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# Antiestrogens from the Cloudforest: Inhibition of Aromatase by Plant Extracts and Essential Oils from Monteverde, Costa Rica

Brittany R. Agius

# ANTIESTROGENS FROM THE CLOUDFOREST: INHIBITION OF AROMATASE BY PLANT EXTRACTS AND ESSENTIAL OILS FROM MONTEVERDE, COSTA RICA

by

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# Abstract

This report discusses the aromatase enzyme and research of the inhibition of the enzyme by natural products from plants. Aromatase is a cytochrome P450 enzyme which catalyzes the conversion of androgens to estrogens. Due to the organization of the aromatase *CYP19* gene, the enzyme undergoes tissue-specific expression, including expression in adipose tissue and the ovary. Estrogen production by aromatase is necessary for normal physiological development and activity in both males and females. However, synthesis of estrogens by aromatase can contribute to the development of estrogen-dependent diseases, such as breast and prostate cancer. Current research in the field of plant natural products focuses on finding compounds from plants which inhibit aromatase activity. Studies which utilized bioassays to test plant compounds on aromatase activity have found that plant natural products are a promising source of aromatase inhibitors. From cytotoxicity screenings, plant natural products have also been concluded as potential new sources of anti-breast cancer drugs. This research leads way to developing new drugs based upon natural products for the treatment of estrogen-dependent disorders.

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# Introduction

Aromatase is the enzyme responsible for the conversion of androgens to estrogens. Androgens and estrogens are steroid hormones found in both males and females. An example of the reactions which aromatase catalyzes includes the conversion of androstenedione to estrone and testosterone to estradiol. Estrogens are present in the ovaries, placenta, and testes, among other tissues. Estrogens have many functions, including the production and distribution of body fat and the development of estrogen-dependent disorders, such as breast and prostate cancer (Monteiro and others 2006). Estrogen interacts with the estrogen receptor in cells which leads to the proliferation of cancer cells (Chen 1998).

Many types of cancers are estrogen-dependent, causing an increase in research interest in the inhibition of aromatase activity. Inhibiting estrogen synthesis by aromatase may lead to new forms of prevention and/or treatment of estrogen-dependent cancers. Natural products from plants is one area of interest in aromatase research. Plants contain many compounds which display biological activity. This suggests that some natural products may possess the ability to inhibit aromatase and, therefore, prevent the development of estrogen-dependent cancers.

This report provides a detailed discussion of the aromatase enzyme, the role of the estrogen receptor in estrogen-dependent cancers, and natural products research of aromatase inhibition. The report begins by describing the mechanics and function of aromatase, including an analysis of aromatase from a genetic perspective. A discussion of the estrogen receptor and its activity in different estrogen-dependent cancers follows. The report concludes with an analysis of research conducted on the inhibition of aromatase by plant natural products, specifically the methods and results of the research.

# Aromatase

Aromatase is the product of the *CYP19* gene and catalyzes the formation of estrogens from androgens via aromatization. The enzyme is found in many different organs and tissues throughout the body, including the ovary, placenta, brain, and adipose tissue. Aromatase activity is essential for normal function in these organs and tissues (Simpson and others 2002).

## *The Aromatase Gene*

The *CYP19* gene encodes aromatase cytochrome P450 (also known as P450arom and aromatase). The location of the *CYP19* gene is 15q21.1, i.e. the long arm of chromosome 15 on sub-band 1 of band 21 (NCBI 2008). The *CYP19* gene is part of the P450 superfamily of genes. This superfamily has over 480 members in 74 families; aromatase is the only member of family 19. The coding region of *CYP19* contains 9 exons which begin with exon II. An exon is a region of a gene which codes for part of the complete protein product of the gene and can contain untranslated regions (NHGRI undated; Zhang 1998). Upstream of exon II at the 5' end are a number of exons (exon I's) in the untranslated region of the gene (see Figure 1). Different tissues

contain a unique exon I in the 5' untranslated region (UTR) of the *CYP19* transcript due to unique promoters. Transcription of *CYP19* at different promoter sites is thus tissue-specific. From Figure 1, the *CYP19* transcript in the placenta, adipose, brain, and bone contain different 5'-UTR exon I's due to their specific promoters. Though the 5'-UTR exons are different, the final transcript and translated aromatase enzyme are the same for each tissue due to splicing which occurs to render the final *CYP19* transcript. As can be seen from Figure 1, this splicing occurs at an AG/GACT splice acceptor site in exon II. This allows the final transcript of the different tissues to contain the same coding region, beginning at exon II. It is important to note that aromatase expression in different tissues is under the control of different transcription factors due to the tissue-specific promoters. Hence, the promoter for adipose tissue may be under a different set of transcription factors than the promoter for bone, for example (Simpson and others 2002).

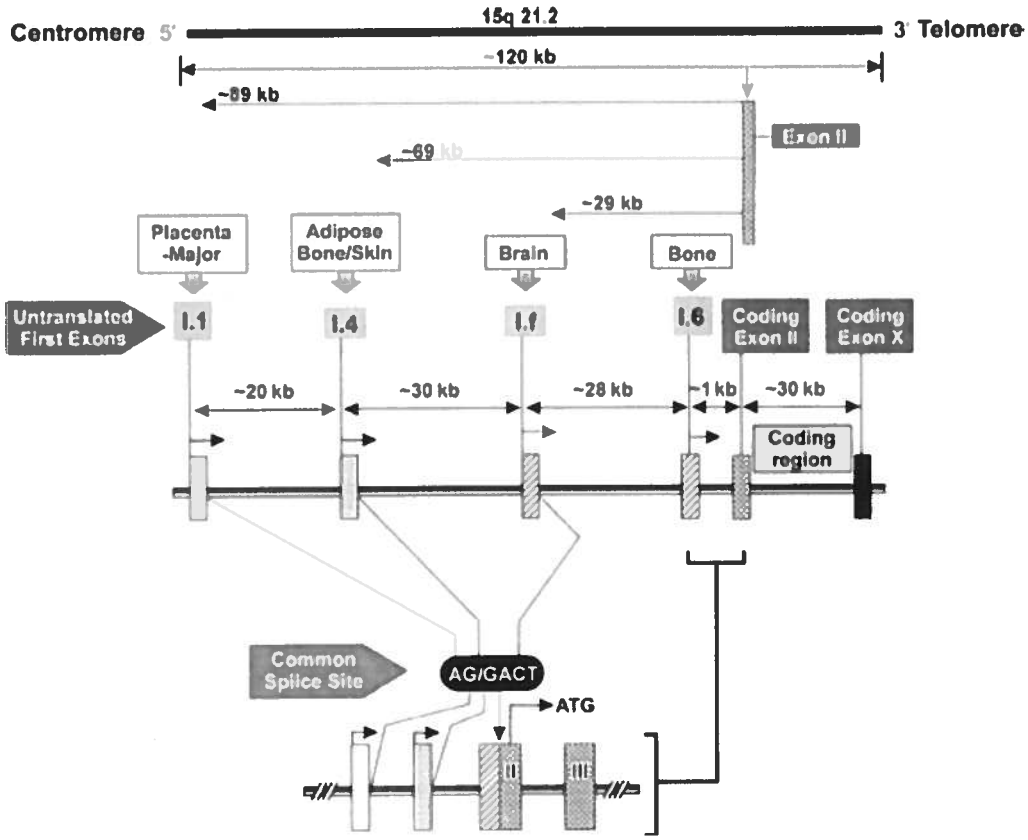



**Figure 1. Exon organization of the human *CYP19* gene located on 15q21.1.** Values in kilobases (kb) indicate exon I distances from exon II. Grey arrows indicate the tissue-specific promoters. Also shown is the AG/GACT splice cite in exon II.

Source: adapted from Simpson E; Clyne C; Rubin G; Chin Boon W; Robertson K; Britt K; Speed C; Jones M. 2002. Aromatase – a brief overview. Annual Review of Physiology 64:93-127.

## *The Structure of Aromatase*

The cytochromes, such as the cytochrome P450 superfamily, are enzymes which participate in electron or proton transfer reactions and contain one or more heme groups (Anonymous 1997). Aromatase has many structural features which are common to all cytochrome P450 proteins. These features include a heme-binding region near the carboxyl end of the protein. This region contains a conserved cysteine residue which acts as a fifth coordinating ligand for the heme iron. A thiolate ion (RS- where R is an alkyl group) in this region is responsible for many of the unique spectrophotometric and catalytic properties of the cytochrome P450 proteins. Upstream of this region are two regions highly conserved in aromatase species. The first is a region of 20 amino acids, while the second region is believed to be part of the substrate binding site which is near the heme prosthetic group where catalytic activity occurs (Simpson and others 2002).

