Université du Québec
Institut National de la Recherche Scientifique
Institut Armand-Frappier

THE EFFECTS OF ESSENTIAL OILS ON HUMAN STEROIDOGENESIS IN PHYSIOLOGICALLY-RELEVANT CO-CULTURE SYSTEMS

Presented by:

Debbie Yancu

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Jury Members:

President of the jury and internal examiner:	Dr. Charles Ramassamy INRS-Institut Armand Frappier
External Examiner:	Dr. Sophie Petropoulos Université de Montréal
External Examiner:	Dr. Pierre Ayotte CHU de Québec-Université Laval
Supervisor:	Dr. Thomas Sanderson INRS-Institut Armand Frappier

ABSTRACT

Essentials oils are naturally-derived compounds that have seen their popularity increase considerably in recent years in an effort to seek alternatives to conventional forms of therapy, particularly by women. Currently, there is very limited scientifically validated information available on the biological activities or potential risks these compounds present, especially at the level of steroidogenesis. The steroidogenic pathway plays a significant role in pregnant women and those suffering from hormone-sensitive breast cancer, yet little is known about the biological actions of essential oils and their active ingredients on the enzymes involved in steroid hormone biosynthesis. This thesis focused on addressing the current lack of knowledge in this particular area of potential endocrine disruption in women. Specifically, attention was placed on the enzyme aromatase (CYP19): a key enzyme in the synthesis of estrogens, the expression of which is regulated in a tissue-specific manner involving alternate promoters. The general objective of this thesis was to uncover any potential disruption of steroidogenesis by several commonly used essential oils and to predict their safety in the context of pregnant women or those suffering from or at risk of developing estrogen-dependent breast cancer, using physiologically relevant *in vitro* co-culture systems. The specific objectives were to 1) determine the effects of several common essential oils and their main active ingredients on steroidogenesis in a feto-placental co-culture model formed by H295R human adrenocortical carcinoma and placental trophoblast-like BeWo choriocarcinoma cells. Measurements were made to hormone production levels, promoter-specific expression of *CYP19* and aromatase catalytic activity to determine the signaling pathways involved in the response of the co-culture compared to observations made in monoculture; 2) determine the effects of essential oils on steroidogenesis in a representative model of estrogen-dependent breast cancer in Hs578t human stromal-like breast cancer cells, with a focus on promoter-specific expression of *CYP19*; 3) develop a novel estrogen-dependent breast cancer co-culture model using Hs578t and T47D human estrogen-dependent breast cancer cells as a research tool to assess effects of essential oils and other potential endocrine-disrupting compounds on stromal-tumoral steroidogenic communication in the human estrogen-dependent breast cancer microenvironment.

After a 24 h exposure of cells in either co-culture model to essential oils at concentrations ranging from 0.0005% to 0.005%, we found that only basil (*Ocimum basilicum*) and fennel seed

(*Foeniculum vulgare*) essential oil significantly affected hormone production in co-culture by increasing concentrations of estradiol, estrone, dehydroepiandrosterone (DHEA), androstenedione, progesterone and estriol, but not testosterone. Using real-time quantitative RT-PCR, basil and fennel seed oil were shown to significantly alter the expression of several key steroidogenic enzymes, such as those involved in cholesterol transport and steroid hormone biosynthesis, including *StAR*, *CYP11A1*, *HSD3B1 and -2*, *SULT2A1*, and *HSD17B1*, *-4*, and *-5*. Moreover, we found that both basil and fennel seed essential oils stimulated promoter-specific *CYP19* expression and catalytic activity of the enzyme. We also provided mechanistic insight into the ability of estragole and trans-anethole (the major constituents of basil and fennel seed essential oil, respectively) to stimulate the promoter-specific expression of *CYP19* through activation of the PKA pathway in H295R cells and the PKC pathway in BeWo cells in co-culture, in both cases associated with increases in intracellular cAMP levels. In Hs578t cells, basil and fennel seed essential oils triggered a promoter-switch in the regulation of *CYP19,* in which promoter I.4 activity was decreased and promoter II activity was stimulated, resulting in increased *CYP19* expression and aromatase catalytic activity. Finally, our breast cancer co-culture model has proved to be a promising new tool for the study of endocrine disrupting chemicals or potential therapeutic agents in estrogen-dependent breast cancer. Overall, in light of their potential to disrupt steroidogenesis in two physiologically relevant *in vitro* co-culture models, our results indicate that further study is necessary to determine the potential risks of the use of basil and fennel seed essential oils (and potentially other essential oils) during pregnancy and by those suffering from estrogen-dependent breast cancer .

Keywords:

- Essential oils
- Alternative medicine
- Pregnancy
- Fetal-placental steroidogenesis
- Estrogen-dependent breast cancer
- Aromatase
- Co-culture
- H295R
- BeWo
- Hs578t
- T47D

RÉSUMÉ

Les huiles essentielles sont des composés d'origine naturelle dont la popularité a considérablement augmenté ces dernières années, en particulier chez les femmes, dans leurs efforts pour trouver des alternatives aux traitements conventionnels. Actuellement, il existe très peu d'informations scientifiques validées sur les rôles biologiques ou les risques que ces composés présentent, en particulier au niveau de la stéroïdogenèse. La voie stéroïdogène jouant un rôle important chez les femmes enceintes et les personnes atteintes d'un cancer du sein hormono-dépendant, l'accent de cette thèse était mis sur cet aspect des actions biologiques des composés actifs des huiles essentielles. Une attention particulière, était placée sur l'enzyme aromatase (CYP19) qui est essentielle à la synthèse de l'œstrogène et dont l'expression est régulée de manière tissue-spécifique à l'aide de promoteurs alternatifs. L'objectif général de cette thèse était de découvrir si des huiles essentielles couramment utilisées sont capable de perturber la stéroïdogenèse, et également de prédire leur innocuité chez les femmes enceintes ou atteintes (ou à risque de développer) d'un cancer du sein estrogène-dépendant en utilisant des systèmes de co-culture *in vitro*. Les objectifs spécifiques étaient 1) de déterminer les effets des diverses huiles essentielles communes et leurs composés actifs importants sur la stéroïdogenèse, dans un modèle de co-culture fœto-placentaire formé de cellules H295R dérivées d'un carcinome surrénalien humain et de cellules BeWo issues d'un choriocarcinome humain avec des caractéristiques de trophoblastes placentaires. L'accent était mis sur la production d'hormones, l'expression promoteur-spécifique du *CYP19* et l'activité catalytique de l'aromatase afin de déterminer les voies de signalisation impliquées dans la réponse de la co-culture par rapport aux observations effectuées en monoculture; 2) déterminer les effets des huiles essentielles sur la stéroïdogenèse dans un modèle représentatif du cancer du sein estrogène-dépendant utilisant des cellules humaines du cancer du sein Hs578t avec des caractéristiques stromales, en mettant l'accent sur l'expression promoteur-spécifique du *CYP19*; 3) développer un nouveau modèle de co-culture du cancer du sein estrogène-dépendant utilisant des cellules humaines oestrogènes dépendantes T47D et Hs578t comme outil de recherche pour évaluer les effets des huiles essentielles et d'autres composés susceptibles de perturber la communication stéroïdogène dans le microenvironnement tumoral-stromal du cancer du sein humain.

Après une exposition de 24 h des lignées cellulaires à des concentrations d'huile essentielle comprises entre 0,0005% et 0,005%, seules les huiles essentielles de basilic (*Ocimum basilicum*) et de graine de fenouil (*Foeniculum vulgare*) augmentaient de manière significative les concentrations hormonales d'oestradiol, d'estrone, de déhydroépiandrostérone (DHEA), d'androstènedione, de progestérone et d'oestriol, mais pas la testostérone. En utilisant RT-PCR quantitative en temps réel, il a été démontré que l'huile de basilic et de fenouil modifie de manière significative l'expression génique de plusieurs enzymes stéroïdogènes clés, telles que celles impliquées dans le transport du cholestérol et la biosynthèse des hormones stéroïdiennes, y compris *StAR*, *CYP11A1*, *HSD3B1* et *-2*, *SULT2A1* et *HSD17β1*, *-4* et *-5*. De plus, nous avons constaté que les huiles essentielles de basilic et de graine de fenouil stimulaient l'expression promoteur-spécifique de *CYP19* et l'activité catalytique de l'enzyme aromatase. Nous avons également fourni des informations mécanistes sur la capacité de l'estragole et du trans-anéthole (les composés principaux de l'huile essentielle de basilic et de graine de fenouil, respectivement) à stimuler l'expression promoteur-spécifique du *CYP19* par l'activation de la voie PKA dans les cellules H295R et de la voie PKC dans les cellules BeWo, les deux cas associés à une augmentation des niveaux de cAMP. Dans les cellules Hs578t, les huiles essentielles de basilic et de graine fenouil ont causé un changement de l'usage des promoteurs de *CYP19*, ce qui a donné lieu à une augmentation de l'expression du *CYP19* ainsi que son activité catalytique par la baisse d'activité du promoteur I.4 et une augmentation d'activité du promoteur II. Enfin, notre modèle de co-culture du cancer du sein s'est révélé être un nouvel outil pour l'étude de substances chimiques perturbant le système endocrinien ou d'agents thérapeutiques potentiels pour le cancer du sein dépendant d'œstrogènes. Dans l'ensemble, compte tenu de leur potentiel de perturber la stéroïdogenèse dans deux modèles de co-culture *in vitro* physiologiquement pertinents, nos résultats indiquent que des études supplémentaires sont nécessaires pour déterminer les risques potentiels de l'utilisation des huiles de basilic et de graine de fenouil (et éventuellement d'autres huiles essentielles) pendant la grossesse et chez les personnes atteintes d'un cancer du sein dépendant des œstrogènes.

