2**) does not present a benefit for estrogenicity, unless the only presence of a hydroxyl at the position 7 (the position 6 being better with the presence of a phenol at the position 2. The benefit due to the presence of an hydroxyl at the position 7 outlines the same analogies for flavones than those already evocated for isoflavones. Moreover, if two hydroxyls are present at the positions 5 and 7 (R_3 and R_1, table **2**), the presence of hydroxyl(s) at the positions 3 or 2' (R_4 and R_5, table **2**) does not bring more estrogenicity (for atom numbering, see (Fig.(**5**))). The presence of hydroxyls at the positions 3' and 4' (R_6 and R_7, table **2**), enhance indubitably the estrogenicity. Nevertheless, it may be very possible that all the restrictions for the positions 2', 3, 6 and 8 (R_5 and R_4, R_3 and R_8, table **2**) are due to the steric effects.

Although a lack of data concerning crystallographic studies to determine the exact binding mode of flavones on ER, the importance of hydroxyls for estrogenic activity has been elucidated by QSAR studies. These studies have shown that the electronic and the ionic characteristics of the phenolic hydroxyls play a crucial role for ER binding. The concept of ligand binding on ER relies on the ability of phytoestrogens to create hydrogen bonds. It has been shown that genistein acts as a partial agonist by involving conformation changes of ER by the alignment of the function AF-2 [23]. Then, the binding mode of genistein is now elucidated according to hydrogen bond formations and steric effects since this compound exhibits the same size as E_2 as shown in the figure **3**. Hydroxyl – hydroxyl larger length is 10.8 Å for E_2 and 12.1 Å for genistein [11, 95]. Moreover, the total volume of genistein is 236 $Å^3$ and the total volume of E_2 is about 232 $Å^3$ [11]. Hence, the fundamental importance of the phenolic hydroxyls at the positions 7 and 4' are well understood by their analogy with the hydroxyl at the position 17β of E_2 (D ring) and the phenolic hydroxyl at the position 3 of E_2 (A ring).

The estrogenicity of flavanones allows us to estimate the influence of the double bond at the position 2-3 as shown in table **3**. This study is all the more interesting that 2-3 reduced compounds, i.e. flavanones, are metabolites of phytoestrogens as shown by genistein metabolised into dihydrogenistein or daidzein metabolised into equol (equol being obtained not only by the reduction of the 2-3 double bond but also by the reduction of the function keto at the position 4 [42]). One of the most interesting compounds is naringenin, the reduced 2-3 double bond genistein derivative, the most active flavanone as shown in table **3**.

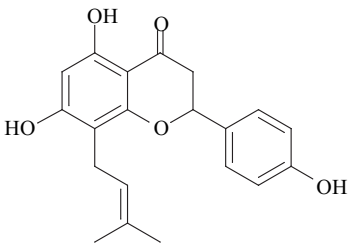
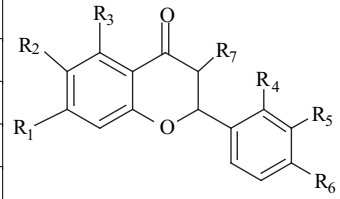


Fig. (4). Structure of 8-prenylnaringenin.

Table 3. Estrogenic Activity of Flavanones

Entry	Compound	Trivial name	R_1	R_2	R_3	R_4	R_5	R6	R_7	Pharmacological properties	Cell line	Ref.
38		Naringenin	OH	H	OH	H	H	OH	H	Estrogenicity*: 40% EC_{50} = 45 μM CAT = 574±235	BT-474 MCF-7 HeLa	[84] [43] [90]
39		Hesperetin	OH	H	OH	H	OH	OCH_3	H	CAT = 86	HeLa	[90]
40		Taxifolin	OH	H	OH	H	OH	OH	OH	CAT = 97±28	HeLa	[90]
41		Pinostrobin	OCH_3	H	OH	H	H	H	H	Estrogenicity*: 0%	BT-474	[84]
42			H	OH	H	H	H	H	H	Estrogenicity*: 0%	BT-474	[84]
43			H	H	H	H	H	OH	H	Estrogenicity*: 0%	BT-474	[84]
44			OH	H	H	H	H	H	H	Estrogenicity*: 0%	BT-474	[84]
45			OH	H	H	H	H	OH	H	CAT = 903±202	HeLa	[90]
46			H	H	H	H	H	H	H	Estrogenicity*: 40% CAT = 40±13	BT-474 HeLa	[84] [90]
47			H	H	H	OH	H	H	H	Estrogenicity* : 0%	BT-474	[84]
48			H	H	OCH_3	H	H	H	H	Estrogenicity: 0%	BT-474	[84]
49			H	OCH_3	H	H	H	H	H	Estrogenicity: 0%	BT-474	[84]
50			OCH_3	H	H	H	H	H	H	Estrogenicity: 0%	BT-474	[84]