Due to the location of mammalian aromatase as a membrane-bound protein and its low concentration in the endoplasmic reticulum of cells which express the protein, obtaining a crystal structure of aromatase has been near impossible. However, Graham-Lorence and others have modeled human aromatase based upon the three-dimensional core structures of soluble bacterial cytochrome P450 proteins, with the addition of secondary structures and other less-conserved features as was rational (1995).

The authors have postulated the hydrophobic membrane-binding region of the protein, which contains aliphatic and aromatic species which participate in recognition of the substrate; a binding site for the redox partner; and residues involved in proper orientation of the substrate for the active site (1995; Simpson and others 2002). Figure 2 illustrates this model.

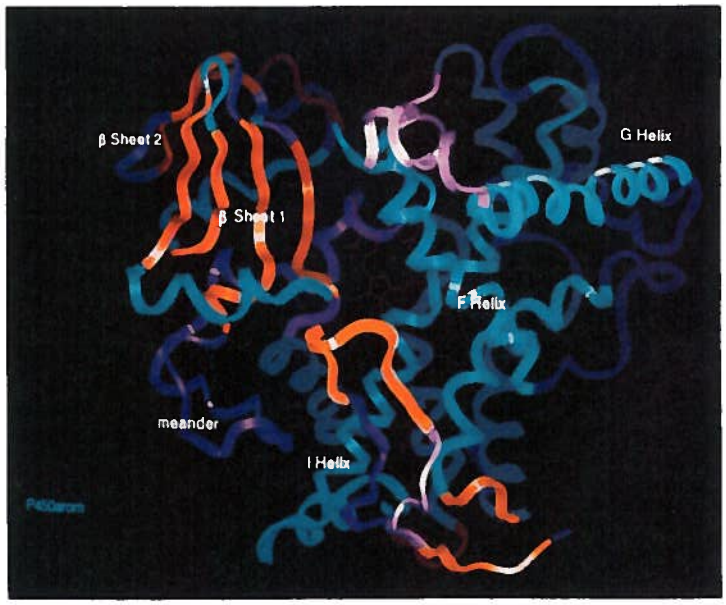



**Figure 2. Structure of the aromatase enzyme.** The structural features are labeled; the heme is the central red structure.

Source: Graham-Lorence S; Amarneh B; White R; Peterson J; Simpson E. 1995. A three-dimensional model of aromatase cytochrome P450. Protein Science 4:1065-80.

The core structure of aromatase, based upon the core structures of the bacterial P450 proteins, contains a bundle of four α-helices (helices D, E, I, and L), separate α-helices J and K, and β sheets 1 and 2. Also in this core structure are the heme binding region and a weaving "meander" region which extends from the N-terminal of the L helix (Graham-Lorence and others 1995). Figure 3 shows the individual core features.

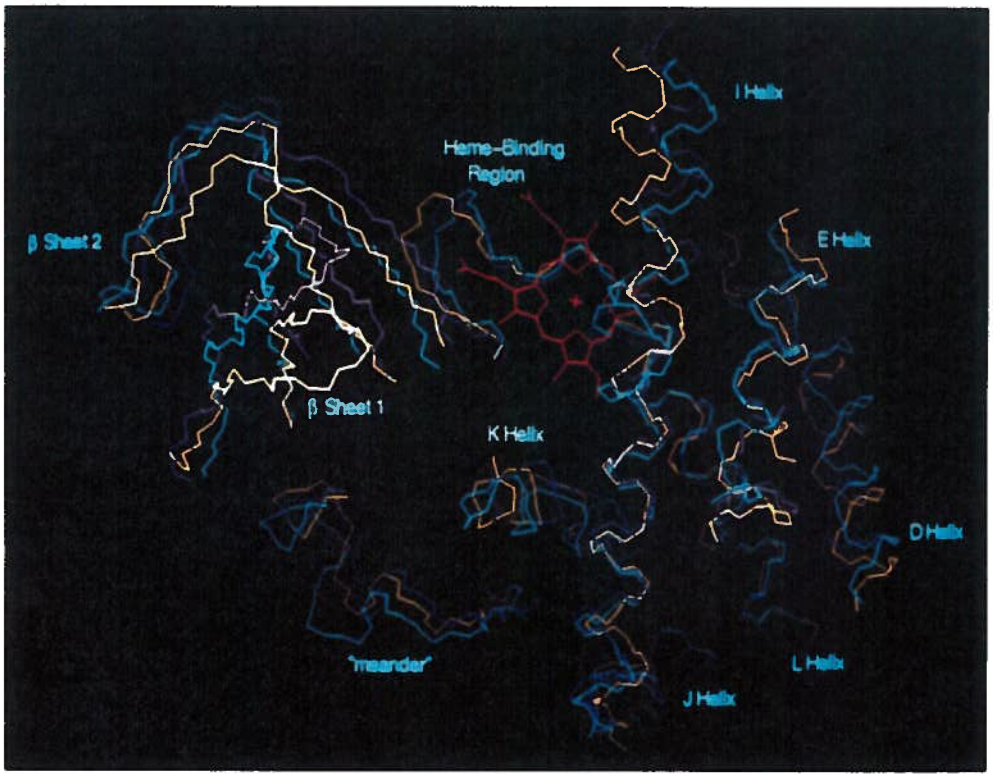



**Figure 3. Core features of the aromatase enzyme.** Note that the red structure is the heme group.

Source: Graham-Lorence S; Amarneh B; White R; Peterson J; Simpson E. 1995. A three-dimensional model of aromatase cytochrome P450. Protein Science 4:1065-80.

## *The Aromatase Reaction*

The cytochrome P450 proteins catalyze monooxygenation reactions. Aromatase catalyzes the aromatization of C19 steroid hormones (the androgens) to estrogens in the endoplasmic reticulum (Rainey and others 2003). This reaction is the sole reaction in vertebrates to produce a phenyl ring. The reaction occurs in the presence of the redox partner NADPH-P450 reductase and converts the A-ring of androgens to the phenyl rings of estrogens, losing the methyl group at carbon-19 as a result (Graham-Lorence and others 1995).

Via the reaction mechanism shown in Figure 4, aromatase converts androstenedione to estrone and testosterone to estradiol (Monteiro and others 2006). The reaction uses 3 moles of oxygen and NADPH for every one mole of androgen substrate converted by aromatase. The first two attacks by molecular oxygen in the reaction cause hydroxylation of the methyl group at C-19 with the loss of water as a byproduct to form a ketone. The third attack is a peroxidative attack on the methyl group which

results in the loss of the methyl group as formic acid and the production of the aromatic phenyl ring of the estrogens. This last step completes the conversion of the androgens to their estrogen counterparts (Graham-Lorence and others 1995).

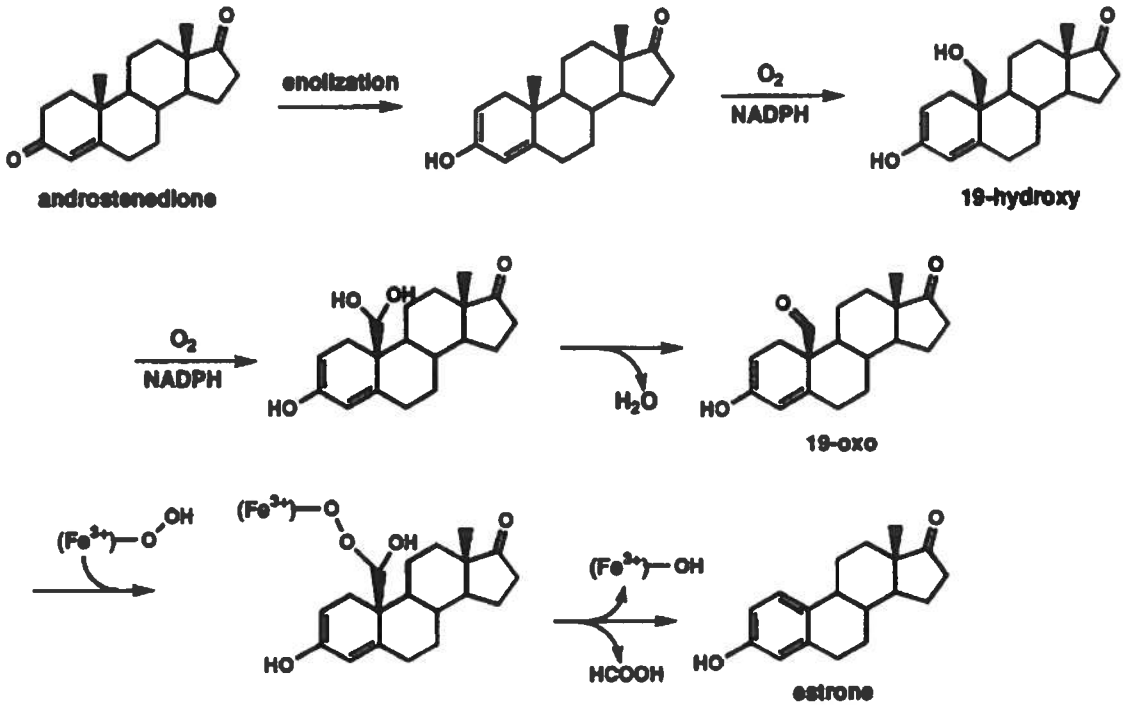



**Figure 4. Conversion of androstenedione to estrone.** $O_2$ and NADPH are required for the reaction to occur, while water and formic acid are released as byproducts.