Mots clés:

- Huiles essentielles
- Médecine alternative

- Grossesse
- Stéroïdogenèse fœtale-placentaire
- Cancer du sein hormono-dépendant
- Aromatase
- Co-culture
- H295R
- BeWo
- Hs578t
- T47D

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16α-Hydroxydehydroepiandrosterone sulfate (16α-OH-DHEA-S)

17α-hydroxylase, 17,20-lyase (CYP17)

17β-Hydroxysteroid dehydrogenase (17β-HSD)

3β-Hydroxysteroid dehydrogenase (3β-HSD)

60S acidic ribosomal protein P0 (RPL0)

Activating function domain (AF-1)

Activating Function domain (AF-2)

Analysis of Variance (ANOVA)
Aromatase (CYP19)

Cholesterol Side Chain Cleavage (CYP11A1)

Cis-regulatory element binding protein (CREB)

Complementary and alternative medicine (CAM)

CREB-binding protein (CBP)

CRE-like sequence (CLS)

Cyclic adenosine monophosphate (cAMP)

Cytochrome P450 (CYP450)

Cytochrome P450 family 21(CYP21)

Cytochrome P450 family 3 subfamily A member 4 (CYP3A4)

Cytochrome P450 family 3 subfamily A member 7 (CYP3A7)

Interleukin 1 beta (IL-1β)

Interleukin 6 (IL-6)

Janus kinase 1 (JAK1)

Ligand-binding domain (LBD)

Low-density lipoprotein (LDL)

Luteinizing hormone (LH)

Mitogen-activated protein kinase (MAPK)

Metastatic lymph node 64 protein (MLN64)

Mitogen-activated protein kinase kinase (MEK)

N-terminal domain (NTD)

Nuclear localization signal (NLS)

Ocimum basilicum (Basil)

Phorbol 12-myristate 13-acetate (PMA)

Phosphatidyl inositol 3 kinase (PI3K)

Porphobilinogen deaminase (PBGD)

Premenstrual syndrome (PMS)

Progesterone receptor (PR)

Prostaglandin E2 receptor 1 (EP1)

Dehydroepiandrosterone (DHEA)

DNA-binding domain (DBD)

Estradiol (E2)

Estriol (E3)

Estrogen receptor alpha (ERα)

Estrogen receptor beta (ERβ)

Estrogen response element (ERE)

Estrone (E1)

Fetal bovine serum (FBS)

Foeniculum vulgare (Fennel seed)

Follicle stimulating hormone (FSH)

Forskolin (Frsk)

Gas chromatography–mass spectrometry (GC/MS)

GATA-binding factor 2 (GATA-2)

Gonadotropin releasing hormone (GnRH)

G-protein coupled estrogen receptor (GPER)

Human chorionic gonadotrophin hormone (hCG)

Human epidermal growth factor receptor 2 (HER2)

Prostaglandin E2 receptor 2 (EP2)

Prostaglandin E2(PGE2)

Protein kinase A (PKA)

Protein kinase C (PKC)

Reactive oxygen species (ROS)

Reverse transcription polymerase chain reaction (RT-PCR)

RNA polymerase subunit 2 (RPII)

Signal transducer and activator of transcription 3 (STAT3)

Steroidogenic acute regulatory protein (StAR)

Steroidogenic factor-1 (SF-1)

Stimulating protein-1 (Sp-1)

Sulfotransferase Family 2A Member 1 (SULT2A1)

TATA-Box Binding Protein (TBP)

Tumor necrosis factor alpha (TNF-α)

Ubiquitin C (UBC)

Untranslated region (UTR)

Vascular endothelial growth factor (VEGF)

Chapter 1 : GENERAL INTRODUCTION

1.1 Complementary and alternative medicine

1.1.1 CAMs and women

There has been a steady increase in the use of complementary and alternative medicine (CAM) over the past decade (Clarke et al., 2015; Frass et al., 2012). Many Western countries report over a third of their population have used some form of CAM, whether for preventive reasons, adjuvant therapies, or as a form of replacement treatment in efforts to combat toxic side-effects associated with traditional therapies (Clarke et al., 2015; Frass et al., 2012). The origin of CAMs can be dated to over 5000 years ago, long before mainstream biomedicine and scientific methodologies, with complex herbal therapies being used in Ayurvedic medicine in India and various forms of herbal, massage, acupuncture, exercise and dietary treatments applied in Traditional Chinese Medicine (Patwardhan et al., 2005). The fastest growing and most controversial sector of CAM is the use of herbal products. Especially since most herbal products do not have to succumb to rigorous scientific studies and clinical trials before being readily available to consumers, with most products generally being accepted for their traditional use for centuries and the public belief that no risk are associated with natural products. Although some herbal products have shown some promise in disease treatment and are widely used, there is a lack of information regarding their mechanisms of action, drug-herb interactions, potential adverse effects, etc. (Ekor, 2014).

Interestingly, women are the primary consumers of these alternative therapies with 67% of women of reproductive age in the United States reporting CAM use (Johnson et al., 2016). Other studies have shown that the primary consumers of herbal medicine are Caucasian women (mostly of ethnic origin) who have a high educational status, high income, and who range from young to middle-aged (Al-Windi, 2004; Harrison et al., 2004; Ni et al., 2002). More troubling are reports that three-quarters of women have reported CAM use during pregnancy or the post-partum period for reasons including perinatal symptoms, nausea/vomiting, pain management, stress, anxiety and induction of labor (Johnson et al., 2016). Is it reported that these women are substituting pharmaceutical treatments, that they anxiously believe will harm their babies, with

herbal products that are generally considered to be “safe” (Ernst, 2002; Louik et al., 2010). Moreover, the lack of CAM research is definitely a growing source of concern in the medical and scientific communities, especially whether certain herbal products can cross the fetal-placental barrier or be transferred to infants during breastfeeding; with almost no teratogenicity studies available (Pelkonen et al., 2017).

There are over 230,000 women diagnosed annually with breast cancer. Many of those adopt lifestyle changes in an effort to increase the probability of long-term survival (Neuhouser et al., 2016). The majority of these patients have reported using natural products and other dietary supplements, although it is mostly unknown whether these alternative treatments contribute positively or negatively to their prognosis (Neuhouser et al., 2016). At present, a major concern is the possibility of drug-herb interactions. To investigate this, the drug and herb would have to be studied together *in vivo* to determine whether the herbal product interferes with activity, metabolism, or toxicity of the drug. Given these facts and recent evidence presented in Mitra and Dash (2018) suggesting that certain natural products may be beneficial in cancer prevention, or even treatment, it is important to evaluate the safety of commonly used herbal products in the context of women's health as they are the most frequent users.

1.2 Essential oils

1.2.1 Isolation and biological role

Essential oils are aromatic liquids isolated from the natural secretions synthesized by different plant organs and stored within specialized secretory cells (El Asbahani et al., 2015). They are commonly extracted via steam- or hydro-distillation from the plant materials. The extraction yields are extremely low, vary depending on climate, soil composition, plant organ, age and vegetative life cycle, and only 10% of plant species, termed aromatic plants, are able to produce them (Svoboda & Greenaway, 2003). Aromatic plants are categorized into families such as the Lamiaceae (mostly culinary herbs), Apiaceae (plants with hollow stem, such as celery (*Apium graveolens*) and parsley (*Petroselinum crispum*)), Lauraceae (the true laurel (*Laurus nobilis*)), Asteraceae (presence of composite flowers), Rutaceae (citruses), Myrtaceae (trees and shrubs found in the tropics or subtropics, such as clove (*Syzygium aromaticum*) and guavua

(*Psidium guajava*)), Poaceae (grasses, such as lemongrass (*Cymbopogon*) and citronella (*Citronella mucronata*)), Cupressaceae (conifers), Piperaceae (peppers), etc. (Bruneton, 1999). The biological role of essential oils is to act as chemical signals through which the plant can communicate with their environment to ensure proper development (Masotti et al., 2003). These actions include inhibiting proximate growth of other plants and their subsequent germination as well as defensive roles against pathogenic microorganisms, herbivores and insects (Masotti et al., 2003). These natural functions have been largely utilized by humans to develop many of the antibacterial, antifungal and insecticidal products currently in use.

1.2.2 Current uses of essential oils

Due to their biological roles and aromatic nature, there are numerous commercial applications of essential oils, in the food, sanitation, cosmetic and perfume industries. In the food industry, there is increasing demand for essential oils as flavoring agents and as food preservatives to prevent growth of pathogens in food packaging (Burt, 2004). New research is also exploring the use of essential oils in the fight against emerging antibiotic-resistant bacteria that may be transmitted by meat products (Oussalah et al., 2007). In the sanitation industry, essentials oils are found in cleaning detergents to disinfect medical equipment or in aerosol sprays in hospitals as means of thwarting airborne contaminants (El Asbahani et al., 2015). In the cosmetic and perfume industries, the use of essentials oils is extremely common, because of their pleasant fragrances and various cleansing, moisturizing or drying, and toning effects (Ali et al., 2015).

Another major and controversial area of the use of essential oils is in aromatherapy. Aromatherapy is a popular alternative therapy that employs essential oils for the improvement of mental and physical health. Essential oils have been recognized for their medicinal properties since ancient civilizations, and were used for analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic purposes (Bakkali et al., 2008). There are many dosage forms of essential oils available in aromatherapy, including capsules, ointments, creams, syrups, suppositories, aerosols and sprays. In terms of applications, specific essentials oils can be ingested after mixing with vegetable oil carriers, massaged on specific areas of the skin, or inhaled. The controversy lies within numerous claims that the use of essential oils in aromatherapy is effective in treating

conditions as diverse as depression, indigestion, headache, insomnia, muscular pain, respiratory problems, skin conditions, swollen joints, labor pains, urinary tract disorders etc., of which most have not yet been scientifically validated (Ali et al., 2015). However, this is changing with renewed interest in using 'environmentally conscious' products and discovering new applications for natural products in an attempt to counter the development of resistance to current pharmaceutical therapies (Herman & Herman, 2015). There is now evidence supporting the use of essential oils for treating neurological disorders (Ayaz et al., 2017), inflammatory diseases (Standen & Myers, 2004), cardiovascular issues (Saljoughian et al., 2018), and cancer with most studies revealing their ability to initiate detoxifying and antioxidant defense systems, activation of anti-inflammatory pathways, and stimulation of cell cycle arrest and/or cell death (Bhalla et al., 2013).

Essential oils are not regulated by the United States Food and Drug Administration (FDA) and safety data for nearly a hundred different essential oils are not readily available. Health Canada (2017) allows for essential oils to be registered as Natural Health Products (NHPs) after being screened to ensure the safety, effectiveness, and high quality nature of the product. Unfortunately, Health Canada allows traditional use claims written in pharmacopoeias as sufficient evidence for product safety and efficacy which many companies can exploit. Ideally, data on acute toxicity, irritation and corrosiveness, sensitization, percutaneous absorption, effects of repeated exposures, phototoxicity, carcinogenicity, reproductive toxicity and teratogenicity should be available for all dosage forms of essential oils (Vigan, 2010). This poses an obvious challenge given that essential oils are complex mixtures that vary in composition according to the soil condition, extraction method, plant vegetative cycle, etc. It is of utmost importance that reports examining essential oils provide gas chromatography and mass spectrometry (GC/MS) analyses so that the components of the mixtures can be easily characterized. Toxicity also varies between species and sex, therefore studies done on rodents, insects and even bacteria do not necessarily forecast effects on humans.

1.2.3 Essential oils and cancer

Essential oils have garnered increasing interest among cancer researchers in light of recent studies reporting that many of their volatile constituents display potent antioxidant, anti-

inflammatory and anti-proliferative effects. Extensive amounts of research have focused on the antioxidant potential of essential oils (Bag & Chattopadhyay, 2018; Purkait et al., 2018; Torres-Martínez et al., 2018). Studies have shown that certain essential oils can combine with reactive oxygen species (ROS) to produce phenoxy radicals that inhibit or prevent increased ROS production after mitochondrial DNA damage and impairment of the electron transport chain, thus inhibiting any further damage to the cell (Bhalla et al., 2013).

An inflammatory response is a key component of many diseases, including cancer, and involves cooperation among many different molecular and cellular signals, ultimately resulting in complex biological cascades that contributes to disease. Compounds in essential oils have been shown to prevent inflammation through interactions with various inflammatory factors involved in these cascades, such as cyclooxygenase 2 (COX-2), prostaglandin E2 (PGE2), nuclear factor-kappa β (NF-κβ) and interleukins, such as interleukin1-β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Lesgards et al., 2014).

The ability of essential oils to activate apoptotic pathways is well documented and appears to be the primary mechanism by which essential oils inhibit the proliferation of cancer cells. It has been shown that essentials oils of key lime, thyme, and carum can stimulate intrinsic apoptosis by decreasing mitochondrial membrane potential, increasing mitochondrial cytochrome c release, increasing caspase activity, which cleaves poly(ADP-ribose) polymerase, which in turn promotes DNA fragmentation (Bhalla et al., 2013) . Certain essential oils have also been shown to stimulate extrinsic apoptosis in human hepatocellular carcinoma cells (Xiao et al., 2008) and in human leukemia cells (Kumar et al., 2008) via death receptors.

