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The Importance of Phytoestrogens in Food Supplements and Phytopharmaceuticals

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Abstract: Different natural products from the plant kingdom including isoflavones, prenylflavones, coumestans, stilbenes, and lignans can cause endocrine perturbations, especially on the estrogenic hormonal pathway because of their structural analogies to estradiol. Several studies report the presence of such compounds in food, the richest source being soy and soy products. These substances, commonly called phytoestrogens, are known to influence the human hormonal profile and soy preparations are being considered for use as post-menopausal estrogen replacement therapy. Numerous clinical and epidemiological reports have studied the influence of phytoestrogens on the development of hormone-dependent cancers. The effect on genital development of postnatal phytoestrogen exposure is still under debate. Consumption of phytoestrogens may also influence the incidence of bone diseases, atherosclerosis, or Alzheimer's disease. This article summarizes the classification of phytoestrogens, their pharmacological evaluation and properties, and the methods used for their identification.

Keywords: Isoflavonoids · Menopause · Phytoestrogens

Introduction

The interest in plant-derived estrogens, or phytoestrogens, has recently increased due to the observation that hormone replacement therapy (HRT) is not as safe or effective as previously thought. Numerous epidemiological studies have shown that nutrients and food products could have an influence on the hormonal system, particularly from studies on Asian populations, which have linked the lower incidence of gynecological cancers, cardiovascular diseases or osteoporosis in post-menopausal women with their high soy and soy products daily intake [1]. Phytochemical and pharmacological studies have demonstrated that particular substances of soy, the isoflavones, do possess a significant affinity to the estrogen receptor and could partly explain the beneficial and preventive effect on the health of Asian post-menopausal women [2][3]. Based on these considerations, phytopharmaceuticals or food complements containing isoflavone-rich soy extracts, purified isoflavones or other medicinal plant extracts are nowadays being commercialized, despite of the lack of large-scale controlled clinical studies.

One of the major challenges of scientific research is the clarification of the pharmacological importance of phytoestrogens. A better insight in the phytochemical and pharmacological phytoestrogen content of food and phytopharmaceuticals is also of prime importance.

Phytoestrogens

Phytoestrogens are naturally occurring non-steroidal plant-derived substances that exhibit estrogen-like bioactivity in vertebrates, whereas their function in plants is quite different, involving herbivore defense, protection against radicals or reactive oxygen forms and a role in growth and development [4]. In the 1940s it was first realized that some plant-secondary metabolites could cause an estrogenic effect [5]. Sheep that were grazing on pastures containing red clover (*Trifolium pratense* L., Leguminosae) had multiple fertility problems. Immature animals showed signs of estrus, ewes were unable to become pregnant and those that were pregnant often miscarried. The clover in these pastures revealed a high amount of isoflavones. Mechanistically, phytoestrogens can bind to the estrogen receptor, and functionally, they may exert both estrogenic effects, such as stimulation of uterus growth and inhibition of bone loss, and anti-estrogenic activities, such as inhibition of breast cancer cell growth [6].

The phytoestrogens have been categorized based on their chemical structures, which resemble estradiol, the natural ligand for the human estrogen receptor (ER). An aromatic ring and a hydroxyl group are important for binding effectiveness. The phytoestrogens are generally composed of a planar ring system that includes a *p*-hydroxy-substituted aromatic ring approximately 12 Å away from a second in-plane hydroxyl group [7]. Two ring structures separated with two carbon atoms as well as spacing between hydrophobic and hydrogen bond interactions are also important in binding affinity to ER [8].

Phytoestrogens have been categorized in five main classes, according to their chemical structures: isoflavones, lignans, coumestans, prenylflavonoids and stilbenes:

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Isoflavones are the most well known phytoestrogens and have received the most attention, especially the aglycones genistein (4',5,7-trihydroxyisoflavone) (**1**) and daidzein (4',7-dihydroxyisoflavone) (**2**), their respective glycosides genistin (**3**) and daidzin (**4**) and their 4'-methylethers derivatives formononetin (**5**) and biochanin A (**6**), found in soy (*Glycine max* L., Leguminosae) and on which the major part of the clinical studies has been realized. They can induce similar responses in breast, ovarian, endometrial, prostate, vascular and bone tissues as estradiol. They can also act as antagonists in some tissues as shown by the inhibition produced on the development and growth of chemically induced tumors in the breast and prostate of mice and rat models [9]. Genistein (**1**), the most active among the phytoestrogens, can produce effects independent of ER, including the ability to inhibit the protein tyrosine kinase and the DNA topoisomerase [10]. Today, isoflavones are considered as the most important class of phytoestrogens, occuring frequently in human diet, although almost exclusively in legumes, beans and derived products.