Source: Graham-Lorence S; Amarneh B; White R; Peterson J; Simpson E. 1995. A three-dimensional model of aromatase cytochrome P450. Protein Science 4:1065-80.

It is readily apparent how important structure of aromatase is to the enzyme's catalytic function. For the reaction to take place, recognition of the androgen substrate must occur and the substrate must be properly bound to the active site. The carbon at position 19 must be within 2 Å of the oxygen bound to the heme region for attack by the oxygen to occur. Also important is the binding of the redox partner NADPH and the activation of oxygen by the heme region (Graham-Lorence and others 1995).

## *The Location of Aromatase*

The aromatase enzyme is expressed in both males and females in a number of different tissues and organs, including the ovary, adipose tissue, and bone. Expression of aromatase is essential for production of estrogen in these organs and tissues. Lack of the enzyme can have severe consequences in terms of regular physiological activity dependent upon estrogen (Simpson and others 2002).

## Aromatase Expression in the Ovary

Due to the tissue-specific promoters for the *CYP19* gene, expression of the gene in different tissues is under the regulation of different transcription factors. Aromatase expression occurs in the granulosa cells of the ovary, cells associated with the oocyte. The gonadotropin follicle-secreting hormone (FSH) causes transcription of *CYP19* by activating a signaling cascade (Bowen 2004). This signaling cascade eventually activates transcription factors which bind to the *CYP19* promoter specific for the ovary to begin transcription of aromatase (Simpson and others 2002).

In mice with an aromatase knockout, the absence of estrogen production causes an impairment in sexual activity and development for both male and female mice. In male mice, decreased sperm production and impaired sexual activity were the result of the aromatase knockout. Similarly, the aromatase knockout in female mice resulted in infertility due to the inability to ovulate (Simpson and others 2002).

## Aromatase Expression in Adipose Tissue

The *CYP19* promoter specific for adipose tissue is under activation by the class I cytokines, tumor necrosis factor-α (TNFα), and glucocorticoids (Simpson and others 2002). Cytokines are small proteins which are secreted in response to certain stimuli; they bind to membrane proteins and cause a response via secondary messengers for the cell to change its behavior (Decker 2006). TNFα is a type of cytokine which has both growth stimulatory and inhibitory effects (Anonymous 2000). Glucocorticoids are compounds which have many different functions, including a role in glucose metabolism and the breakdown of fat in adipose tissue. Certain glucocorticoids bind to their receptors in the cell cytoplasm and translocate to the nucleus where they directly regulate expression of their target gene (Bowen 2006).

In previous studies, aromatase deficiency in mice resulted in obesity, increased number of fat droplets in the liver, and impairment of the fatty acid β-oxidation pathway in liver cells. Obesity was also a result of aromatase deficiency in humans. Also observed were increased cholesterol and high-density lipoprotein (HDL) blood levels in aromatase knockout mice (Simpson and others 2002).

## Aromatase Expression in Bone

Like expression in adipose tissue, expression of *CYP19* in osteoblast-like cells from human bone samples is also under regulation by class I cytokines, TNFα, and glucocorticoids. Also contributing to the regulation of aromatase expression is the transforming growth factor-β1 (TGF-β1) which has a role in cell proliferation and differentiation (Hyytuäinen and others 2004). Aromatase knockout in male and female mice caused abnormal bone metabolism and growth, as well as decreased bone density in the lumbar spine (Simpson and others 2002; WebMD 2006).

# The Estrogen Receptor and Estrogen-Dependent Cancers

Estrogens play a key role in the proliferation of estrogen-dependent disorders. Roughly 60% of premenopausal patients and 75% of postmenopausal patients suffer from estrogen-dependent carcinomas. Estrogen regulates the development of estrogen-dependent disorders via its interaction with the estrogen receptor (ER) in cells. Examples of estrogen-dependent cancers include breast, ovarian, and prostate cancer (Chen 1998).

## *The Estrogen Receptor (ER)*

Two mechanisms describe the function of the estrogen receptor and how estrogen binds to the receptor. In the first mechanism, estrogen diffuses into the cell and binds to the estrogen receptor which is in the nucleus. The estrogen-ER complex then binds to estrogen response element (ERE) regulatory sequences in target genes. This binding occurs directly or indirectly with the aid of activator protein 1 (AP1) or the transcription factor SP1. Binding to the ERE sequences recruits coregulatory proteins (whether coactivators or corepressors) which either activate or repress the promoter of the target gene, leading to increased or decreased production of the mRNA and protein corresponding to that gene. This cascade of events typically takes hours to occur before producing a physiological response (Deroo and Korach 2006).

The second mechanism of the interaction of estrogen with the estrogen receptor normally occurs within seconds or minutes. In this mechanism, estrogen either binds to the estrogen receptor associated with the plasma membrane or binds to non-estrogen receptor proteins which are also associated with the plasma membrane. Adaptor proteins play a role in locating the estrogen receptor to the cytoplasmic side of the plasma membrane. This binding causes an increase in the levels of $Ca^{2+}$, NO, and other small molecules, and causes kinase activation, all of which take part in signaling (Deroo and Korach 2006). Figure 5 illustrates the two mechanisms of estrogen action in the cell.

There are two forms of the estrogen receptor: ER $\alpha$ and ER $\beta$. These ER forms have different expression patterns in tissues. The genes which encode ER $\alpha$ and ER $\beta$ – *ESR1* and *ESR2*, respectively – have different chromosomal locations (Deroo and Korach 2006). The DNA-binding sites of ER $\alpha$ and ER $\beta$ are 97% similar, differing in only two amino acid residues. Likewise, the ligand-binding site of the two proteins is 59% similar in terms of amino acid sequence. Thus, ER $\alpha$ and ER $\beta$ share DNA and ligand binding characteristics (Parl 2000). Studies observing the results of mutations in ER $\alpha$ and ER $\beta$ have found that mutations produce distinct phenotypes in the animal models used, allowing for a better understanding of the physiological role of these forms of the estrogen receptor (Deroo and Korach 2006).

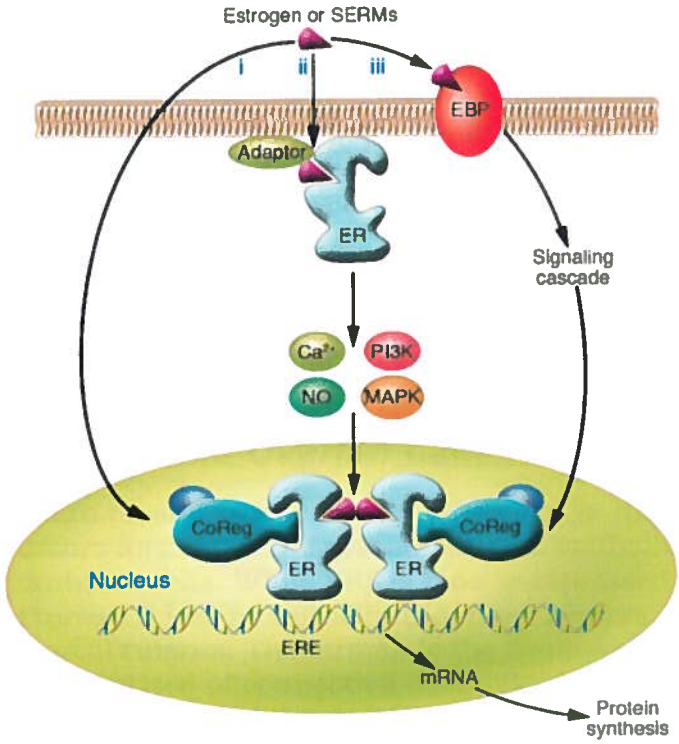



**Figure 5. The two mechanisms of interaction between estrogen and the estrogen receptor (ER).** Estrogen or selective estrogen receptor modulators (SERMs) enter across the cell membrane and interact with the estrogen receptor in the nucleus or bound to the plasma membrane. The mechanisms are described in the text. EBP, estrogen-binding protein; CoReg, coregulatory protein; ERE, estrogen response element.