1.2.4 Chemical composition of essential oils

Essentials oils are composed of a complex mixture of volatile odorous compounds with good bioavailability. The chemical components are characterized by low molecular weight of which the majority are classified as terpene and terpenoid hydrocarbons. The building blocks of these compounds is the isoprene unit (2-methyl-1,3-butadiene) with the general formula $(C_5H_8)_n$ where n represents the number of isoprene units linked together (El Asbahani et al., 2015). Terpenes consist of two major classes, monoterpenes and sesquiterpenes. Monoterpenes contain

10 carbon atoms and possess the general formula $C_{10}H_{16}$ (2 isoprene units) and sesquiterpenes possess the general formula $C_{15}H_{24}$ (3 isoprene units). Structurally, both classes of terpenes can be arranged in a mono-, bi- or tricyclic manner (El Asbahani et al., 2015). The class of terpenoids includes the isoprenoids, which are oxygenated derivatives of monoterpenes and sesquiterpenes that take the form of alcohols, acids, aldehydes, ethers, esters, ketones and oxides. Phenylpropanoids, such as cinnamates (and estragole and trans-anethole, subjects of this thesis), are another group of oxygenated hydrocarbons found in only a limited number of aromatic plants. This group of molecules contain a phenyl ring in addition to the allyl group common to other terpenoids (Bakkali et al., 2008). Through oral, cutaneous and inhalation routes, terpenes and terpenoids are well absorbed and metabolized via oxidation, hydroxylation, or converted into glucoronides or sulfates, but many remain unchanged as they travel through the body to exert their effects (El Asbahani et al., 2015). Table 1-1 includes a comprehensive list of the major chemical groups found in essential oils as well as any currently known biological properties.

Despite the many constituents found in essential oils, sometimes up to 100 in differing concentrations, they are typically categorized by their major components present at the highest concentrations (20-70%) according to GC/MS analysis. It is generally believed that the most abundant compound(s) within the essential oil are responsible for its biological activity (Bakkali et al., 2008). However, the concept of synergism has not been sufficiently evaluated, as very few studies address the subject. It is not known whether a combination of several or all components of the essential oil define its bioactivity or only one or a few individual molecules. This is true for the determination of its specific odor, texture, density, etc., as well as for possible synergistic activities among the molecules that may affect bioavailability and distribution within the body and within cells, thus determining the ultimate biological responses that are elicited (Bayala et al., 2014).

Table 1-1: Constituents found in essential oils (Martin, 2007)

Name of chemical group:	**Examples found in Essential Oils**	**Effects, Characteristics, and known Safety Issues**
Monoterpernes	α-pinene, β-pinene, camphene, careen, dipentene, fenchene, limonene, menthene, mycrcene, p-cymene, phellandrene, sabinene, terpinene, terpinolene, thujen, tricyclene	Skin penetrating, tonic, and immune supportive, antiseptic, volatile Possibly skin irritating

Sesquiterpenes	Aromadendrene, bergamotene, bisabolene, bourbonne, cadinene, β-caryophyllene, cedrene, chamazulene, copaene, cubene, curcumene, elemene, farnesene, guaiene, gurgujene, humulene, italicene, longifolene, muurolene, patchoulene, santalene, selinene, seychellene, viridiliflorene, ylangene, zingiberene	Anti-inflammatory, calming
Monoterpene alcohols	Borneol, carveol, citronellol, fenchol, geraniol, lavandulol, linalool, menthol, myrtenol, nerol, pinocarveol, pulegol, terpinen-4-ol, terpineol, thuyan-4-ol	Antimicrobial, immune supportive (some are sedative, hypotensive, antispasmodic)
Sesquiterpene alcohols	Atlantol, bisabolol, cadinol, carotol, caryophyllene alcohol, cendrol, elemol, eudesmol, farnesol, globulol, guaiol, manool, muurolol, nerolidol, patchoulol, pogostol, salviol santolol, sapthulenol, sclareol, viridiflorol,	Anti-inflammatory, antispasmodic, sedative
Phenylpropanoids	Carvacrol, cresol, eugenol, estragole, guiacol, safrole, thymol, trans-anethole	Antiseptic, antifungal, anti-inflammatory
Aldehydes	Acetaldehyde, arisaldehyde, benzaldehyde, caproic aldehyde, citral, citronellal, cinnamadelhyde, cuminaldehyde, decanal farnesal, geranial, myrtenal, neral, nonanal, periladehyde, phellandral, piperonal, sinensal, teresantal, valeranal	Antimicrobial terpenoids, possibly sedative Skin irritating in large amounts
Ketones	Acetophenomen, atlantone, artemisia ketone, camphor, carvone, cryptone, dione, fenchone, ionone, irone, isopinocamphone, jasmine, menthone, methyl heptone, nootkalone, octanone, pinocamphone, pinocarvone, piperitone, pulgone, tagetone, thujone, valeranone, verbenone	Mucolytic, antimicrobial, skin healing Possibly hepatoxic and neurotoxic
Esters	Cinnamyl acetate, eugenyl acetate, geranyl acetate, linalyl acetate, methyl salicylate, neryl acetate, terpinyl acetate	Antispasmodic, sedative, adaptogenic, anti-inflammatory
Ethers	Trans-anethole, apiol. asarone, chamospiroether, elemicin, isosafrole, methyl carvacrol, estragole, methyl eugenol, myristicin, phenylethyl, methyl ether, safrole	Antispasmodi and carminative. Trans-anethole is potentially estrogenic Possibly hepatoxic
Oxides	1,8 cineole, ascaridole, bisabolol oxide, bisabolone oxide, caryophyllene oxide, geranyl oxide, linalool oxide, nerol oxide, manool oxide, pinene oxide, piperitone oxide, rose oxide, sclareol oxide	Expectorant, skin penetrating, antispasmodic
Acids	Anisic acid, atlantic acid, benzoic acid, cinnamic acid, citronellic acid, geranic acid, lauric acid, myristic acid, rosmarinic acid, valerenic acid, velveric acid	***Only found in very small amounts in essential oils

1.2.5 Fennel seed essential oil

Fennel, or *Foeniculum vulgare*, is a popular aromatic herb that belongs to the Apiaceae family. Traditionally cultivated in Europe and the Mediterranean regions, it is now grown worldwide. Its essential oil, extracted from the seed, is very commonly used in the food industry and in folk medicine. In the food industry, fennel seed essential oil is used as a flavoring agent for different baked goods, drinks and cheeses owing to its black licorice aroma (Gori et al., 2012a; Zeng et al., 2015). In folk medicine, fennel seed essential oil is considered to have several biological actions, including antispasmodic, diuretic, anti-inflammatory, analgesic, antioxidant, expectorant, etc. (Gori et al., 2012a; Zeng et al., 2015). However, it has been shown in cellular and rodent models to have antioxidant, antithrombotic, antidiabetic, and antifungal purposes (Choi & Hwang, 2004; Mimica-Dukic et al., 2003; Zeng et al., 2015).

With regards to hormonal activities, a study from Devi et al. (1985) first alluded to the estrogenic nature of fennel seed. In that study and in a study by Malini et al. (1985), fennel seed extracts fed to rats resulted in a significant increase in the weights of their mammary glands, oviducts, cervix and endometrium, and triggered the start of the menstrual cycle. A study by Ostad et al. (2001) later revealed that fennel seed essential oil inhibits the contraction of isolated uterine smooth muscle cells induced by oxytocin or PGE_2. The authors of this study suggested that the uterine relaxation by fennel seed essential oil was most likely due to its estrogenic activity, since isolated rat uterine strips had been previously shown to be relaxed by estrogens (Bengtsson, 1978). Several studies have found fennel seed essential oil to act as a galactagogue, a compound that increases maternal milk supply and lactation (Ayers, 2000; Brodribb, 2018; Dennehy et al., 2010; Petrie & Peck, 2000). Fennel seed essential oil concoctions were also considered safe enough to be administered in clinical trials to breastfed infants suffering from colic (Alexandrovich et al., 2003; Savino et al., 2005; Weizman et al., 1993). In all cases, colic symptoms were significantly improved in infants compared to placebo-controlled groups, sometimes within 1 week of treatment. A study by Fariba et al. (2006) has also showed that fennel seed essential oil treatment has the ability to reduce the development of osteoporosis in ovariectomized rats (considered a model for post-menopausal osteoporosis) by significantly reducing bone loss. In the study, the action of fennel seed essential oil was compared to that of

estradiol with suggestions that the natural compound may possess bone resorption capabilities and anabolic effects. More recently, a clinical study found that fennel seed essential oil is an effective treatment for women suffering from a moderate to severe case of premenstrual syndrome (PMS) (Delaram & Heydarnejad, 2011). The possible estrogenicity of fennel seed essential oil has still not clearly been elucidated beyond claims that the natural compound exhibits activity at the estrogen receptor level. However, it has been reported that the estrogenic nature of fennel seed essential oil should be linked to that of its major compound: trans-anethole (Mazaheri et al., 2013).

1.2.5.1 Trans-anethole

Essential oils differ in chemical constitution based on the extraction process and various factors related to the cultivation of the aromatic plant, but trans-anethole has been identified by GC/MS to be the major constituent of fennel seed essential oil produced by steam distillation. Trans-anethole is a phenolic ether and belongs to the phenylpropane family (Fig. 1-1). The popularity of fennel seed essential oil as a flavoring agent and its importance in herbal medicine for thousands of years has resulted in various *in vitro* and *in vivo* toxicology studies to determine whether trans-anethole was potentially genotoxic, mutagenic or immunotoxic (Nakagawa & Suzuki, 2003). Initially, trans-anethole was considered a carcinogenic agent since rats and mice treated with the compound had dose-dependent increases in the weight of their livers and developed hepatocarcinomas (Truhaut et al., 1989). However, several follow-up studies have reported that trans-anethole is not carcinogenic in humans; a conclusion based on the high concentrations (1mM) of trans-anethole that is required to induce cytotoxic effects in rat hepatocytes (Marinov & Valcheva-Kuzmanova, 2015; Marshall & Caldwell, 1992, 1996). With regards to steroidogenesis, Dhar (1995) was the first to suggest trans-anethole may possess estrogenic activity as it resulted in implantation failure in pregnant female rats as well as increasing the uterine weight of immature female rats to similar levels as an estradiol treatment (2 g/kg and 3 g/kg, respectively, compared to control values of 0.5 g/kg). A study in human MCF-7 breast cancer cells showed that the hydroxylated intermediates of trans-anethole competitively displaced estradiol from the estrogen receptor and enhanced cell proliferation, with lower concentrations proving more effective (greatest effect occured at 10^{-6} M) (Nakagawa & Suzuki, 2003). The Joint FAO/WHO Committee on Food Additives (JECFA) has listed the

acceptable daily intake of trans-anethole at 0.2 mg/kg body weight when used as a flavoring agent, while the Council of Europe have evaluated trans-anethole as a flavoring agent and considers a daily intake of 1.5 mg/kg to be safe (Marinov & Valcheva-Kuzmanova, 2015).