*Estrogenicity compared to genistein (100%) by dosing the estrogen-regulated protein *p*S2

**CAT: The estrogenicity is obtained using HeLa cells transfected by the plasmid pERE-TK-CAT (chloramphenicol acetyl transferase). These assays are expressed in terms of pmoles/min – mg protein (ethanol vehicle: CAT = 82±12).

This compound has shown a conclusive AF2 inhibitory activity, which may explain its antagonist potential [96]. Recently, 8-prenylnaringenin (Fig. (**4**)) has revealed to be the most potent phytoestrogen *in vitro* [97].

Regarding to the Structure-Activity Relationships (SAR), we can give some rules required to optimise the estrogenicity of a flavovonid phytoestrogen (Fig. (**5**)) [84].

1. The benzopyranic core structure is required for estrogenicity.

2. The 4'-phenolic hydroxyl mimics the phenolic hydroxyl at the position 3 of E_2 and is an absolute requirement for estrogenic activity since the deletion of any phenolic at this position diminishes tragically the estrogenicity. Moreover, the replacement of the 4'-hydroxyl by a methoxy strongly decreases the estrogenicity, proving the fundamental importance of hydrogen interactions in the binding process of ligands in the ER-LBD.

3. The presence of a hydroxyl at the position 7 enhances significantly the estrogenic potency since it may mimic the 17β hydroxyl of E_2.

4. The presence of a hydroxyl in the position 5 usually enhances significantly the estrogenic potency.

5. The 2-3 double bond is implicated in the estrogenic and in the progestin activities. Nevertheless, the reduction of the double bond 2-3 decreases strongly the estrogenicity of about 60% [84].

6. The substitution at the position 2 is not recommended. Nevertheless, the disappearance of the phenyl at the position 3 for the benefit of the position 2 decreases the estrogenic activity and enhances the progestational activity.

7. The presence of a function keto at the position 4 enhanced significantly the estrogenicity and points towards the unoccupied face β of the ligand binding pocket [11]. Actually, 7-hydroxyflavan is devoid of estrogenicity compared to 7-hydroxyflavanone [84].

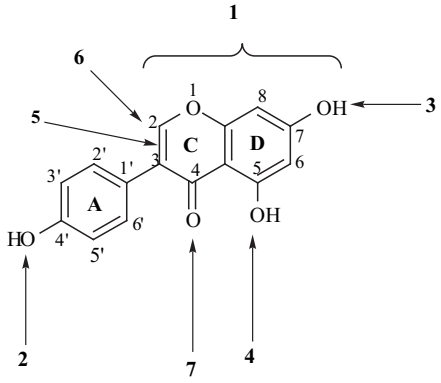


Fig. (5). Schematic representation of the most important features required for estrogenic activity in the family of benzopyrans.

(B) Coumarins

Coumarins exist as phytochemicals in many plants and are known for their pharmacological activities [98]. Among these derivatives, 6*H*-benzofuro[3,2-*c*]benzopyran-6-ones, or coumestans, are of a great interest because of their cytotoxicity and their estrogenicity [99]. Two derivatives, coumestrol [100] and the parent phenol of trifoliol (Table **4**) are the two principal coumarinic phytoestrogens [101]. These derivatives are the most potent phytoestrogens and display a high affinity for ERs [102]. Coumestans act not only directly on ERs (estrogenic activity) but also by inhibiting on topoisomerase II (antiproliferative activity). Coumestans are also involved in DNA strand breaks and deletions. For this reason, coumestrol is mutagenic and clastogenic [83]. Nevertheless, the synthesis of coumestrol arouses still interest, even if its synthesis is still difficult [103-106]. Recently, Jacquot *et al.* [107] have discovered a new family of estrogens associated to coumarins, i.e. benzopyrano-benzothiazin-6-ones, obtained easily and with good yields by a one step synthesis. The lead compounds of this new family are 6,12-dihydro-3-methoxy-1-benzopyrano[3,4-*b*] [1,4]benzothiazin-6-one and 6,12-dihydro-3-hydroxy-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-one, a little bit less potent than the 3-methoxy derivative (Fig. (**6**)). The interest of these derivatives is their similar pharmacological profile compared to coumestrol and their high affinity for ERs *in vivo*. Nishimura *et al.* [108] have also characterized a new family of phytoestrogens associated to coumarins and structurally closed to 4-arylcoumarin dimers (neoflavones, compound 54, table **4**) with a 7 fold IC_{50} compared to the one of genistein.