Source: Deroo BJ; Korach KS. 2006. Estrogen receptors and human disease. The Journal of Clinical Investigation 116(3):561-70.

## *The Estrogen Receptor and Breast Cancer*

Previous studies have shown that increased exposure to estrogen causes an increased risk of developing breast cancer. There are two hypotheses which explain this relationship between estrogen and breast cancer. The first hypothesis explains than estrogen causes an increase in the growth of mammary cells. Due to the increased number of target cells, increased cell division will take place, allowing for a higher chance of mutations to occur during replication. Detrimental effects to cell processes such as apoptosis, cell growth, and DNA repair could result from mutations. Cancer cells would thrive due to inhibition of apoptosis and DNA repair which naturally kill or repair cancer cells, respectively. Mutations preventing regulation of cell growth would allow uncontrolled proliferation of the cancerous cells. The second hypothesis describes the metabolic byproducts of estrogen as causing DNA damage, resulting in point mutations which can lead to cancer. Estrogen may participate in formation of breast cancer by one or both mechanisms (Deroo and Korach 2006).

Previous studies conducted on animal models and cell cultures have revealed that the estrogen receptor is a factor in the development of mammary glands and in mammary cancer. Adult female mice with an ERα knockout exhibited mammary glands which were similar to those of prepubescent female mice, i.e. mammary glands that were not fully developed. In other mouse studies, the ERα knockout either prevented, delayed, or decreased tumor formation. Thus, ERα clearly has a role in regulating the activity of estrogen in mammary gland maturation and cancer onset. Transcriptional coregulators are also known to participate in estrogen-dependent tumor formation. The coregulator amplified in breast cancer-1 (AIB1) has increased expression in certain breast tumors and affects tumor initiation and growth. The combined activity of the estrogen receptor and transcriptional coregulators in the development of breast tumors is still being understood (Deroo and Korach 2006).

## The Estrogen Receptor and Ovarian Cancer

Ovarian cancer is the fifth most common cancer in women in the Unites States. The majority of ovarian tumors form from the ovarian surface epithelium, while a smaller number form from granulosa cells. While ERβ is mostly expressed in tumors from granulosa cells and expressed less in epithelium-derived tumors, ERα is expressed in both epithelial and stromal tumors. The stroma is the framework which supports an organ and is normally comprised of connective tissue (MedicineNet.com 2003). In cell culture studies, estrogens stimulated the growth of cancerous and normal ovarian cells, while estrogen agonists inhibited growth of both cell types. As of yet, the exact function of estrogens in cancerous and normal ovarian cell growth is not fully comprehended (Deroo and Korach 2006).

## The Estrogen Receptor and Prostate Cancer

Since the 1980s, the second leading cause of death in men due to cancer has been prostate cancer. ERβ is more prominent in human and rodent prostates than ERα. While ERβ is present in epithelial cells and, in lower quantities, in stromal cells, ERα is only localized to stromal cells. Studies have shown that ERβ expression levels decrease in prostate tumors. Also observed in studies has been the formation of prostatic epithelial hyperplasia, the excessive growth of normal cells, in ERβ knockout mice (MacDonald 2000). Similarly, rodents treated with agonists specific for ERβ developed prostate atrophy as a result of apoptosis. These findings suggest that ERβ has a necessary function in the healthy prostate (Deroo and Korach 2006).

Conversely, ERα is known to mediate the effect of estrogens in the prostate. Neonatal rodents exposed to estrogens suffered from abnormal adult prostate function and morphology, as well as inflammations, hyperplasia, and dysplasia (cells which are abnormal but not cancerous) (Johns Hopkins Health Alerts 2007). Neonatal ERα knockout rodents were not found to develop prostate hyperplasia upon exposure to estrogens. Though studies have shed light on the contribution of the estrogen receptor to prostate function, more research must be conducted to fully understand the role of estrogens in prostate development (Deroo and Korach 2006).

# Aromatase Inhibition by Plant Natural Products

The inhibition of aromatase for medicinal purposes has been studied quite extensively in the field of natural products research, especially in regards to inhibition by natural products from plants. Whether from animals, microbes, or plants, natural products have historically been sources of therapeutic agents. This section begins with a brief introduction to natural products drug discovery and discusses the methods, results, and conclusions of studies conducted upon aromatase inhibition by plant natural products.

## *Natural Products Drug Discovery*

Natural products drug discovery is the study of chemical products from nature for new medicinal treatments. Research of drugs from natural products by the Western pharmaceutical industry reached a climax between 1970 to 1980. Approximately 49% of the 877 small molecules with pharmaceutical possibilities introduced between 1981 and 2002 were natural products, semi-synthetic natural products, or synthetic compounds based upon natural products. Approximately 25% of drugs prescribed since the 1980s are natural products or based upon natural products (Koehn and Carter 2005; Stokes 2007). As shown in Figure 6, drugs from natural products treat a range of illnesses.

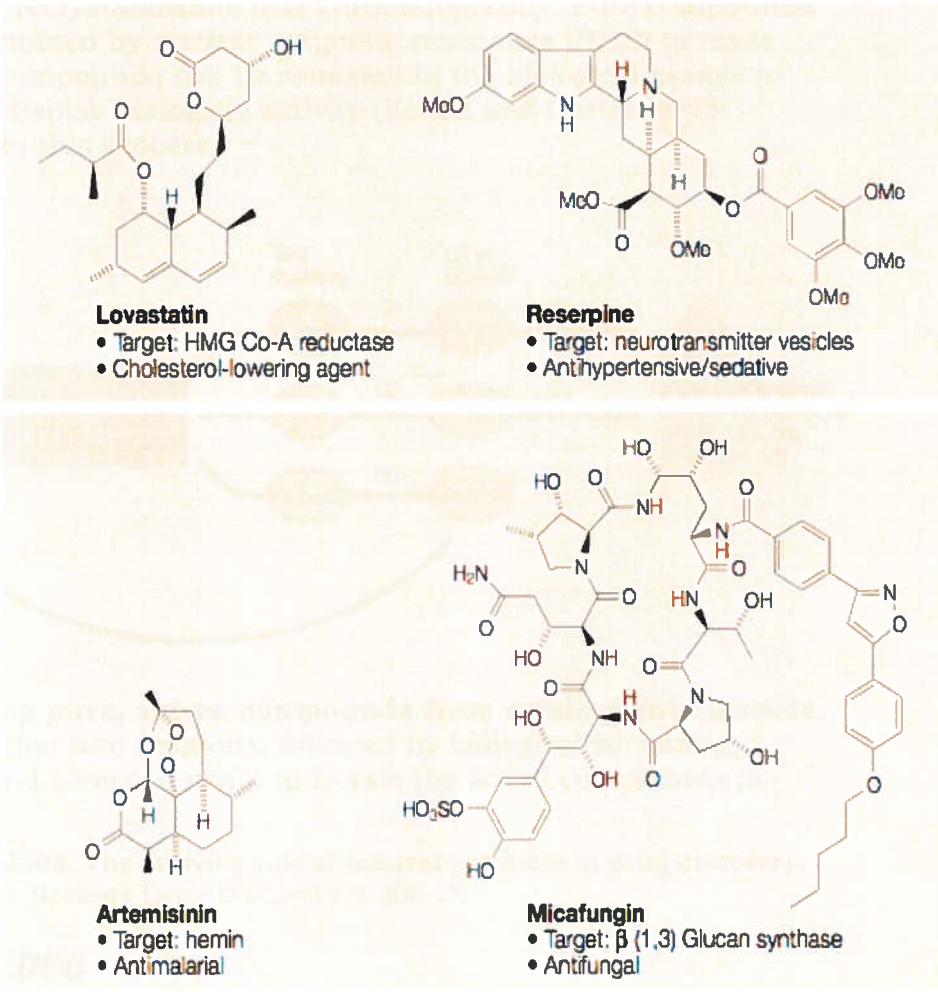



**Figure 6. Examples of natural products used for drug purposes.** Note the variety in the structures and their different drug qualities.

Source: Koehn FE; Carter GT. 2005. The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery 4:206-20.

## Natural Products from Plants

Due to their immense diversity, plants offer many drug possibilities. The initial step in determining whether a natural product is active against a particular disease is to isolate the natural product, i.e. isolate the pure compound. Many steps are involved in the isolation process. First, an extract or essential oil is obtained from a plant sample. Extracts are collected by extraction of plant material, such as bark or root samples, using a solvent. The solvent is typically somewhat polar like dichloromethane or chloroform. Essential oils are obtained by hydrodistillation of finely chopped leaves, followed by extraction with dichloromethane. In the next step, extracts are separated into fractions using separation techniques which commonly include column chromatography. Individual essential oils and fractions can then be tested in biological screenings to determine which samples are active. Since the essential oils and fractions are mixtures of compounds, this identifies which samples contain the active compounds of interest. The active essential oils and fractions are then purified by various techniques, including recrystallization and chromatography. Pure compounds can then be structurally determined by nuclear magnetic resonance (NMR) or mass spectrometry (MS). The pure compounds can be re-tested in the biological assays to determine which compounds display biological activity (Koehn and Carter 2005; Stokes 2007). Figure 7 outlines this process.