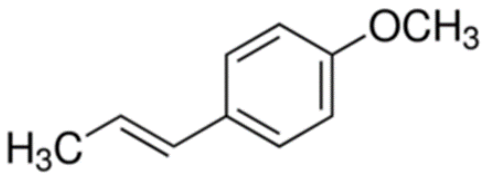

Figure 0-1

Figure 0-2

Figure 1-1: *Trans-anethole, a major constituent of fennel seed essential oil.*

1.2.6 Basil essential oil

Sweet basil, or *Ocimum basilicum*, is a popular culinary herb belonging to the Lamiaceae family. Originally found in India and other Asian regions, it now is cultivated all around the world resulting in over 200 different botanic varieties and forms (Joshi, 2014). It is the most common variety found in food products as a flavoring agent (Akgül, 1989; Joshi, 2014). Basil essential oil has applications throughout the cosmetic industries as well as in folk medicine. In the cosmetic industry, it has been used in perfumes since the beginning of the 20th century and is prevalent in lotions, toothpastes and mouthwashes (Aburjai & Natsheh, 2003; Sumit et al., 2012). Basil essential oil has numerous applications in folk medicine, especially in Chinese medicine, with reports recommending its usage to combat gastrointestinal disturbances, renal dysfunction, irregular blood flow (including menses) as well as relieving headaches, acne, and pain from insect bites (Leung, 1980). Currently, basil essential oil has been shown *in vitro* and in mice to have antimicrobial, antifungal, and insect-repelling, anticonvulsant, and antioxidant activities (Joshi, 2014). Most toxicological studies of basil essential oil have focused on the suspected carcinogenicity of its major chemical constituent, estragole. To date, little to no research, neither *in vitro* nor *in vivo* has been performed to determine the effects of basil essential oil on human steroidogenesis.

1.2.6.1 Estragole

Estragole is a phenolic ether and belongs to the phenylpropane class (Fig. 1-2) and, in fact, an isomer of trans-anethole, differing only in the location of the double bond. Extensive research in rodents found that treatment with estragole resulted in hepatocarcinoma formation in rats and the formation of DNA adducts in mouse livers (Anthony et al., 1987; Miller et al., 1983; Phillips, 1994). However, according to Gori et al. (2012a) and Kanno (2014), the doses required to induce the genotoxic responses in rodents are far greater than physiologically relevant human concentrations (1000mM compared to an exposure level of approximately 4uM in a cup of tea); therefore the relevance of the rodent studies is questionable. In fact, the biotransformation of estragole into the toxic metabolites that are responsible for its carcinogenicity is thought not to occur in humans at doses normally consumed (Kanno, 2014). The Joint FAO/WHO Expert on Food Additives (2009) has not established an acceptable daily intake for estragole but report an average exposure per person of 5 μg/day as a flavouring agent in the USA population. Despite the lack of human studies on carcinogenicity and reproductive toxicity, the European Medicine Agency has established a daily acceptable intake limit of estragole at 0.5 mg/person/day and suggestion that children and pregnant or breastfeeding women avoid exposure entirely. In light of this, there has been a renewed interest in studying estragole which has led to the discovery of antioxidant, antilipase, and anti-toxoplasmosis activities of estragole *in vitro*, *in vivo*, and *in silico.* (Oliveira et al., 2016; Santos et al., 2018)

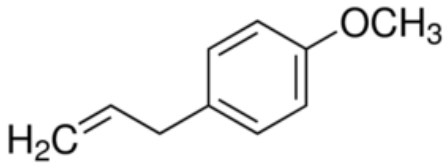

Figure 1-2: *Estragole, major constituent of basil essential oil*

1.2.7 Black Pepper Essential Oil

Black pepper, or *Piper nigrum*, belongs to the Piperaceae family of aromatic plants. Black pepper is grown mostly in South India and other tropical regions. The peppercorn, known as the fruit of black pepper, when dried is a very commonly utilized spice in cuisine. The aroma of black pepper is attributed to the volatile oil found within the dried peppercorn. In the Indian culture, black pepper essential oil is used to treat a variety of ailments, including rheumatism,

muscle pain and fever as well as to improve blood circulation and for gastrointestinal purposes (Pruthi, 1993). Currently, black pepper essential oil has been studied for its antioxidant potential using chemical systems which does not account for cellular constituents, antioxidant enzymes or antioxidant activity against reactive oxygen species (ROS) (Bagheri et al., 2014).

The chemical composition of black pepper essential oil differs largely dependent on the type of distillation process used and whether the fruit was fresh or dried at the time of extraction. Through steam distillation of dried peppercorn, the major component of the resultant pepper essential oil is β-caryophyllene; a sesquiterpene hydrocarbon (Fig. 1-3) (Pathasaranthy et al, 2008). Despite a lack of studies on its effects on steroidogenesis, this sesquiterpene has been studied in pregnant rats, where it showed promise as a potential therapy for endometriosis by reducing cyst size and producing apoptosis (Abbas et al., 2013), and in MCF-7 human breast cancer cells, as it inhibited several important signaling cascades involved in tumorigenesis in a concentration range of 30 to 50 μM (Park et al., 2011).

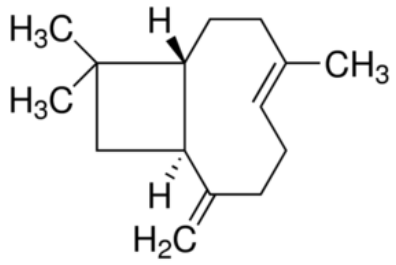

Figure 1-3: *β-caryophyllene, major constituent of black pepper essential oil*

1.2.8 Sweet Orange Essential Oil

Sweet orange (*Citrus × sinensis*) is a citrus of the Rutaceae family grown in tropical regions all around the world and is a hybrid of *Citrus reticulata* (mandarin) and *Citrus maxima* (pomelo). Sweet orange essential oil is extracted from the peel of the fruit via a process known as cold compression. Sweet orange essential oil has a wide variety of applications in the food, perfume, cosmetics and sanitation industries, mostly for its favorable aroma and sweetness (Navarra et al., 2015a). Folk medicine employs sweet orange essential oil for its detoxifying effects, for treatments of digestive disorders, stress or anxiety and as an immune system stimulant (Arias & Ramon-Laca, 2005). The focus of most recent studies has been to explore the

anti-inflammatory and anti-proliferative effects of sweet orange essential oil, which may provide insight into potential anticancer activity (Navarra et al., 2015b).

Limonene, a monoterpene hydrocarbon (Fig. 1-4), is the major volatile compound found in sweet orange oil and in all other essentials oils derived from citrus fruits, such as bergamot, lemon, lime, grapefruit, etc. (Arias & Ramon-Laca, 2005). It is found in either D- or L- isomer forms. D-limonene has been studied in pregnancy, where it was shown to stimulate uterine contractions in pregnant mice by activating adenosine receptors and opening voltage-gated Ca^{2+} channels (Hajagos-Toth et al., 2015), and in a breast cancer clinical study involving newly diagnosed women with an operable cancer electing to take 2 g of limonene daily for 2-6 weeks prior to surgery (Miller et al., 2013). The purpose of the latter study was to determine drug/metabolite concentrations in the blood and breast tissue that was collected post-surgery and it was shown that limonene induced cell-cycle arrest and decreased cell proliferation (Miller et al., 2013). The potential effects of limonene on steroidogenesis have, so far, not been yet been fully explored.

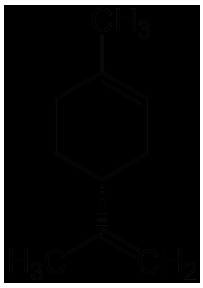

Figure 1-4: *D-Limonene, a major constituent of sweet orange essential oil*

1.2.9 Common Sage Essential Oil

Common sage, or *Salvia officinalis*, is an evergreen shrub of the Lamiaceae family and native to the Mediterranean region, but now cultivated in various countries around the world (Raal et al., 2007). *Salvia officinalis* implies its use for medicinal properties (salvia = to save or cure, officinalis = medicinal). The essential oil is extracted from the leaves, typically via steam distillation, and its recommended therapeutic application differs greatly based on cultural differences in varying countries. However, Middle Eastern countries appear to have the most

applications for this essential oil, with indications ranging from eye infections to digestive disorders (Lima et al., 2005). At present, scientific research has attributed only anti-bacterial and anti-fungal activity to sage essential oil (Pinto et al., 2007).

The *Salvia* genus comprises approximately 900 different species of sage, mostly depending on geographical location. Common sage, as the name suggests, is one of the most widely used essential oils; its chemical composition has been well documented, with camphor, a terpenoid, being considered the active component (Fig. 1-5) (Abu-Darwish et al., 2013). Studies have shown that camphor crosses the fetal-placental barrier of rats and rabbits. This has led to fears camphor may be potentially toxic to the human fetus and it is suggested to be avoided during pregnancy (Leuschner, 1997). A study of camphor metabolites demonstrated they stimulated the proliferation of human MCF-7 estrogen-dependent breast cancer cells, which is considered to be estrogenic activity mediated by the estrogen-receptor (Schlumpf et al., 2004). However, the potential interference of camphor with human steroidogenesis is entirely unknown.

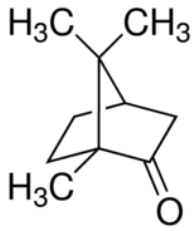

Figure 1-5: *Camphor, major constituent of common sage essential oil*

1.3 Steroidogenesis

1.3.1 Steroidogenesis in pre- and post-menopausal women

In the ovaries of pre-menopausal women, steroidogenesis proceeds in a unique manner requiring the interaction between ovarian theca and granulosa cells, termed the two-cell-two-gonadotropin theory (Fig. 1-6). Biologically active steroid hormones rely on cholesterol as major substrate for their biosynthesis. The major source of this cholesterol is low-density lipoproteins (LDLs), a storage and transport form of dietary cholesterol as well as cholesterol produced *de novo* by the liver; other human tissues that produce cholesterol *de novo* for use *in situ* are the adrenal cortex and the gonads. Cholesterol biosynthesis starts from acetate in the endoplasmic

reticulum and relies on the carefully tuned polymerization of isoprene units (Samavat & Kurzer, 2015). One of the most critical steps in steroidogenesis involves the movement of cholesterol in the form of esters to the mitochondria (Fig. 1-7). In humans, this process involves uptake of circulating LDLs through LDL receptor-mediated endocytosis. Once internalized in endocytic vesicles, fusion occurs with lysosomes and the cholesterol esters within the LDL particle are hydrolyzed to free-cholesterol by a lysosomal acid lipase. Free cellular cholesterol, either from the metabolism of LDLs in the endosomes or from intracellular synthesis *de novo*, binds to StAR-related lipid transfer domain proteins (StarD). StarD enables the entry of free cholesterol into the outer mitochondrial membrane. Unique to steroidogenic cells, is the steroidogenic acute regulatory protein (StAR). This protein is essential for the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. The next step in the steroidogenic process requires conversion of cholesterol to pregnenolone by the mitochondrial cytochrome P450 (CYP450) cholesterol side-chain cleavage enzyme CYP11A1. Pregnenolone is the precursor for the biosynthesis of all steroid hormones (Miller & Auchus, 2011). Pregnenolone is converted into androgens: dehydroepiandrosterone (DHEA) and androstenedione by two different enzymes: CYP17 and 3β-HSD1/2. CYP17 has dual 17α-hydroxylase and 17,20-lyase activity, and in thecal cells the first activity converts pregnenolone into 17α-hydroxypregnenolone followed by further conversion by its lyase activity into DHEA. Pregnenolone may also be converted into progesterone by 3β-HSD1/2 which is then converted by CYP17 into 17α-hydroxyprogesterone and subsequently androstenedione. It is important to note that 3β-HDS1/2 is also able to synthesize androstenedione from DHEA. The enzyme aromatase (CYP19) is required for the conversion of androgens into estrogens. Theca cells do not possess this enzyme, granulosa cells do. Working in communication, the androgens produced by the theca cells travel to the granulosa cells, where CYP19 converts androstenedione into estrone (E1). There is also interconversion between androstenedione and testosterone via different types of 17β-HSD enzymes depending on the cell-type: 17β-HSD types 3 and 5 produce testosterone from androstenedione, whereas 17β-HSD2 and 4 do the reverse. Testosterone is converted by CYP19, into estradiol (E2), and E1 is also able to be converted into E2 by 17β-HSD types 1 and 7. Estrone and estradiol are the main estrogen responsible for normal development and maintenance of female sex characteristics by acting at estrogen receptors at distant target sites throughout the body. Estrone is considered a less abundant,

weaker estrogen, but is the main estrogen in post-menopausal women, whereas estradiol is the predominant and most potent estrogen that is produced during reproductive years. (Simpson, 2003). The enzymes and hormones involved in ovarian steroidogenesis are shown in Fig. 1-6. In post-menopausal women, steroidogenesis in the ovaries ceases, and circulating androgens originate from the adrenal glands. Essentially, these circulating androgens are needed for estrogen biosynthesis that now occurs solely in extra-gonadal sites, such as the breast, bone, vascular endothelium, aortic smooth muscle cells, and brain, where it acts a paracrine or intracrine factor (Simpson, 2003b). Local estrogen synthesis is made possible by tissue-specific regulation of the aromatase enzyme.