Coumestans are structurally similar to isoflavones and possess an ether bridge between positions 4 and 2' of the 3-phenylcoumarin nucleus. A large number of these compounds has been isolated from dietary plants, but only a few of them have shown estrogenic activity. The most common are coumestrol (7,12-dihydroxycoumestan) (**7**) and 4'-methoxycoumestrol (**8**) found in alfalfa (*Medicago sativa* L., Leguminosae), clover and soy. Coumestrol exhibits a bright blue fluorescence with an emission maximum at 438 nm when excited at 340 nm. When bound to the ER, this fluorescence emission shows a blue shift to 410 nm while the intensity grows about four times [11]. Due to this interesting fluorescent property and its high and selective affinity for the ERs, coumestrol can serve as marker in bioassays. Coumestans are not current in the human diet; they appear predominantly in fodder crops and during germination, as for example in sprouting beans [4].

Lignans are minor constituents in many plants, especially cereals, seeds, fruits and vegetables, as precursors of lignin formation in plant cell walls. Secoisolariciresinol (**9**) and matairesinol (**10**) are two lignan dimers, isolated principally from flaxseeds (*Linum usitatissimum* L., Linaceae), and representing the most well known phytoestrogenic lignans. These are not estrogenic by themselves, but are readily converted by the gut microflora to the mammalian lignans, enterodiol (**11**) and enterolactone (**12**), respectively, which are readily absorbed and possess estrogenic properties [12].

Prenylflavonoids are flavanones found in the beer ingredient hops (*Humulus lupulus* L., Cannabaceae), and are mainly represented by 8-prenylnaringenin (**13**) and 6-prenylnaringenin (**14**) isolated from the female flowers [13]. This may provide an explanation for the account of menstrual disturbance amongst female workers when hops were picked by hand [14]. Prenylflavonoids and prenylisoflavonoids are widely distributed in higher plants and more specifically in the Leguminosae family, as illustrated by the numerous prenylisoflavonoids isolated from *Erythrina vogelii* Hook. f. (Leguminosae) [15][16].

Among the ***stilbene derivatives***, resveratrol (trans-3,4',5-trihydroxystilbene) (**15**) is certainly the most important. It occurs naturally in the skin of grapes (*Vitis vinifera* L., Vitaceae), peanuts and a variety of medicinal plants where it functions as a phytoalexin (protecting substance) and is synthesized under stimulation by UV light, injury and fungal infections [17]. Because of the high concentration of resveratrol in grape skin, significant amounts are also present in red wine, while very low levels appear in white wine [18]. The estrogenic activity and antioxidant properties exhibited by this compound have been proposed to explain, at least in part, the apparent ability of a moderate red wine consumption to reduce the risk of cardiovascular disease. Resveratrol possesses a high bioavailability and physiological levels can be obtained through drinking red wine. This effect is also known as the 'French paradox', the name given to the phenomenon observed in certain population, *e.g.* the French and the Greeks, suffering little heart diseases despite a relatively fatty diet, but a regular and moderate consumption of red wine [19].

Beside these classes, ***additional compounds*** have been claimed, with varying degrees of supportive evidence, to be responsible for estrogenic activities. For example, anthraquinones [20], chalcones [21], flavones [13], saponins [22], terpenoids [23], resorcylic acid lactones, described as mycotoxins and mainly represented by zearaleone [24]. Finally, considering the human diet, it could be useful to keep in mind that residues of pesticides and insecticides may also be a source of endocrine disrupters [25].