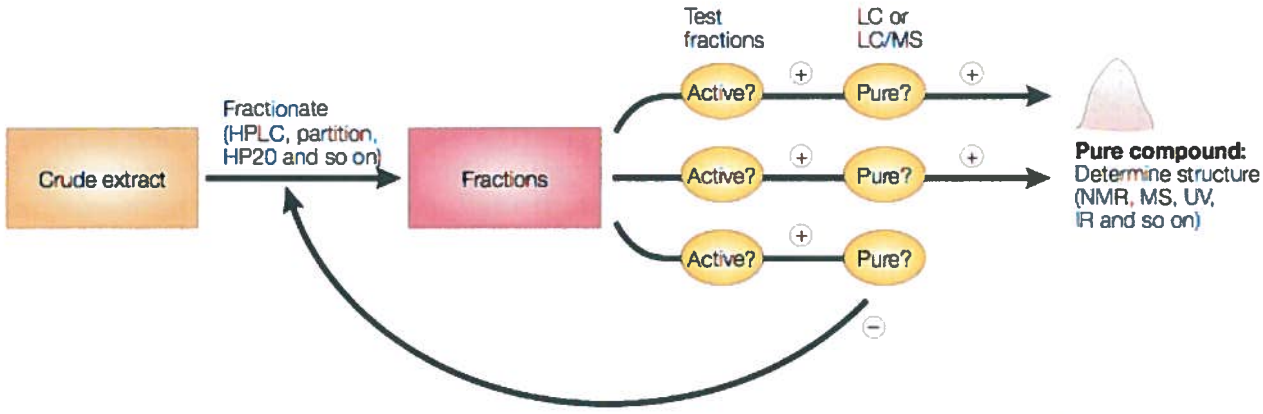



**Figure 7. Process of obtaining pure, active compounds from crude plant extracts.** The extract undergoes separation into fractions, followed by biological screening. Active fractions are purified and screened again to isolate the active compounds in their pure form.

Source: Koehn FE; Carter GT. 2005. The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery 4:206-20.

## Monteiro and Others, 2006

In their study, Monteiro and others determined the effect of diet polyphenolic compounds on the inhibition of aromatase. Polyphenolic beverages, such as wine and tea, were also tested. Aromatase inhibition was measured using the tritiated water release assay. The authors concluded that polyphenolic compounds do indeed inhibit aromatase activity, but do not inhibit expression of the enzyme or cell viability (2006).

## Background

The authors wished to study the effects of polyphenols on the inhibition of estrogen synthesis by aromatase. Polyphenols are present in almost all plant-based foods. The compounds contain the phenol structural unit, and many classes of polyphenols share structural similarity with estrogens. This structural similarity opens the possibility that polyphenols may be able to interfere with certain estrogen-dependent cellular processes. Many polyphenol-rich beverages, such as red wine and tea, have been related to anticancer activity, including cell cycle regulation and impairment of cancer cell activation (2006).

## Methods

Monteiro and others measured aromatase inhibition by using the tritiated water release assay. The assay works by measuring the release of tritiated water ($[^3H]H_2O$) during the conversion of tritiated androstenedione ([$^3$H]androstenedione) to estrone. Tritium, $^3H$, is a radioactive isotope of hydrogen. JAR cells were incubated with samples of polyphenolic compounds and polyphenolic beverages, including red and white wine, alcohol-free red and white wine, and black and green tea. The JAR cell line originates from a placental tumor; the cell line was chosen for the experiment since JAR cells are rich in aromatase (IST 2005). JAR cells were initially treated with [$^3$H]androstenedione and then incubated with the various polyphenol samples. After incubation, the cells were vortexed and centrifuged. The supernatant was collected and the amount of $[^3H]H_2O$ present in the supernatant was measured using liquid scintillation counting. Liquid scintillation counting involves addition of the supernatant to a scintillation cocktail which contains an aromatic solvent. The beta particles released from the decay of the radioactive isotope transfer their kinetic energy to the solvent particles. When this energy excites the solvent particles, the solvent particles emit UV light. This UV light then excites fluor molecules in the solution which emit blue light upon relaxation. A liquid scintillation counter measures the pulses of blue light from the fluor molecules in the sample and converts the data to a measure of the radioactivity of the sample (UWM undated; 2006).

To ensure that the polyphenols and beverages were not decreasing cell viability or inhibiting expression of aromatase, the authors tested lactate dehydrogenase (LDH) activity and conducted Western blotting and reverse-transcription polymerase chain reaction (RT-PCR). Cell viability was determined by the release of LDH into the media, which correlated linearly with cell death. LDH activity was measured by determining the oxidation of NADH when pyruvate is reduced to lactate; LDH is the enzyme which catalyzes the reduction reaction. RT-PCR and Western blotting demonstrated whether aromatase was being expressed at the mRNA and protein level, respectively (2006).

## Results

Figure 8 shows the structure of the polyphenols tested in the study. Table 1 gives the $IC_{50}$ values for a set of the polyphenols. The $IC_{50}$ value is the concentration of sample required to inhibit aromatase activity by 50%. Thus, the lower the $IC_{50}$ value, the more potent the sample. From Table 1, chrysin, naringenin, and quercetin were found to have the most inhibitory effect against aromatase, while kaempferol, myricetin, resveratrol had moderate inhibitory effect. Epigallocatechin-3-gallate had the least inhibitory effect (Monteiro and others 2006).

Table 2 shows the aromatase inhibition of a second set of polyphenols; aromatase activity is presented as a percent of the control. The solvent control (DMSO or ethanol, depending on which solvent was used for that particular polyphenol) was taken as 100% aromatase activity. Catechin caused the most decreased aromatase activity, followed by genistein and rutin. Epicatechin also had a significant decrease in activity, but it was tested at double the concentration of the other compounds in the table. None of the polyphenols reduced cell viability or inhibited aromatase expression (Monteiro and others 2006).

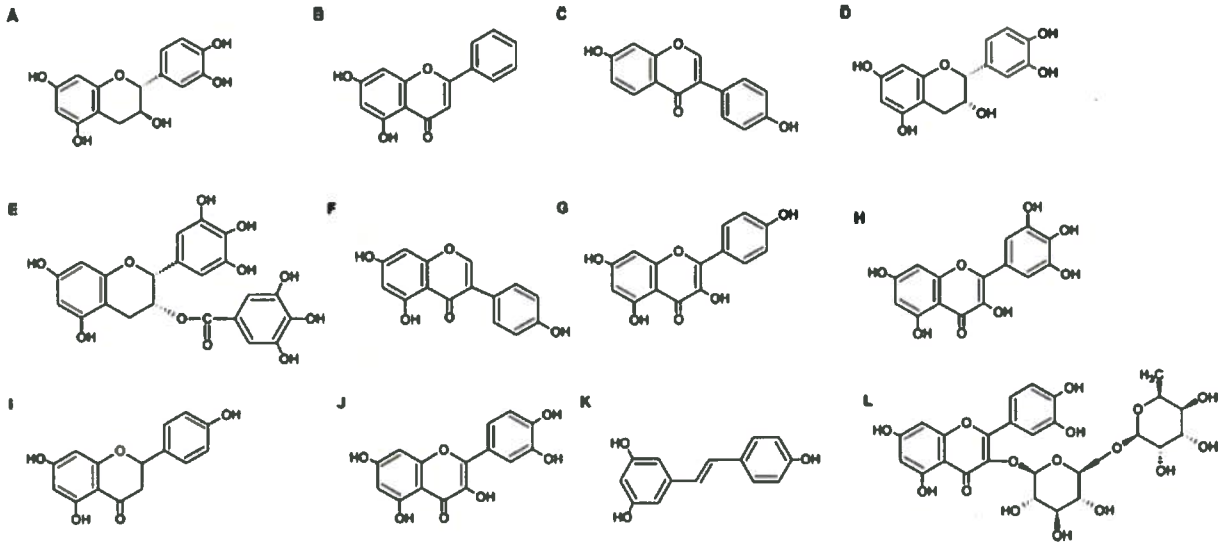


**Figure 8. Structures of the polyphenols tested in the study. (a)** catechin, **(b)** chrysin, **(c)** daidzein, **(d)** epicatechin, **(e)** epigallocatechin-3-gallate, **(f)** genistein, **(g)** kaempferol, **(h)** myricetin, **(i)** naringenin, **(j)** quercetin, **(k)** resveratrol, and **(l)** rutin.