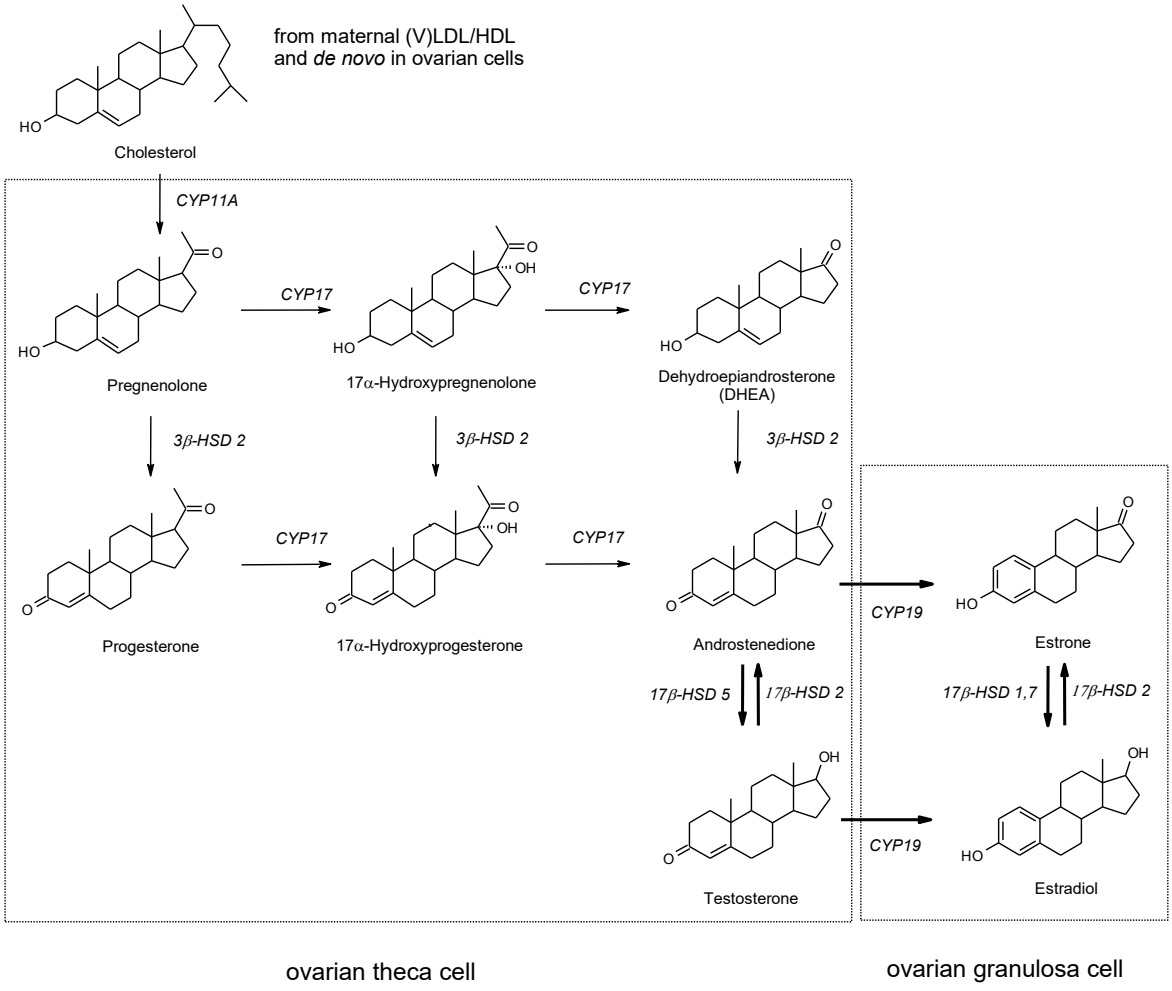

Figure 1-6: *Ovarian steroidogenesis: the two-cell two-gonadotropin theory*

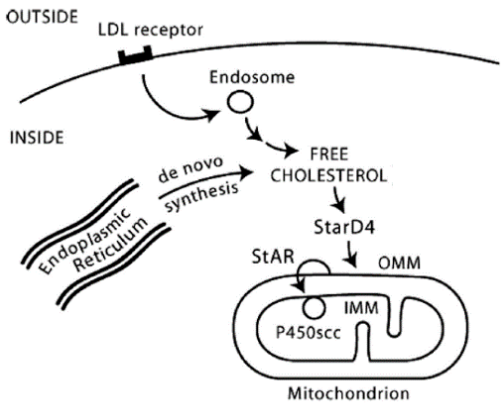

Figure 1-7: *First step in steroidogenesis: entry of cholesterol into the mitochondria*

(Miller & Auchus, 2011)

1.3.2 Feto-placental steroidogenesis

Feto-placental steroidogenesis, like ovarian steroidogenesis, requires communication among several different cell-types. The finely tuned cooperation between the placental trophoblast, fetal adrenal zone and fetal liver is essential for maintaining appropriate steroid hormone production throughout pregnancy (Fig. 1-8). Firstly, cholesterol must be supplied from either maternal dietary LDLs, synthesized *de novo* in the placenta or from the fetal adrenal zone. In the trophoblast, cholesterol is converted into pregnenolone and progesterone via the action of CYP11A1 and 3β-HSD1. Similar to the granulosa cells of the ovaries, the trophoblast is unable to synthesize androgens from progestogens (pregnenolone and progesterone) because they lack CYP17 enzyme activity. Thus, the progestogens are brought into the fetal adrenal zone, where pregnenolone is converted by CYP17 into DHEA and progesterone is used as a substrate for the production of fetal adrenal mineralocorticoids and glucocorticoids via the adrenocortex-specific enzyme CYP21. The DHEA produced in the fetal adrenal zone undergoes sulfatation by sulfotransferases (SULT2A1) and is sent back to the trophoblast as well as being transferred to the fetal liver in the form of DHEA-S, where it is converted into 16α-OH-DHEA-S by the fetal enzyme CYP3A7. The DHEA-S that arrives in the trophoblast is de-sulfated and used as precursor for the synthesis of estrone and estradiol by the same steroidogenic enzymes that are used by granulosa cells of the ovary. In a step unique to pregnancy, a third estrogen, estriol (E3), is produced from fetal hepatic 16α-OH-DHEA-S. As estriol synthesis is dependent on a viable fetus, E3 is used as a marker of fetal health and well-being (Mucci et al., 2003). Estrogens in the

placenta are essential for providing the rapidly growing fetus with enhanced utero-placental blood flow to ensure optimal exchange of gases and nutrients (Albrecht & Pepe, 2010).

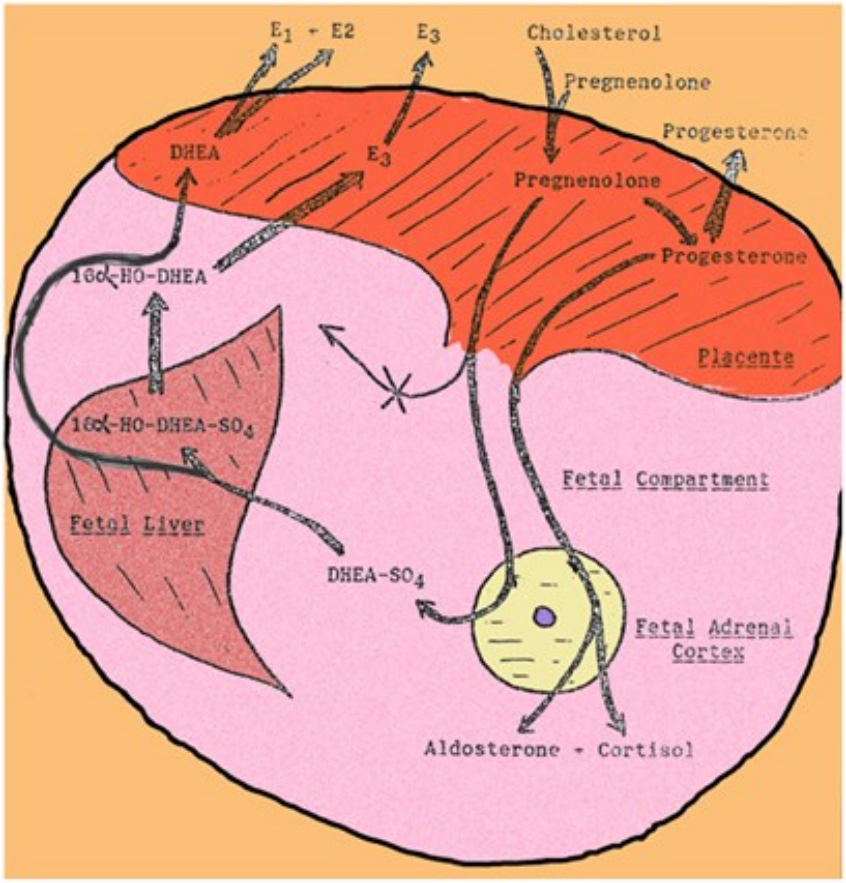

Figure 1-8: *Feto-placental steroidogenesis*

(Campbell, 2009)

1.3.3 Role of estrogen in women and during pregnancy

Estrogen is an instrumental hormone in the regulation of female reproductive cycle: menstruation, pregnancy and menopause. During reproductive years, the major estrogen produced by the ovaries is estradiol. In post-menopausal women, estrone becomes the dominant estrogen as aromatase is no longer expressed in the ovaries. During pregnancy, the placenta produces large amounts of estriol, considered to be the weakest among estrogens as it has the lowest affinity for estrogen receptors (Sasson & Notides, 1983). Nevertheless, its importance should not be underestimated as it is considered a crucial marker of fetal viability with low levels

being associated with chromosomal or congenital abnormalities, such as Edward's syndrome and Down syndrome (Cuckle, 1992). The physiological roles of estriol during pregnancy are otherwise poorly understood.