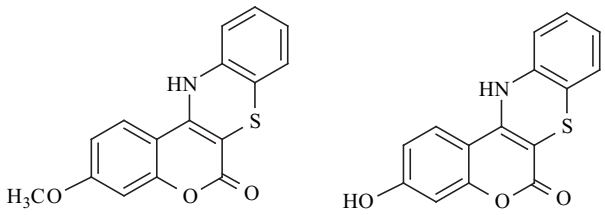
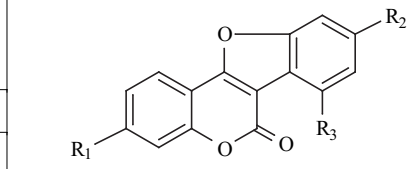
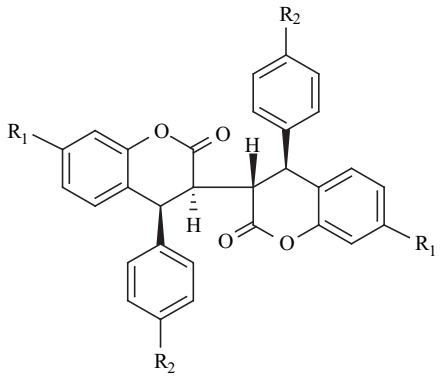


Fig. (6). Structure of 6,12-dihydro-3-methoxy-1-benzopyrano [3,4-*b*][1,4]benzothiazin-6-one and 6,12-dihydro-3-hydroxy-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-one.

It is interesting to note for the active compounds that the presence of a phenolic hydroxyl or a methoxy at the position 3 is analogous to the hydroxyl at the position 17β of E_2 (ring D) as suggested by Jacquot *et al.* [95,107]. Actually, the disappearance of this hydroxyl induces a dramatic lost of the estrogenicity. Moreover, it has been shown that trifoliol, i.e. 7- methoxycoumestrol (compound 52, table **4**), was inactive, probably because of the steric restrictions induced by the methoxy in the ligand pocket in this region. This hypothesis has been approved by the demethylation of trifoliol into its parent phenol, 7-hydroxycoumestrol which exhibits an activity as potent as coumestrol. This observation proves that the presence of a hydroxyl at the position 7 does not increase estrogenicity [101]. As for the implication of the coumarinic carbonyl and the double bond at the position 6a-11a of coumestans, the comparison of the luciferase induction on MVLN cells by coumestrol and medicarpin (Fig. (**7**)) as for their effect on the proliferation on MCF-7 cells have shown that these two factors are important. First, the carbonyl at the position 6 may be involved in the ligand binding by hydrogen bonds with

Table 4. Estrogenic Activity of Coumarins

Entry	Compound	Trivial name	R1	R2	R3	Pharmacological properties	Cell line	Ref.
51		coumestrol	OH	OH	H	Proliferation*: 93.1% (at 10 nM) Luciferase : 0.7% EC_{50} = 10 µM	MCF-7 By measure of uterin weight in mice	[78] [78] [101]
52		trifoliol	OH	OCH_3	H	No activity		[101]
53			OH	OH	OH	As active as coumestrol		[101]
54			OH	OH		Proliferation **: 145% EC_{50} = 500 nM	T47D	[108]

* Proliferation and transcriptional activity compared to E_2 = 100% (EC_{50} = 1 nM).

** Proliferation compared to $E_2 \approx 180\%$.

** Proliferation compared to control (100%, E_2 = 49.2%).

amino acids residues of the LBD. Secondly, the double bond strongly involved in the rigidity of the coumarinic structure contributes to the a lipophilicity of the ligand that facilitates its passage across biological membranes. As for the methoxy at the position 9 instead of the hydroxyl, it is probable that this structural modification has no decisive repercussion on the estrogenicity by analogy with benzopyranobenzothiazinones [95].

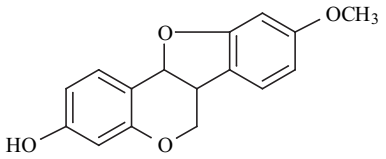


Fig. (7). Structure of medicarpin.

Regarding to the Structure-Activity Relationships (SAR), we can give some rules required to optimise the estrogenicity of a coumarinic phytoestrogen (Fig. (**8**)) .