Source: Monteiro R; Azevedo I; Calhau C. 2006. Modulation of aromatase activity by diet polyphenolic compounds. Journal of Agricultural Food Chemistry 54:3535-40.

**Table 1. $IC_{50}$ values for a selection of the polyphenols.**

| phenolic compound | $IC_{50}$ (95% CI) μmol/L |
|---|---|
| chrysin | 6.1 (3.0–12.4) |
| naringenin | 7.5 (3.2–17.5) |
| quercetin | 84.6 (58.4–122.4) |
| kaempferol | 161.6 (86.0–304.0) |
| myricetin | 164.1 (87.2–309.1) |
| resveratrol | 296.3 (196.5–446.6) |
| epigallocatechin-3-gallate | 1770 (1501–2088) |

Source: Monteiro R; Azevedo I; Calhau C. 2006. Modulation of aromatase activity by diet polyphenolic compounds. Journal of Agricultural Food Chemistry 54:3535-40.

**Table 2. Aromatase activity, reported as a percent of the control, for a selection of the polyphenols.**

| phenolic compound | aromatase activity (% of control) | conc mmol/L |
|---|---|---|
| catechin | 64.2± 5.5%[b] | 1 |
| epicatechin | 60.5 ± 2.1%[b] | 2 |
| genistein | 74.0 ± 6.0%[b] | 1 |
| rutin | 77.9 ± 3.7%[b] | 1 |

Source: Monteiro R; Azevedo I; Calhau C. 2006. Modulation of aromatase activity by diet polyphenolic compounds. Journal of Agricultural Food Chemistry 54:3535-40.

The beverages tested were red wine (RW), white wine (WW), alcohol-free red and white wine (AF-RW and AF-WW, respectively), green tea (GT), and black tea (BT). Aromatase activity was reported in units of pmol mg $prot^{-1}$ $h^{-1}$ for RW, WW, and the controls (cells with no treatment, C, and cells treated with ethanol, C'). These results are shown in Figure 9(a). RW significantly decreased aromatase activity with respect to the cells that were not treated. The ethanol control displayed the next highest decrease in activity, followed by WW. Figure 9(b) shows aromatase activity, reported as a percent of the control, for AF-RW, AF-WW, GT, and BT. The controls were cells with no treatment, C, and cells treated with sodium chloride, C'. The controls were set to 100% aromatase activity. AF-RW caused the highest decrease in aromatase activity, followed by GT, BT, and AF-WW. None of the beverages inhibited expression of aromatase or reduced cell viability (Monteiro and others 2006).

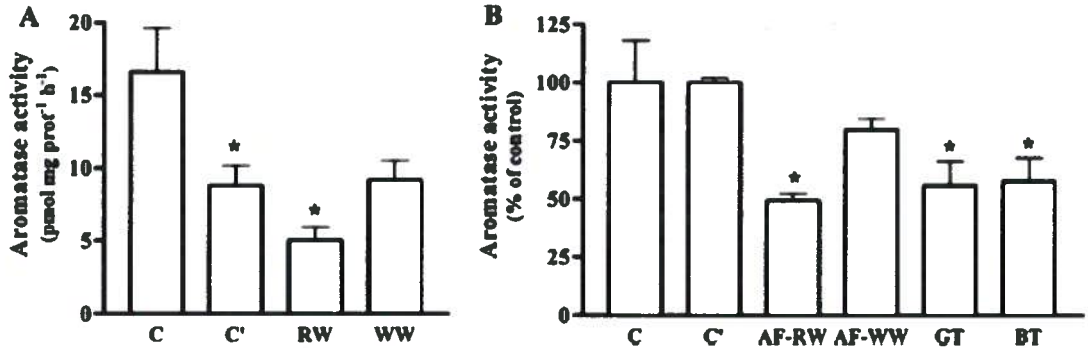



**Figure 9. Aromatase inhibitory activity of the polyphenolic beverages.**
**(a)** Aromatase activity for red wine (RW) and white wine (WW). C, untreated cells; C', cells treated with ethanol. **(b)** Aromatase activity for alcohol-free red and white wine (AF-RW and AF-WW), green tea (GT), and black tea (BT). C, untreated cells; C', cells treated with NaCl.

Source: Monteiro R; Azevedo I; Calhau C. 2006. Modulation of aromatase activity by diet polyphenolic compounds. Journal of Agricultural Food Chemistry 54:3535-40.

## Conclusions

While most of the polyphenols tested can be found in varying levels in both wine and tea, chrysin is not found in any of the beverages tested. Likewise, naringenin is most abundant in citrus fruits and is found in small quantities in red wine. The inhibitory activity of the beverages can be attributed to their polyphenol content. RW and AF-RW were found to be more active than WW and AF-WW, respectively. Generally, red wine has approximately twenty times the content of polyphenols as WW. This occurs due to the wine making process: grape skins are kept in contact with ethanol for a longer period of time when making red wine versus white wine, allowing for more polyphenols to be present in red wine. Due to the inhibitory activity of AF-RW and AF-WW, ethanol was not concluded to be necessary for wine activity. Independent of wine, ethanol does display its own inhibitory activity against aromatase (Monteiro and others 2006).

The mode of inhibition of these polyphenols is not clear. The authors hypothesize that the compounds may interact with the aromatase enzyme, inhibiting aromatase activity by blocking substrate binding. This is a possibility since the polyphenols are structurally similar to the aromatase substrate. Also, the antioxidant properties of the polyphenols may reduce NADPH and $O_2$ availability, both of which are necessary for the aromatase reaction to proceed. No matter the mechanism of the polyphenols, their inhibitory activity against aromatase makes the compounds possible drug candidates for treatment of estrogen-dependent disorders (2006).

# *Ta and Walle, 2007*

In their study, Ta and Walle tested the aromatase inhibitory activity of the methylated flavones 5,7-dimethoxyflavone, 7-methoxyflavone, and 7,4'-dimethoxyflavone versus their non-methylated counterparts chrysin, 7-hydroxyflavone, and 7,4'-dihydroxyflavone, respectively. The authors used a high-throughput aromatase assay for their study (2007).

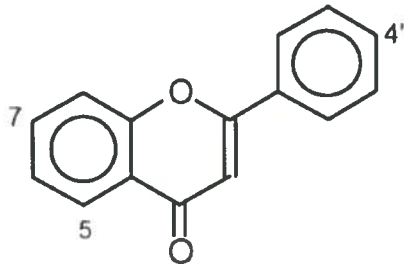
## Background

Flavonoids are naturally occurring compounds in plants. It is known that methylated flavones, a class of flavonoids, have better intestinal absorption and resistance to metabolism than their non-methylated counterparts. As such, methylated flavones would be better pharmaceuticals. In this study, the authors tested the inhibitory effect of methylated flavones against aromatase in comparison to the non-methylated equivalents which are known to inhibit aromatase. The methylated flavones could prove to be new drug candidates if they are effective against aromatase (2007).

## Method

The compounds tested in the study were chrysin, 7-hydroxyflavone (7-HF), and 7,4'-dihydroxyflavone (7,4'-DHF) and their methylated counterparts 5,7-dimethoxyflavone (5,7-DMF), 7-methoxyflavone (7-MF), and 7,4'-dimethoxyflavone (7,4'-DMF). The structures of the compounds are shown in Figure 10. The aromatase assay conducted was an enzymatic assay. The aromatase enzyme was obtained from recombinant human CYP19. The compounds were diluted by serial dilution to concentrations of 0.05–100 μM and then added to an NADPH-generating system in phosphate buffer. After a brief incubation period, aromatase and dibenzylfluorescein (DBF) substrate

were added. The procedure was conducted in 96-well plates. The reaction was terminated by the addition of sodium hydroxide and, two hours later, fluorescence was measured using a plate reader. A triplicate test was performed for each concentration of the flavones. The assay gave a measure of aromatase activity since DBF fluoresces upon metabolism by aromatase. Thus, the more fluorescence, the more aromatase activity (Ta and Walle 2007).




| | Substituent | | |
|---|---|---|---|
| Flavone | 5 | 7 | 4' |
| 5,7-DMF | $OCH_3$ | $OCH_3$ | H |
| Chrysin | OH | OH | H |
| 7-MF | H | $OCH_3$ | H |
| 7-HF | H | OH | H |
| 7,4'-DMF | H | $OCH_3$ | $OCH_3$ |
| 7,4'-DHF | H | OH | OH |

**Figure 10. Structures of the flavones tested in the study.** Substituents at the 4', 5, and 7 positions vary for each compound.