During the first stage of menstruation (Fig 1-9), known as the follicular phase, the endometrium, the inner lining of the uterus, is thick and full of nutrients to support a fertilized egg. However, if fertilization does not occur, there is a significant drop in estradiol and progesterone levels which results in shedding of the endometrium layer. This drop in hormone levels also triggers the hypothalamus to release gonadotropin releasing hormone (GnRH), which acts on the pituitary gland to secrete follicle-stimulating hormone (FSH) (Reed & Carr, 2018). The rise in FSH levels stimulates growth of ovarian follicles (surrounded by granulosa and theca cells). Eventually, a dominant follicle is formed and FSH stimulates aromatase activity in the granulosa cells to result in a rise in estradiol production. Estradiol in the presence of FSH stimulates the formation of luteinizing hormone (LH) receptors on granulosa cells which results in the release of a small quantity of progesterone into the blood circulation, subsequently triggering the pituitary gland to release LH (Fink, 1988). This is the beginning of the ovulatory phase of menstruation. LH released from the pituitary gland binds to receptors located on theca cells and stimulates androstenedione production. This androstenedione is transported to the granulosa cells where aromatase increases estradiol levels; a process known as the two-cell, two-gonadotropin hypothesis of estrogen synthesis in the human ovary (Fig. 1-6) (Reed & Carr, 2018). Eventually, the LH-surge triggers the ovarian follicle to rupture and release of the egg. In the luteal phase of menstruation, the follicle that is left in the ovary forms the corpus luteum. The corpus luteum continuously secretes estradiol and progesterone to thicken the endometrium. If pregnancy does not occur, the corpus luteum is degraded and the menstrual cycle begins anew. However, if the pregnancy does occur, the corpus luteum is maintained by human chorionic gonadotrophin hormone (hCG) released from the fertilized egg after it has implanted in the endometrium (Reed & Carr, 2018). In later stages of pregnancy, the corpus luteum will degrade and steroid hormone production is taken over by the fetal-placental unit.

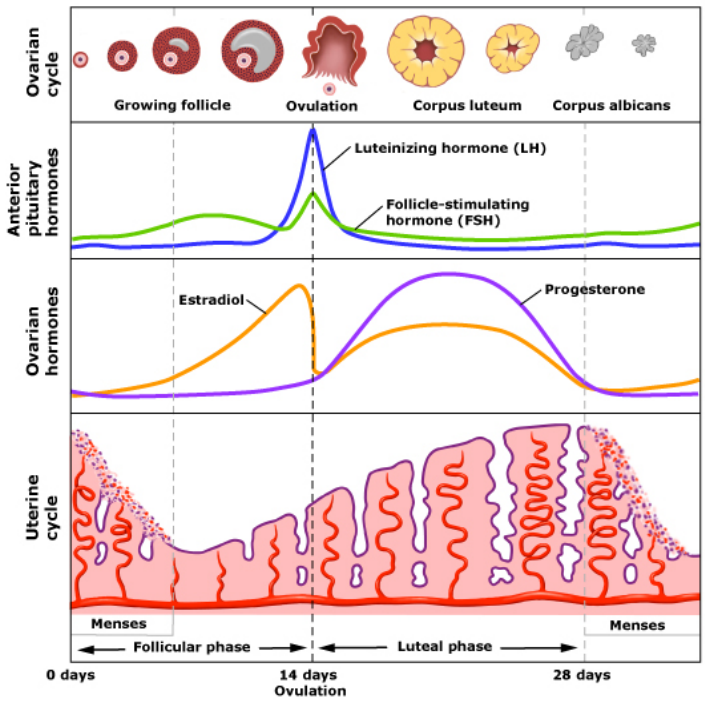

Figure 1-9: *The menstrual cycle*

Image from: Nguyen (2017)

1.3.4 Tissue-specific aromatase expression

Local estrogen synthesis is made possible by tissue-specific regulation of the aromatase enzyme. The *CYP19A1* gene that encodes aromatase in humans is found on chromosome 15q21.2 and is approximately 123 kb long consisting of a 93 kb 5'-untranslated region (UTR) and a 30 kb coding region localized at the 3'-end (composed of exons II-X) (Fig. 1-10) (Boon et al., 2010; Bulun et al., 2005). Upstream of the 30 kb coding region lie several untranslated exons I that are each associated with a tissue-specific promoter. However, the protein expressed at the tissue-specific sites are identical due to splicing of the RNA transcript, which produces a common junction in the 5'-untranslated region upstream of the translation start site (Bulun et al., 2005; Simpson et al., 1993). At present and unique to humans, numerous tissue-specific aromatase promoters have been uncovered that regulate aromatase expression, including I.1 (major) and I.2 (minor) in the placenta; I.8 in placenta, fetal liver, and thyroid; I.4 in adipose tissue and skin; I.5 in fetal tissues; I.7 in endothelial cells and adipose tissue; I.f in the brain, I.6 in the bone; I.3 and PII in the ovary and testis (Fig. 1-10) (Demura et al., 2008; Zhao et al., 2016). Studies looking at tissue-specific aromatase expression can be challenging as one cannot

use rodent models; in lower vertebrates aromatase expression is limited to gonads and brain and is regulated by only two tissue-specific promoters (II and I.f) (Zhao et al., 2016). In humans, the activity of each promoter is regulated by a unique set of hormones, cytokines and second messengers that recruit different transcription factors, leading to the transcription of various alternatively spliced mRNA species. Each mRNA species has a distinct promoter-specific first untranslated exon, but identical coding region, thus enabling a tissue-specific expression of aromatase (Fig. 1-10) (Boon et al., 2010; Bulun et al., 2005; Zhao et al., 2016).

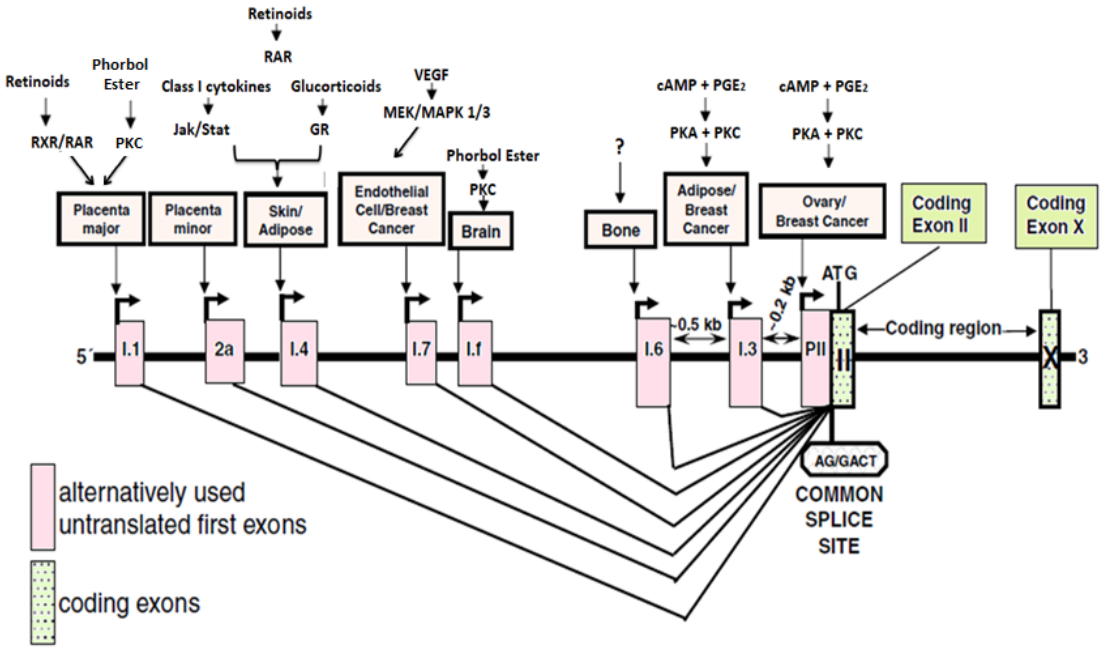

Figure 1-10: *The human CYP19 gene is located on chromosome 15 and its expression is controlled by tissue-specific promoters located upstream of the coding region.*

Image adapted from Sanderson (2006)

1.3.5 Aromatase in the pre- and post-menopausal women

In pre-menopausal women, estradiol levels fluctuate throughout the menstrual cycle. The highest estradiol levels are reached immediately before ovulation, levels are moderate in the luteal phase and reach their lowest levels in the follicular phase (Zhao et al., 2016). Estrogen levels increase during puberty, are highest throughout reproductive years and gradually decline to lower levels during the post-menopausal phase. During pre-menopausal years, estrogen levels are maintained by the cyclic expression of ovarian aromatase (Zhao et al., 2016). Post-

menopausal women produce estrogens through the regulation of aromatase in extra-gonadal sites. Ovarian aromatase expression is regulated by follicle stimulating hormone (FSH) which activates FSH receptors on granulosa cells, thus stimulating cyclic AMP (cAMP) production and protein kinase A (PKA) activity; this signaling pathway stimulates promoter PII-mediated aromatase expression (Findlay et al., 2010; Zhao et al., 2016). FSH-stimulated PKA activity increases phosphorylation of a cis-regulatory element binding protein (CREB) at position Ser133 (Findlay et al., 2010; Michael et al., 1997). Phospho-CREB then associates with co-activator protein CREB-binding protein (CBP) or its homolog p300, forming a complex that then binds to CRE-like sequences in promoter II of the *CYP19* gene, resulting in increased gene transcription (Ghosh et al., 2005). PKA is also capable of phosphorylating and activating steroidogenic factor-1 (SF-1), which binds to a cis-acting SF-1 binding site in the proximal region of promoter II of the *CYP19* gene to initiate transcription (Ghosh et al., 2005). The surge in FSH-induced genes is decreased during the luteal phase when the granulosa cells transition from a proliferative to a differentiated state (Ghosh et al., 2005). In post-menopausal women, where adipose tissue is the main source of circulating estrogens, aromatase expression is primarily mediated by the adipose-specific I.4 promoter of *CYP19* (Findlay et al., 2010). Promoter I.4 is activated by cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNFα) in the presence of glucocorticoids, and involves the JAK1/STAT3 regulatory pathway (Findlay et al., 2010).

1.3.6 Aromatase expression during pregnancy

During pregnancy, the primary site of estrogen synthesis is the placenta with the essential support of androgen precursors produced by the fetal adrenal gland and the fetal liver. Placental aromatase is expressed primarily via the tissue-specific promoter I.1 (Sun et al., 1998). Stimulation of promoter I.1 and subsequent increased *CYP19* transcription has been reported to occur via ligands of the RXR and RAR retinoid receptors and vitamin D receptor (Simpson, 2003a; Sun et al., 1998). Promoter I.8, although having its highest presence in the placenta, plays only a minor role in overall placental-specific aromatase expression. Located only 2 kb upstream of promoter I.4, they share similar activation mechanisms initiated by the glucocorticoids that activate the glucocorticoid receptor-mediated signaling pathway in the presence of cytokines (IL-6, IL-11, leukemia inhibitory factor and oncostatin M), resulting in activation of JAK/STAT

signaling and subsequent activation of glucocorticoid response elements in the upstream region proximal to the I.8 promoter of *CYP19*. (Demura et al., 2008; Zhao et al., 1995).