Pharmacological Aspects

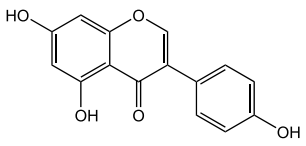
Steroid estrogens (mainly 17β-estradiol) play an important role in the growth and differentiation of various target organs or tissues, including the female and male reproductive systems, such as mammary glands, uterus, ovaries, testes or prostate, but also bone metabolism, the central nervous system, and the cardiovascular system. The activity of estrogens is mediated through estrogen receptors alpha (ERα), and a second ER subtype, ERβ. Studies on the tissue distribution of the ERs indicated a broad expression pattern for ERα, whereas ERβ is more concentrated in breast, ovary, epididymis, lung and hypothalamus [26]. As suggested by their name, phytoestrogens bind to the ERs and several studies compared the affinities of various compounds to ERα and ERβ [3][27][28]. Usually, phytoestrogens have a lower ERα affinity compared to estradiol, except for coumestrol that binds at a concentration of the same order of magnitude. On the other hand, affinity to ERβ is higher with phytoestrogens especially with isoflavones. However, it is likely that phytoestrogens produce more antagonistic effects on ERα and more agonistic effects on ERβ. Such results, together with the differential tissular distribution of both ER subtypes, may provide an explanation for the protective effect of isoflavones against breast and prostate cancers [3].

However, estrogen receptor binding can hardly provide an explanation for all the numerous pharmacological and metabolic effects of phytoestrogens, such as in gynecological cancer chemoprevention, osteoporosis, cardiovascular disease and menopause.

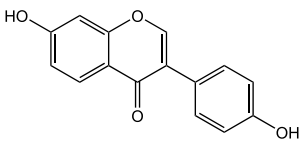
Phytoestrogens act as weak estrogens, they exhibit estrogenic activity in a low-estrogen environment and show anti-estrogenic activity when the estrogen concentration is high. This explanation suggests that prior to menopause, when there is a high level of estrogen, phytoestrogens may protect against breast cancer, and after menopause, when the estrogen concentration has decreased, they may stimulate breast cancer [29]. Different mechanisms of action have been suggested for the action of phytoestrogens on breast tissues, among them, the heterodimerisation of ERβ with ERα resulting in a decreased estrogenic effect, the inhibition of tyrosine and other protein kinases, the inhibition of the angiogenesis, the alteration of growth-factor activity and the lowering of the circulation levels of unconjugated sex hormones [30].

Most of the studies suggest that phytoestrogens are somewhat effective in maintaining bone mineral density in postmenopausal women. The mechanisms involved include preventing urinary calcium loss, beneficial effects on osteoblasts, and influences on the secretion of calcitonin which suppresses bone resorption [31].