Source: Ta N; Walle T. 2007. Aromatase inhibition by bioavailable methylated flavones. Journal of Steroid Biochemistry and Molecular Biology 107:127-9.

## Results

Figure 11 shows the results of the study. Graphs are depicted as aromatase activity as a percent of the control (the control taken as 100%) versus concentration of the compound in μM units. The authors found that 5,7-DMF had very poor inhibition against aromatase in comparison to its counterpart, chrysin, which is a very potent aromatase inhibitor. Conversely, 7-MF and 7,4'-DMF had very similar inhibition in comparison to their respective counterparts, 7-DF and 7,4'-DHF, which are the two most potent aromatase inhibitors of the flavones (Ta and Walle 2007).

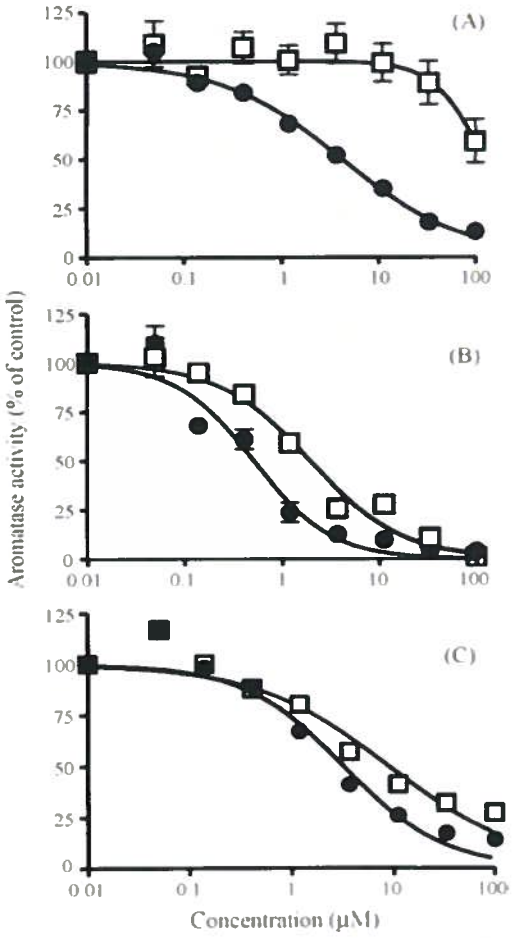



**Figure 11. Aromatase activity as a function of concentration of the flavone samples.** Aromatase activity for **(a)** 5,7-dimethoxyflavone and chrysin, **(b)** 7-methoxyflavone and 7-hydroxyflavone, and **(c)** 7,4'-dimethoxyflavone and 7,4'-dihydroxyflavone. The methylated flavones are represented by the white squares, while the black dots represent the unmethylated counterparts.

Source: Ta N; Walle T. 2007. Aromatase inhibition by bioavailable methylated flavones. Journal of Steroid Biochemistry and Molecular Biology 107:127-9.

## Conclusions

The methylated flavones, such as 7-MF and 7,4'-DMF, have better resistance against metabolism in the human liver and have better intestinal absorption versus their non-methylated equivalents. Due to the high aromatase inhibition of 7-MF and 7,4'-DMF, the compounds are excellent candidates as anti-aromatase drugs in humans. Though the compounds used in the study were synthetic, both compounds are found naturally in plants. 7-MF is found in Meliaceae and Rutaceae plants, while 7,4'-DMF has been found in neotropical nutmeg species and propolis, the resin created by bees from plant products (Ta and Walle 2007; NLM 2008).

# Inhibition of Aromatase by Plant Extracts and Essential Oils from Costa Rica

A study was performed test the inhibitory effect of plant extracts and essential oils obtained from Monteverde, Costa Rica, against aromatase activity. The study was conducted by the author of this report through the Natural Products Drug Discovery Group at the University of Alabama in Huntsville, under the direction of Dr. William N. Setzer. The natural products were previously tested by the author for cytotoxic activity against breast cancer cells. An enzymatic fluorescence assay was the method of choice for testing aromatase inhibition by the plant samples. While the study concluded the natural products to have excellent cytotoxic abilities, results of the aromatase inhibition assay are, as of yet, inconclusive.

## *Background*

The natural product samples tested in this study were obtained from the rainforests of Monteverde, Costa Rica. Natural products from Costa Rica are of particular interest due to the many drug possibilities which exist in the rainforests. Between 1983 and 1995, more than 60% of new drugs approved for the treatment of cancer and infections were based on natural products from tropical rainforest plants. Multiple surveys of the bioactivity of plants collected in Monteverde, Costa Rica, have yielded cytotoxic, antibacterial, antifungal, and antiviral activity. The wealth of biodiversity in the Monteverde region lends credence that the rainforests yet contain many potential medicinal agents. (Setzer and others 2003)

## *Method*

Plant materials – including bark, roots, and leaves – were collected in the rainforests of the Monteverde region in May 2008. All materials were finely chopped. From bark and roots, crude extracts were obtained by Soxhlet extraction with solvents including acetone, chloroform, dichloromethane, and methanol. Essential oils were collected by the hydrodistillation of leaves with chloroform using a Likens-Nickerson apparatus. The extracts and essential oils were prepared as 1% w/w dimethylsulfoxide (DMSO) solutions for subsequent testing in cytotoxicity and aromatase inhibition assays.

### MTT Assay

The DMSO samples of the crude extracts and essential oils were initially tested for cytotoxicity against the MDA-MB-231 breast cancer cell line. Cytotoxicity was determined by testing for cell viability using the MTT cell proliferation assay. A total of sixty-six DMSO samples were tested in the assay. The assay works by measuring product formation from a chemical reaction carried out by living cells. Live cells treated with the yellow compound MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) reduce MTT to the purple compound formazan, a reaction catalyzed by the enzyme mitochondrial reductase. This reaction is shown in Figure 12. Thus, this test offers an easy method by which to detect live cells since dead cells cannot produce formazan. (Biocompare 2009)

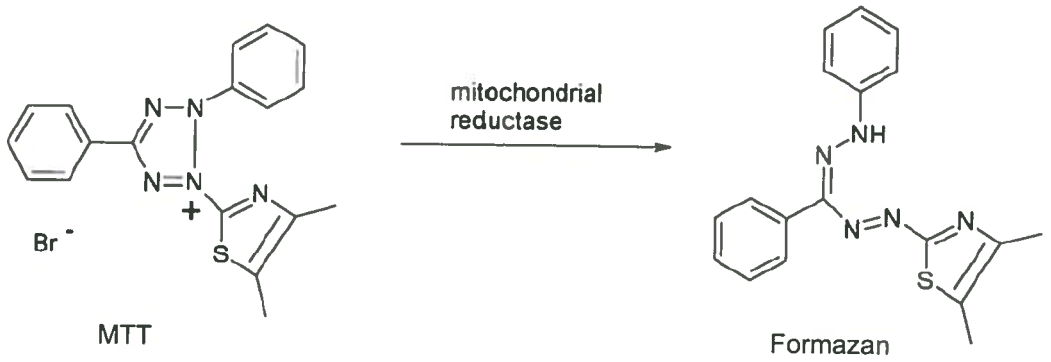



**Figure 12. Conversion of MTT to formazan by mitochondrial reductase.** MTT is a bright yellow compound, while formazan is deep purple in color.

Source: Biocompare. 2009. Influence of washing steps on cell attachment: Comparison of PDL coated and cell culture treated microplates. http://www.biocompare.com/Articles/TechnicalArticle/1605/Influence-Of-Washing-Steps-On-Cell-Attachment-Comparison-Of-PDL-coated-And-Cell-Culture-Treated-Microplates-from-Greiner-Bio-One.html. Accessed 2009 April 21.

The author used the following method for performing the MTT assay. Breast cancer cells grown in a 96-well plate were treated with a selection of DMSO samples. The samples were tested in quadruplicate at a concentration of 100 µg/mL. The negative controls in the assay were growth media and DMSO, while the positive control was the compound tingenone. Tingenone was the positive control due to its highly cytotoxic nature, being cytotoxic even at relatively low concentrations (Setzer and others 1998). The cells were then incubated with the samples for 48 hours. After incubation, MTT was added to each well and the plate was allowed to incubate for a further 4 hours. Before incubation, however, the plate was pre-read in a spectrophotometer to obtain initial absorbance readings at a wavelength of 570 nm. After the 4-hour incubation period, the production of purple formazan was observed if live cells were present. The plate was read once more in the spectrophotometer to obtain final absorbance readings at the same wavelength. Initial and final absorbance readings were tabulated in a spreadsheet and entered into equations to determine the percentage of viable cells remaining after treatment with each sample. From the percent viable cells, the percent kill of the sample was calculated.