1.4 Estrogen receptor signaling

1.4.1 Estrogen receptors (ERα and ERβ)

Estrogens exert their biological activity primarily through activation of estrogen receptors ERα and ERβ. Both receptors belong to the nuclear receptor superfamily of ligand-activated transcription factors. ERα and ERβ reside in the cytoplasm prior to ligand binding and form a complex with heat shock protein 90 (HSP90). Estrogens enter the cytoplasm via diffusion across the cell membrane and displace HSP90 from the estrogen receptors in order to bind to the ERs (Girgert et al., 2019). Upon ligand binding, the ER dimerizes followed by translocation to the nucleus. In the nucleus, the ligand-bound receptor recruits coactivators to form a complex that is able to bind to estrogen response elements (EREs) on the DNA which initiates transcription of target genes that influence growth and proliferation as well as numerous other cellular functions (Hayes & Lewis-Wambi, 2015).

ERα and ERβ are encoded by two distinct genes, but the receptors share overlapping structural characteristics. The receptors are divided into 6 domains (Fig. 1-11) starting with domain A/B which is the N-terminal domain (NTD) and contains an Activating Function domain (AF-1) which can activate transcription of target genes without the need for ligand binding (Kumar et al., 2011; Vrtačnik et al., 2014). In this case, the receptor is activated by binding to co-regulators or after being phosphorylated on certain residues (Vrtačnik et al., 2014). The C domain comes after the NTD and contains the DNA-binding domain (DBD) which is the most conserved sequence between ERα and ERβ (Kumar et al., 2011; Vrtačnik et al., 2014). A nuclear localization signal (NLS) is contained in domain D and it connects to the ligand-binding domain (LBD) located in domain E. Importantly, the hormone-dependent Activating Function domain (AF-2) is also located in domain E and is responsible for the recruitment of co-activators to initiate gene transcription (Kumar et al., 2011). Finally, the F domain is localized to the C-terminus and despite its role not having been fully elucidated in ERβ, in ERα it has been shown

to regulate, in a ligand-dependent manner, transcription activity, co-activator interaction, dimerization and receptor stability (Yaşar et al., 2016).

ERα and ERβ also possess different isoforms. ERα has two isoforms without an NTD that can repress the activity of full-length ERα through heterodimerization (Vrtačnik et al., 2014). A third isoform of ERα (ERα36) has been shown to mediate ER activity at the cellular membrane by interacting with the GPER1 (G protein-coupled estrogen receptor) (Vrtačnik et al., 2014). Four isoforms of ERβ differ from the full-length ERβ receptor with regard to their LBD. These isoforms lack transcriptional activity, but still dimerize with ERα resulting in silencing of ERα signaling (Vrtačnik et al., 2014).

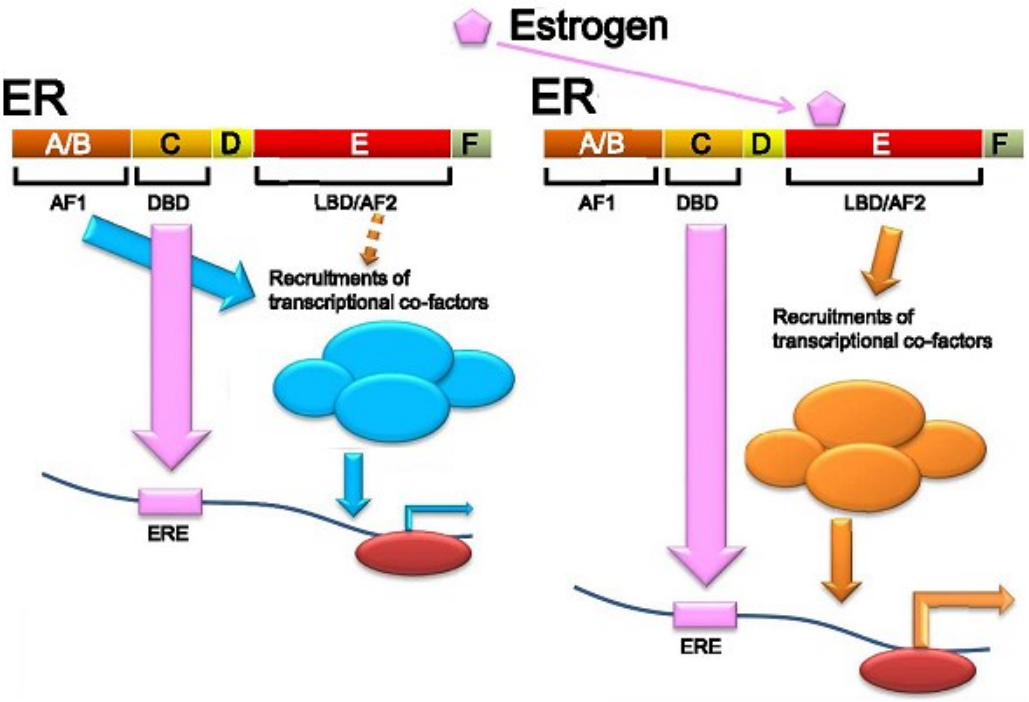

Figure 1-11: *Domains of the estrogen receptor (ER) and their function*

Estrogen receptors, ERα and ERβ, are divided into 6 domains: A/B is the N-terminal domain (NTD) and contains an Activating Function domain (AF-1), C domain contains the DNA-binding domain (DBD), D domain contains a nuclear localization signal, E domain contains the ligand-binding domain (LBD) and hormone-dependent Activating Function domain (AF-2), and F domain is localized to the C-terminus. Upon ligand binding, the ER dimerizes followed by translocation to the nucleus. In the nucleus, the ligand-bound receptor recruits coactivators to form a complex that is able to bind to estrogen response elements (EREs) on the DNA

Image adapted from: Wada et al. (2013)

1.4.2 Estrogen receptor signaling

The canonical model of estrogen signaling involves direct binding of estrogens to the ER in the cytoplasm, dimerization of the receptor, followed by translocation to the nucleus where it binds to EREs on in the DNA to initiate transcription of ER target genes. Additionally, estrogen receptors are capable of initiating transcription of genes without DNA binding in a process known as "transcriptional cross-talk" (Marino et al., 2006). Here, the ER associates to DNA with the help of transcription factors such as Specificity protein-1 (Sp-1). Essentially, estrogen binding to the Sp-1 stimulates the recruitment of co-activators and DNA binding (Marino et al., 2006). Approximately 35% of ER genes are transcribed through this indirect association to DNA, including the low-density lipoprotein (LDL) receptor, endothelial nitric oxide synthase (eNOS), cyclin D1, etc. (Marino et al., 2006; O'Lone et al., 2004).

ER-mediated effects can also occur by a non-genomic mechanism to initiate rapid events such as the four main signaling cascades: protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phosphatidyl inositol 3 kinase (PI3K), and protein kinase A (PKA) (Marino et al., 2006; Vrtačnik et al., 2014). Non-genomic estrogen signaling is mediated by a plasma membrane bound ER, GPER1, as well as certain isoforms of ERα and ERβ (Vrtačnik et al., 2014).

1.5 Breast cancer

1.5.1 Role of hormones in the normal breast

The normal breast is composed of the stroma, which contains adipocytes, fibroblasts, blood vessels, inflammatory cells and the extracellular matrix and of the epithelium, which is composed of a system of branching ducts and lobes. Ducts and lobes are lined by a single layer of luminal epithelial cells surrounded by a basal cell layer comprised of myoepithelial cells that possess contractile properties and play an important role in lactation (Hilton et al., 2018). The epithelium of the breast is further separated from the stromal region via a basement membrane (Fig. 1-12).

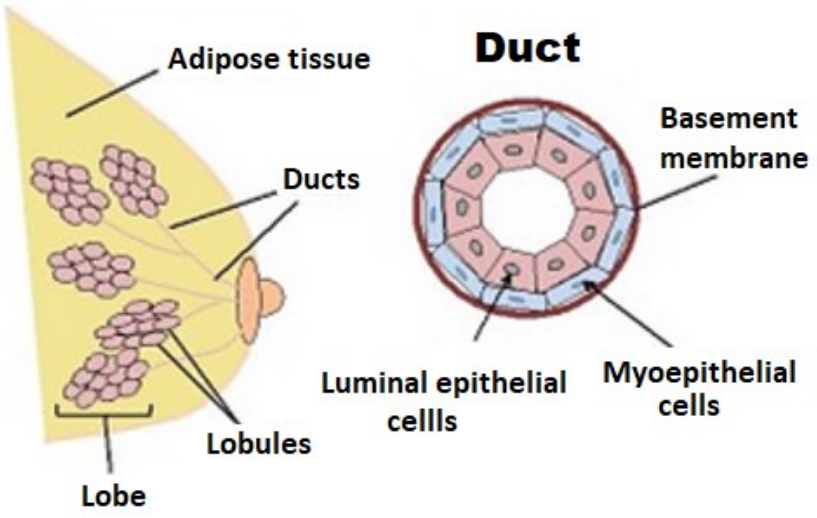

Figure 1-12: *The female breast composition*

Mammogenesis or mammary gland development, occurs primarily after birth during three stages of a woman's life cycle: puberty, menstrual cycle and pregnancy (Hilton et al., 2018). Breast tissue development is mediated by the action of the estrogen receptor (ER) and the progesterone receptor (PR) which are primarily localized to luminal epithelial cells (Clarke et al., 1997). Postnatally, the mammary gland is an under-developed ductal system, whereas at the onset of puberty, estrogens released from the ovaries along with pituitary growth factors act on receptors in the mammary gland to stimulate ductal morphogenesis, whereby ducts become elongated and start to branch. Additionally, estrogens stimulate the growth of the stromal and adipose tissue.

Progesterone released from the ovaries also plays an important role in side-branching and enlargement of the mammary ducts (Hilton et al., 2018; Macias & Hinck, 2012). ERα is considered to be the most active estrogen receptor during development of the mammary gland; ERα knockout mice have impaired breast development, whereas ERβ knockout mice are only slightly impacted (Förster et al., 2002; Hilton et al., 2018). PR knockout mice also lack a complete ductal network and have limited lobular development (Lydon et al., 1995).

The next stage of mammogenesis occurs during the menstrual cycle, when brief proliferation spurts take place. Essentially, the estrogen surge during the follicular phase stimulates duct elongation and expansion of the fat pad, while progesterone released during the

luteal phase is responsible for the formation alveolar buds, which regress when progesterone levels drop at the end of each cycle (Atashgaran et al., 2016; Macias & Hinck, 2012).

During pregnancy, estrogen and progesterone levels remain elevated and work together to contribute to growth of the ducts and glandular buds. Milk production is ensured by the cooperation of many other hormones, including FSH, LH, prolactin, oxytocin and human placental lactogen (Lee & Kelleher, 2016; Macias & Hinck, 2012).

Finally, when estrogen and progesterone production by the ovaries ceases, the epithelial component of the breast is replaced with stromal cells and adipose tissue (Hutson et al., 1985).