1. The benzopyranic core structure is required for estrogenicity.

2. The phenolic hydroxyl or a methoxy at the position 3 are critical for estrogenicity since it mimics the hydroxyl at the position 17β of E_2.

3. A phenolic hydroxyl at the position 9 of coumestans is implicated in the binding but less in the estrogenicity than in the case of the 3 hydroxylated derivatives.

4. The lactonic carbonyl is probably involved in estrogenicity and in the ligand binding affinity.

5. The double bond 6a-11a seems to bring a significant contribution for estrogenicity and ligand binding, probably because it participates actively to the rigidity of the molecule.

6. The region of the B ring is relatively flexible in terms of steric effects because of the large space in the B and C ring regions of the receptor.

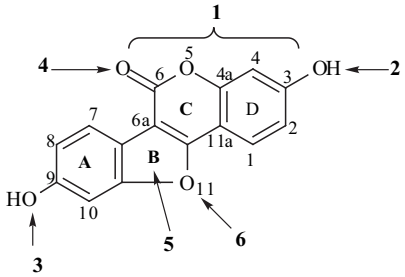


Fig. (8). Schematic representation of the most important features required for estrogenic activity in the family of coumarins.

(C) Chalcones and Dihydrochalcones

Chalcones exhibit a moderate estrogenicity. Actually, polyhydroxylated chalcones have shown an estrogenic activity at 1 μM that may be explained by their structural analogies with flavones (Fig. (**9**), table **5**) [90].

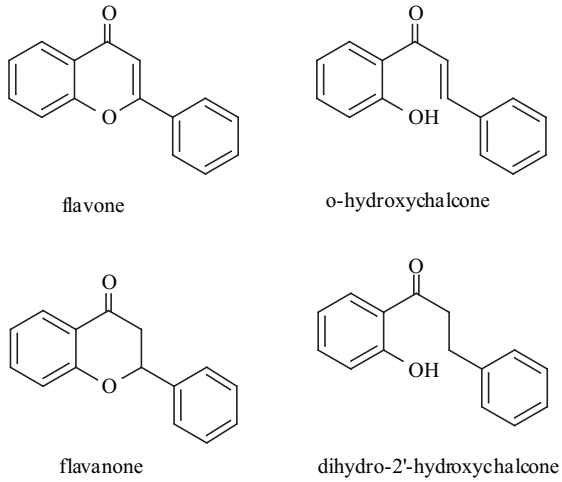


Fig. (9). Structural analogies between o-hydroxychalcones and flavones and dihydro-2'-hydroxychalcones and flavanones.

These data show that the presence of a hydroxyl at the position 4 (R_4, table **5**) is involved in the estrogenic activity. Moreover, the presence of a secondary hydroxyl, particularly at the position 4' (R_2, table **5**) which mimics the hydroxyl at the position 7 of flavonoids and exhibits a three folds estrogenicity compared to 4-hydroxychalcone. Nevertheless, if the hydroxyl at the position 4' is methylated, the disappearance of the quasi totality of the estrogenicity is observed. The study of the estrogenicity displayed by chalcones shows that the presence of a hydroxyl at the position 2' (R_1, table **5**) decreases the activity as shown by the comparison of the transcriptional activity of isoliquiritigenin and naringenin chalcone. The presence of a hydroxyl at the position 6' decreases significantly the estrogenicity by a factor 7 when it did not influence the estrogenicity in the case of flavonoids. Finally, this study shows clearly that dihydrochalcones exhibit more estrogenicity than chalcones.

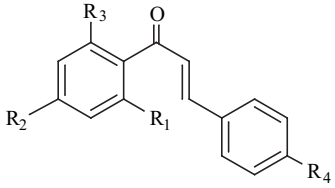
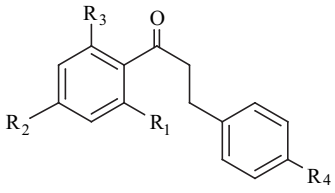
Regarding to the Structure-Activity Relationships (SAR), we can give some rules required to optimise the estrogenicity of a phytoestrogenic chalcone (Fig. (**10**)).

1. Even if a hydroxyl at the position 4 is involved in the estrogenicity of chalcone derivatives, the presence of a hydroxyl at the position 4' enhances considerably the activity.

2. The replacement of the most important phenolic hydroxyl at the position 4' by a methoxy decreases dramatically the estrogenicity, because of the strong implication of this hydroxyl in hydrogen bonds formation in the LBD.