## Aromatase Inhibition Assay

The aromatase inhibition assay conducted in the study was performed using a CYP19/MFC high throughput inhibitor screening kit purchased from BD Gentest Corporation (Cat #459520). The assay is an enzymatic fluorescence assay, measuring the production of a fluorescent compound by active aromatase. Aromatase converts the compound methoxy-4-trifluoromethyl-coumarin (MFC) to the fluorescent compound 7-hydroxy-4-trifluoromethyl coumarin (7-HFC). This reaction is shown in Figure 13. (BD Biosciences 2009)

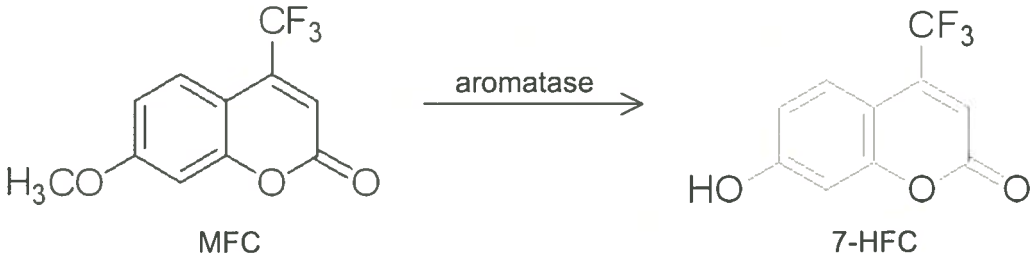



**Figure 13. Conversion of MFC to 7-HFC by aromatase.** 7-HFC is a highly fluorescent compound.

Source: Sigma-Aldrich. 2009. 7-Methoxy-4-(trifluoromethyl)coumarin. http://www.sigmaaldrich.com/catalog/ProductDetail.do?N4=T3165|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC. Accessed 2009 Apr 21.

Sigma-Aldrich. 2009. 7-Hydroxy-4-(trifluoromethyl)coumarin. http://www.sigmaaldrich.com/catalog/ProductDetail.do?N4=368512|ALDRICH&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC. Accessed 2009 Apr 21.

The assay works by treating the aromatase enzyme with the various samples to be tested in a solid black 96-well plate. This particular type of plate is necessary for measuring fluorescence readings. For the amounts of reagents present in the kit, thirty samples can be tested in duplicate at the initial concentration of choice; serial dilution of the samples is performed in the plate. The negative control includes all of the reagents minus an inhibitor, while the positive control is the known aromatase inhibitor ketoconazole (KTZ). MFC is then added to the mix and the reaction between MFC and aromatase is allowed to occur. After addition of a stop reagent to halt the reaction, the plate is placed in a spectrophotometer to measure fluorescence readings. The excitation and emission wavelengths are 409 nm and 530 nm, respectively. Fluorescence indicates the presence of 7-HFC and, therefore, active aromatase not inhibited by the samples. From the fluorescence readings, the concentration of sample to cause 50% inhibition of aromatase activity ($IC_{50}$) can be calculated. (BD Biosciences 2009)

## *Results*

Table 3 shows the results for the cytotoxicity screenings using the MTT assay. From a total of sixty-six samples of crude extracts and essential oils, eleven samples were found to show activity against the MDA-MB-231 breast cancer cells. An "active" sample was defined as any which displayed a percent kill of 90% or higher. Certain samples were tested in multiple trials, hence multiple percent kill values are available for those samples.

**Table 3. MTT assay results for the active crude extract and essential oil samples.** Results are given as % kill values with standard deviations (σ). A total of sixty-six samples were tested in the assay.

| Crude Extract | % Kill | σ (%) |
|---|---|---|
| *Tapirira mexicana* bark (chloroform) | 83.11 | 7.21 |
| | 87.71 | 0.97 |
| | 90.98 | 2.09 |
| | 97.35 | 0.86 |
| *Salacia* sp. "liana" bark (dichloromethane) | 95.45 | 0.31 |
| | 95.67 | 1.01 |
| *Verbesina turbacensis* bark (acetone) | 96.83 | 0.35 |
| | 96.99 | 0.88 |
| *Bursera ovalifolia* bark (chloroform) | 99.41 | 1.07 |
| | 99.89 | 2.12 |
| *Verbesina turbacensis* root (acetone) | 100.05 | 0.83 |
| | 102.16 | 1.55 |

| Essential Oil | % Kill | σ (%) |
|---|---|---|
| *Rhodostemonodaphne kunthiana* | 90.61 | 14.58 |
| *Guatteria dispyroides* | 98.83 | 1.67 |
| *Desmopsis bibracteata* | 99.31 | 1.10 |
| *Schefflera rodrigueziana* | 99.60 | 0.68 |
| *Montanoa guatemalensis* (leaf essential oil #2) | 100.55 | 1.55 |
| *Cymbopetalum costaricense* | 101.54 | 0.89 |

For the aromatase inhibition assay, results have not yet been confirmed by the author's efforts. Errors have been detected in the fluorescence readings for the controls in trial runs. Fluorescence readings for the positive and negative controls have been very similar, when readings for the positive control should be significantly lower than readings for the negative control due to decreased production of 7-HFC. Possibilities for these observed results include solvent and enzyme issues. The solvent used in the assay to dissolve the samples, DMSO, may be exhibiting inhibitory effects against the aromatase enzyme. Also, the aromatase enzyme itself used in the assay may be faulty and, thus, not catalyzing the conversion of MFC to 7-HFC as expected.

## Conclusions

From the results of the MTT assays, the crude extracts and essential oils obtained from Costa Rica can be concluded as promising sources of new anti-breast cancer drugs. However, to determine the inhibitory effect of the samples against aromatase,

further testing using the aromatase inhibition assay must be conducted. Results of the aromatase assay will be conclusive once errors with fluorescence readings are resolved.

In terms of what is responsible for the cytotoxicity of the active samples, the compounds present in the samples must be known. The activity displayed by the *Salacia* "liana" species may be due to the presence of tingenone in the extract, while bornyl caffeate may contribute to the activity of the *Verbesina turbacensis* extracts. Tingenone, which has previously been isolated from the bark extract of the *Salacia* "liana" species, is highly cytotoxic. $IC_{50}$ values for tingenone against different cancer cell lines are 1.9 μM (H4IIE), 2.7 μM (Hep-G2), and 1.7 μM (SK-Mel-28) (Setzer and others 1998). Similarly, bornyl caffeate, which has been isolated from *Verbesina turbacensis*, has been shown to display antitumor activity in other studies utilizing the MTT assay (Amaro-Luis and others 2002; Xia and others 2008).

Further research must be conducted to determine which specific compounds in the extracts and essential oils are responsible for observed activity. This will involve isolation and purification of compounds from the active samples. Isolation and purification techniques include column chromatography and gas chromatography-mass spectrometry (GC-MS), for example. Structural determination for purified compounds would then be conducted using nuclear magnetic resonance (NMR). Purified compounds would undergo subsequent screening in MTT assays to conclude individual cytotoxic activities. Synergism studies would also be beneficial to determine if any compounds present in the samples work in synergy to cause cytotoxicity.

# Conclusion

The role of aromatase in estrogen synthesis makes the enzyme a prime target for drugs treating estrogen-dependent diseases. The interaction of estrogen with the estrogen receptor in certain tissue cells can increase the development of cancer such as breast, ovarian, and prostate cancer. Thus, regulating estrogen production by aromatase to allow for normal cell development but decreased development of tumor cells is key in preventing and treating these estrogen-dependent cancers.

Increasingly, researchers are looking towards natural products from plants as sources of new medicines. Plants offer an immense diversity of natural products, increasing the likelihood of discovering bioactive compounds from the vast amount of natural products available. For example, from cytotoxicity screenings conducted by the author, natural products from Costa Rica were found to be promising sources of new anti-breast cancer drugs. In terms of targeting aromatase, such natural products from plants would need to inhibit aromatase catalytic activity. In other words, inhibit aromatase production of estrogens. From previous studies of aromatase inhibition by natural products from plants – such as the study by Monteiro and others and the research conducted by Ta and Walle – various compounds from plants were found to show aromatase inhibitory activity. This affirms that plant are a promising source of aromatase inhibitors.

Further research is still in order before creating an anti-aromatase drug from a natural product. More bioactivity tests are required, as well as research of the *in vivo* activity of bioactive plant compounds. Indeed, the activity and efficacy of a drug once

it is in the human body is of immense importance in determining drug safety and dosage amounts. Though so much research is still to be done, the discovery of new drugs as treatment, and hopefully a cure, for devastating illnesses like cancer is well worth the journey.

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