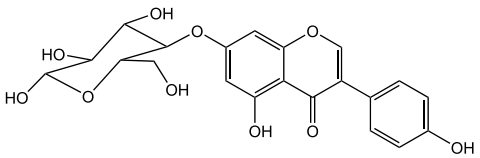
CHIMIA **2005**, *59*, No. 6



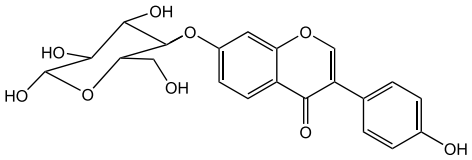
(1) Genistein



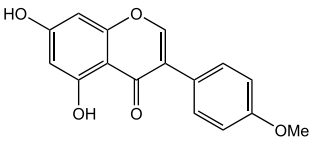
(2) Daidzein



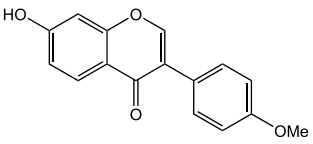
(3) Genistin



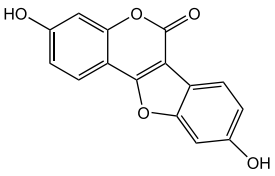
(4) Daidzin



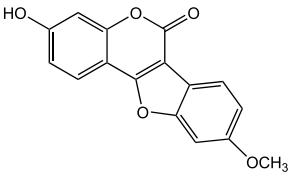
(5) Formononetin



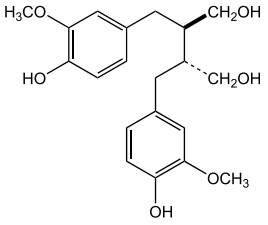
(6) Biochanin A



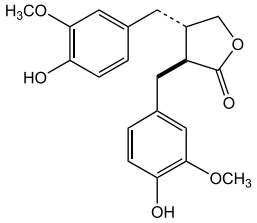
(7) Coumestrol



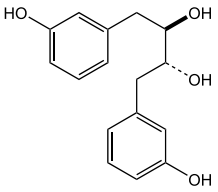
(8) 4'-methoxycoumestrol



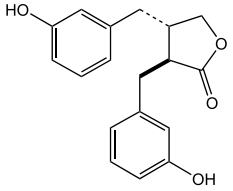
(9) Secoisolariciresinol



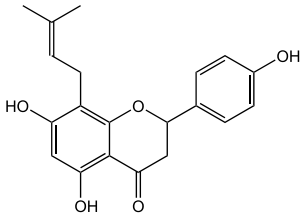
(10) Matairesinol



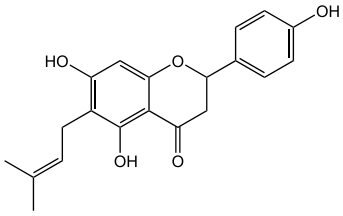
(11) Enterodiol



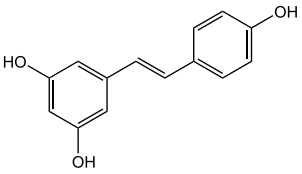
(12) Enterolactone



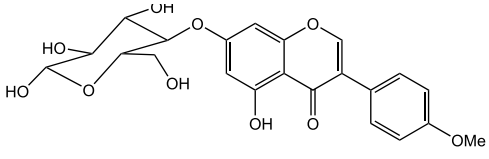
(13) 8-prenylnaringenin



(14) 6-prenylnaringenin



(15) Resveratrol



(16) Ononin

CHIMIA **2005**, *59*, No. 6

Phytoestrogens have shown beneficial effects on lipid profiles, vascular reactivity, cellular proliferation and thrombosis; these being factors that affect coronary heart disease. The improvement of plasma lipid concentrations, reduction of thrombus formation through inhibition of platelet action, the improvement of systemic arterial compliance and the antioxidant activity are the mechanisms suggested to explain the prevention of cardiovascular disease and the reduction of atherosclerosis [29].

Analytical Methods

Originally, quantification of phytoestrogens was limited to the high isoflavone content in soy-derived products and HPLC-UV methods were used. However, measurements of low concentration samples and analysis of other chemical entities strongly limit the use of HPLC-UV, due to the lack of sensitivity and specificity. Therefore, a sensitive and selective isotope dilution GC-MS method was widely used by Adlercreutz [32]. This method was applied to the determination of the above-mentioned compounds and to coumestrol [33]. Recently, a very sensitive method by time-resolved fluoroimmunoassay was developed for the analysis of genistein, daidzein, and enterolactone [34]. High sensitivity was also achieved with capillary electrophoresis coupled to electrospray mass spectrometry (CE-MS) in the analysis of soy isoflavones [35]. The above-mentioned methods have been the mainstay of phytoestrogen analysis for the past 20 years. Although providing very high chromatographic resolution and a high sensitivity, these are very labor-intensive methods and have been supplanted by HPLC-MS methods which require very little sample work up and provide the opportunity to determine the chemistry of the phytoestrogens and their metabolites, even in the conjugated form. HPLC-MS quantification methods are known to date for the quantitative analysis of resveratrol in red wine [18][36] and the genistein and daidzein physiologic conjugates (glucuronides and sulfates) [37]. Detailed quantitative HPLC-MS studies of soy isoflavones, lignans and coumestrol have been published [38–40] and numerous other reports describe new HPLC-MS methods to detect isoflavones [38][41–44]. This is also the method we are using for the qualitative analysis of plant material assumed to contain phytoestrogens. An example is illustrated in Fig. 1, with a HPLC-UV-MS chromatogram of a clover extract and the UV and MS spectra of an isoflavonoid glycoside identified (ononin (**16**)).