3. The presence of a hydroxyl at the position 6' – which mimics the phenolic hydroxyl at the position 5 in isoflavonoids derivatives - enhances considerably the activity. This observation shows that this position is more crucial with chalcones than others benzopyranic derivatives. In the LBD, this hydroxyl may position

Table 5. Estrogenic Activity of Chalcones and Dihydrochalcones

Entry	Compound	Trivial name	R1	R2	R3	R4	Pharmacological properties	Cell line	Ref.
55			H	H	H	OH	CAT* : 152±94	HeLa	[90]
58			H	OH	H	OH	CAT* : 471±156	HeLa	[90]
59			H	OCH_3	H	OH	CAT* : 86	HeLa	[90]
60		Isoliquiritigenin	H	OH	OH	OH	CAT* : 994±219	HeLa	[90]
61		Naringenin chalcone	OH	OH	OH	OH	CAT* : 156±35	HeLa	[90]
62		Phloretin	OH	OH	OH	OH	CAT* : 402±68	HeLa	[90]

*CAT: The estrogenicity is obtained using HeLa cells transfected by the plasmid pERE-TK-CAT (chloramphenicol acetyl transferase). These assays are expressed in terms of pmoles/min – mg protein (ethanol vehicle: CAT = 82±12).

estrogenic chalcones in a reliable conformation thanks to hydrogen bonds formation.

4. The presence of a hydroxyl at the position 2' is not a benefit for estrogenicity. Actually, such hydroxyl decreases the estrogenicity. Since the hydroxyl at the position 2' mimics the heterocyclic oxygen of the benzopyranic structure, this observation suggests that the presence of a free hydroxyl at this position is a disadvantage.

5. The suppression of the double bound enhances the estrogenic activity.

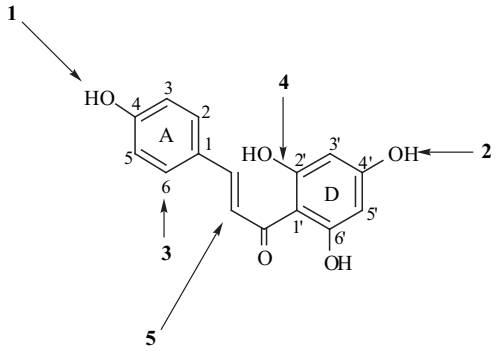


Fig. (10). Schematic representation of the most important features required for estrogenic activity in the family of chalcones.

(D) Lignans and Resorcylic Acid Lactones

As for others phytoestrogens without benzopyranic core structure, the two best known compounds are enterolactone and zearalenone.

Zearalenone is an organic acid like abietic acid issued from pine wood. These derivatives act not only as direct ER agonist [86, 89] but also as non-hormonal compounds by interacting with 17β-hydroxysteroid dehydrogenase type 5 [79]. Most of phytoestrogenic lignans such as enterolactone are isolated from cereals and oilseeds such as flaxseed [46, 71].

Nevertheless, in the regard of the actual knowledge about lignans and resorcylic lactones, it is not possible to establish a rule about the SAR of these derivatives.

CONCLUSION AND PERSPECTIVES

The knowledge of phytoestrogens is of a great interest for HRT development. These natural compounds act not only by binding directly on the hormone receptor, but also by interfering with key enzymes of the hormonal metabolism. Nevertheless, they act also by interfering with a large variety of targets. Despite of the very interesting therapeutically perspectives of phytoestrogens not only as estrogens-like for the treatment of pathologies associated to osteoporosis but also as anti-estrogenic derivatives for the treatment of hormono-dependent cancers, it is very difficult to extrapolate at present to HRT use because of their large activities through non-hormonal targets. Nevertheless, the more and more well known chemical requirements for pharmacological activity opens the way to a large pharmacomodulation and to the development of more selective potential drugs.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

E_2 = 17β-estradiol

ER = Estrogen receptor

CNS = Central nervous system

HRT = Hormonal replacement therapy

LBD = Ligand binding domain

HSP = Heat shock protein

AF = Activating function

DBD = DNA binding domain

TF = Transcriptional factor

TBP = TATA binding protein

SRC-1 = Steroid receptor coactivator 1

TIF = Transcriptional intermediary factor

ERE = Estrogen response element

SERM = Selective estrogen receptor modulator

CVD = Cardiovascular disease

NO = Nitric oxide

HSD = Hydroxysteroid dehydrogenase

PTK = Protein tyrosine kinase

EGF = Endothelium growing factor

QSAR = Quantitative structure activity relationship

SAR = Structure activity relationship

SHBG = Sex hormone binding globulin

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