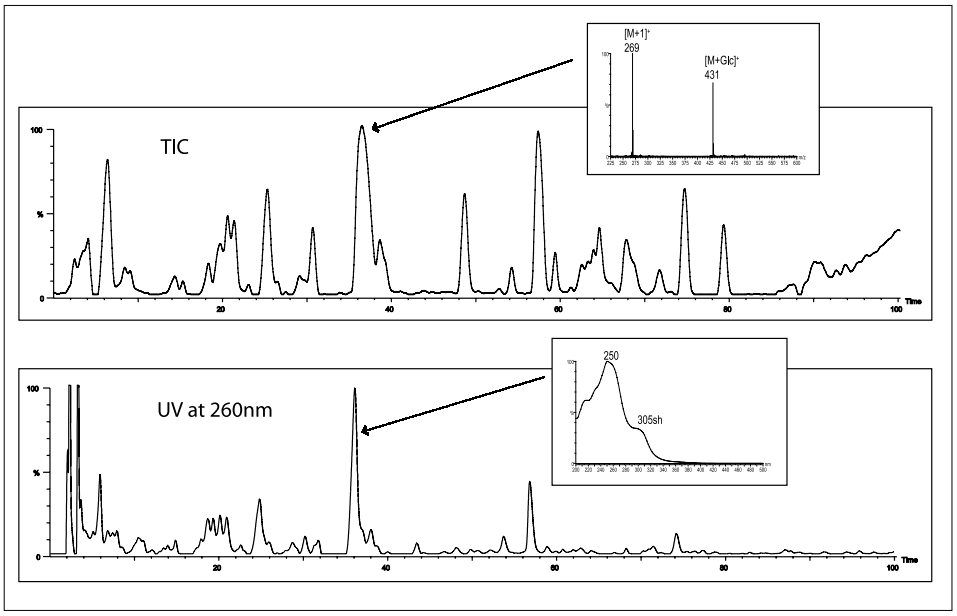


Fig. 1. Simultaneous LC-UV at 260 nm and LC-ESI-MS total ion chromatograms of the methanol extract of *Trifolium alpinum* L. (Leguminosae) and the UV and MS spectra of Ononin (**16**) (formononetin 7-O-β-D-glucoside)

Pharmacological Assays

Direct binding of phytoestrogens to the estrogen receptor (ER) seems to be a key event in the mode of action of these endocrine disruptors, and several studies have been performed on the binding of these compounds to ERα and ERβ [3][45–47]. They have mainly shown that isoflavones and other estrogenic chemical classes bind to ERβ with higher affinity, but are unable to differentiate between agonist and antagonist action.

A lot of other animal and *in vitro* test systems have been developed in the last decades to determine the estrogenic potency of synthetic compounds and these systems are also suitable to detect and quantify estrogenicity of natural products. The most commonly used *in vitro* test systems, are, beside the above-mentioned receptor binding assay, the cell proliferation assays (E-screen) [48] and the reporter gene assays [49][50]. The arrival of robotics has allowed the development of high-throughput strategies in the search for bioactive compounds. However, major drawbacks appear: lack of selectivity due to cross-reactivity of structurally related compounds; matrix components in high concentration may produce false-positive or false-negative results. One way to overcome these problems is the separation of the sample constituents prior to testing. This can be performed by direct coupling of the separation technique, most of the time liquid chromatography (LC), with the biochemical detection (BCD) or receptor affinity detection (RAD). The theoretical basis for on-line LC-RAD has been performed with the estrogen receptors ERα and ERβ involving a fluorescent hormone as competitor [51].

Conclusion

Phytoestrogens represent at present a topic of major interest, as they concern healthcare or health prevention problems. However, scientific data are still lacking, particularly at the chemical, pharmacological and clinical levels. Such data are of prime importance in order to corroborate epidemiological studies and elaborate dietary and healthcare recommendations. The search for new natural products with estrogenic activity is also a crucial issue. Such compounds can serve as leads for the development of new estrogenic drugs or they can be used as pharmacological tools to elucidate mechanisms of action involved in healthcare effects produced by phytoestrogens. Diet and nutrition contribute to the different rates of cancer and other diseases throughout the world. Diets rich in plant-derived products may supply a variety of phytoestrogens capable of producing a range of pharmacological effects in the human body. As people live longer, women are spending more of their lives in menopause, affected by a variety of estrogen-related conditions that decrease the overall quality of life. Regrettably, the understanding of the potential health effects of phytoestrogens, plant-derived cousins of estradiol, is far from complete. Many factors contribute to this lack of understanding that persists despite the growing number of clinical trials. For instance, the great diversity of phytoestrogens makes it difficult to make general

conclusions about their health effects, since different members of this class may have different activities, pharmacokinetic properties and metabolic fate. Clinical trials that study phytoestrogens are often performed with a variety of botanical formulations and thus cannot be compared directly. Non-estrogenic compounds present in phytoestrogen-rich plant sources used in clinical research may interact with phytoestrogens and either potentiate or interfere with their activity and bioavailability. In addition, some phytoestrogens may act as estrogen agonists or antagonists depending on their structure and concentration. These factors underscore the difficulties of studying the clinical effects of bioactive mixtures over the single-ingredient pharmaceuticals.

In conclusion, much research is required to clearly define the pharmacological effect of dietary and environmental phytoestrogens. It is essential that future studies be performed with standardized and structurally characterized mixtures of compounds or with isolated phytoestrogens.

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