1.5.2 Role of estrogen in breast cancer initiation

Lung and breast cancers were the most common cancers worldwide in 2018, each contributing 12.3% of the total number of new cases diagnosed (World Cancer Research Fund). Breast cancer is also the most common cancer worldwide in women, contributing 25.4% of the total number of new cases diagnosed in 2018 (World Cancer Research Fund). In Canada, breast cancer represented 25% of all new cancer cases in women in 2017 and 13% of all cancer deaths in women (Canadian Cancer Society). Although the exact etiology is unknown, about 70% of the cases are estrogen-dependent (Lumachi et al., 2015). Estrogen has been suggested to play a carcinogenic role in breast cancer initiation. First, it is widely accepted that estrogen carcinogenicity in the breast is associated to its binding of ERα and subsequent stimulation of cellular proliferation, either directly or indirectly, with help of growth factors (Russo et al., 1999; Russo & Russo, 2006). Secondly, there is evidence to suggest that the metabolism of estrogen generates catechol intermediates that can bind directly to DNA and form adducts (Cavalieri et al., 1997; Rogan et al., 2004). Third, there is now evidence that estrogen can cause mutations in certain chromosomes of human breast epithelial cells and initiate breast cancer (Russo & Russo, 2006).

ERα and ERβ are both normally expressed in breast tissue. ERα is localized to a small subset of epithelial cells, while ERβ is found in epithelial and stromal cells. One of the hallmarks of estrogen-dependent breast cancer is that the expression of ERα is upregulated in the breast tumor cells and ERβ's expression is decreased (Palmieri, 2004; Saji et al., 2000). Estrogen

promotes breast cancer growth by binding to ERα which subsequently activates genes that increase cellular proliferation of breast tumor cells. ERβ's role in breast cancer remains elusive. Structurally speaking, it is similar to ERα save for minor amino acid differences inside and around the ligand-binding pocket (Haldosen et al., 2014). The little evidence available of ERβ's function suggests it plays a bi-faceted role (Jonsson et al., 2014). It is thought that ERβ activation may have anti-proliferative effects in breast cancer when ERα levels are elevated, and proliferative effects with low or absent levels of ERα. Essentially, different isoforms of ERβ, which differ from full-length ERβ, may dimerize with ERα and silence its activity (Vrtačnik et al., 2014) while other isoforms may enhance ERα activity (Jonsson et al., 2014). Due to its uncertain mechanistic activity, ERβ status is not considered a reliable tool for the diagnosis or treatment of breast cancer (Jonsson et al., 2014).

1.5.3 ER-dependent breast cancer microenvironment

The heterogeneity of breast cancer is reinforced by the discovery of multiple gene expression signatures representing the various cancer cells of the mammary gland involved, some of which are more prominent than others at different stages of tumor growth. Cross-talk between cells of the ER-dependent breast cancer environment has been heavily studied over the past decade; especially the communication of stromal and tumor cells of post-menopausal women. In normal breast adipose tissue, 90% of the residing cells are fibroblasts, precursors to mature adipocytes, and another 7% represent epithelial cells (Bulun et al., 2005). Aromatase levels are maintained at a low level by fibroblasts via the activation of promoter I.4 (Bulun et al., 2005). I.4 in adipose tissue can either be activated through glucocorticoid-binding and initiation of the Jak/STAT signaling cascade or by retinoids (highly expressed in adipose tissue) binding to the retinoid acid receptor (RAR) (Wilde et al., 2013). Stromal cells, including fibroblasts, inflammatory, and endovascular cells, have been shown to support ERα-positive breast cancer progression to metastasis. Essentially, fibroblasts have inducible aromatase activity before differentiating into mature adipocytes (Meng et al., 2001; Zhou et al., 2001). In breast cancer, a desmoplastic reaction is initiated when malignant epithelial tumor cells secrete a variety of factors (tumor necrosis factor-α, prostaglandins E2, interleukin-6, and interleukin-11) which are able to stimulate aromatase activity in fibroblasts and prevent their differentiation into mature adipocytes, subsequently increasing estrogen production (Meng et al., 2001; Zhou et al., 2001).

A positive feedback mechanism is created when the estrogen synthesized in the tumor microenvironment binds to ERs on the epithelial tumor cells, which in turn stimulates further production of factors to activate fibroblast aromatase expression leading to ER-positive breast cancer progression (Fig. 1-13) (Bulun et al., 2005; Saha Roy & Vadlamudi, 2012).

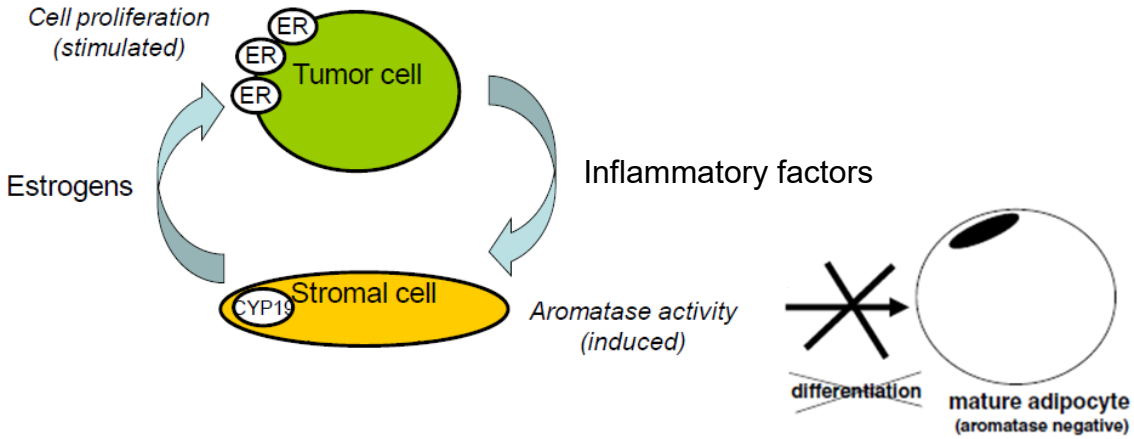

Figure 1-13: *Stromal-tumor microenvironment: a positive feedback mechanism for hormone-dependent mammary tumor growth in humans*

1.5.4 Aromatase promoter switch in breast cancer

In normal breast tissue, aromatase is expressed at low levels in adipose tissue and fibroblasts, under the control of promoter I.4. In breast cancer, a promoter-switch takes place whereby promoter I.4 is repressed and CYP19 becomes activated via promoters II, I.3, and I.7, resulting in a significant rise in aromatase mRNA and estrogen levels due to the combined action of these promoters (Bulun et al., 2009; Khan et al., 2011). Promoter I.3 and II are the most proximal to the coding region and possess similar patterns of activation. Firstly, PGE_2 activates PKA and PKC through binding of the prostanoid receptors, EP_1 and EP_2, located on breast adipose fibroblasts (Chen et al., 2009). PKA activation is crucial for aromatase expression via promoter I.3/II, but the PKC pathway is considered unessential, and rather used to potentiate PKA-dependent aromatase expression (Chen et al., 2009). Once PKA and PKC pathways are activated, phosphorylation of cAMP responsive element binding protein 1 (CREB1) occurs, which results in its translocation to the nucleus and binding to CRE-like sequences in PI.3/II and transcription of the promoter-specific *CYP19* gene (Chen et al., 2011).

In regards to activation of the promoter I.7, it is known that aromatase is overexpressed in vascular endothelial of breast tumor tissue (Bulun et al., 2005). This is thought to contribute to angiogenesis of the tumor (Bulun et al., 2005). The binding of transcription factor GATA-2 to a regulatory element of promoter I.7 is essential for its regulation of aromatase expression and estrogen production in endothelial cells of the breast tumor tissue (Bulun et al., 2005; Sebastian et al., 2002). The signaling pathway involved in promoter I.7 activation has still not been clearly elucidated, but may involve the vascular endothelial growth factor (VEGF) which plays a role in angiogenesis in breast cancer (Sebastian et al., 2002). Recent evidence suggests that VEGF may bind to receptors present on breast cancer endothelial cells and initiate the MEK/MAPK 1/3 signaling pathway and result in promoter I.7 overexpression and upregulation in the endothelial cells of a breast tumor (Caron-Beaudoin et al., 2018).

1.5.5 Endocrine disruptors

Endocrine disruptors are chemicals found in a wide-range of commercial products that are present in the environment sometimes at relatively low levels and can interfere with proper hormonal activity in male and female biological systems resulting in adverse effects. Exposure to endocrine disruptive chemicals can be either direct with phytoestrogens found in consumable plants to indirect exposure with pesticides and fungicides. Primary routes of exposure include inhalation, dermal, and oral while others have shown endocrine disrupting compounds reaching embryonic tissues (Yang et al., 2015). In regards to the latter, it has been shown that organic pollutants present in China, and to which mothers are exposed to, are capable of transplacental transfer to reach the fetus (Li et al., 2013).

The molecular mechanisms by which endocrine disruptive chemicals interfere with hormone regulation is quite varied because they can impact every level of an endocrine system. One of the most common mechanisms is binding to sex hormone receptors needed for steroidogenesis, as either agonists or antagonists, which can result in deleterious increases or decreases of important downstream estrogens and androgens (Yang et al., 2015). Another critical mode of action is their ability to mimic endogenous hormones and bind to endocrine receptors, displaying agonist or antagonistic activity in a tissue-specific manner (Celik et al., 2008). This is of particular relevance when considering the diverse activity and distinct tissue distribution of

the estrogen receptors ERα and ERβ. Furthermore, endocrine disruptors when bound to ERs can induce a conformational change so that the recruitment of coactivators/corepressors is halted and gene expression of ER-targeted genes is affected (Yang et al., 2015). Additionally, endocrine disruptors may act indirectly by stimulating the activation of endocrine receptors through protein kinases (Watson et al., 1991). Endocrine disruptors can also interfere with non-hormonal pathways by inducing epigenetic changes, such as alterations in DNA methylation patterns and histone modifications, which subsequently alters expression of genes important to steroidogenic pathways (Skinner et al., 2011).

1.6 Cellular models

1.6.1 Feto-placental co-culture model

A recently established feto-placental co-culture system that enables the *in vitro* study of steroidogenesis during human pregnancy was utilized in this thesis (Thibeault et al., 2014), as it is important to understand the potential impact on steroidogenic activity of compounds that pregnant women may be consuming. This model utilizes two different cell lines, in a trans-well system, to mimic the cooperation between the placental trophoblast, fetal adrenal zone, and the fetal liver that is essential for maintaining steroid hormone production throughout a healthy pregnancy. The fetal compartment (adrenal zone and liver) is represented by the H295R cells isolated from a human adrenocortical carcinoma. This cell line possesses all the necessary steroidogenic enzymes to mimic the fetal adrenal zone, such as CYP17 (lacking in the placental trophoblast region), CYP21, sulfotransferase, etc (Samandari et al., 2007). They also have the ability to produce DHEA via 16α-hydroxylase activity (representative of the fetal liver compartment) therefore providing the necessary precursors for estriol production in the placental region and required for healthy pregnancy (Thibeault et al., 2014). BeWo cells isolated from a human choriocarcinoma are used to represent the trophoblast compartment of the placenta due to the ability for the cells to fuse and form a syncytiotrophoblast structure (Nampoothiri et al., 2007) as well as express high levels of basal CYP19 activity. More importantly, BeWo cells have relatively low basal levels of estradiol and estrone because they possess almost negligible levels of CYP17; which is necessary for the *de novo* synthesis of estrogens from androgens. The basic principle of this co-culture system is that the BeWo cells and H295R cells communicate

through the interchanging of molecules that are necessary for proper feto-placental steroidogenic activity, as is the case *in vivo*. Here, H295R cells, which possess CYP17 activity, produce androgen precursors that are utilized by BeWo cells. In turn, BeWo cells contain endogenous placental aromatase (CYP19) activity that converts these androgen precusors into essential estrogens. The placental estrogens will then exhibit autocrine activity on BeWo cells by allowing the differentiation to form a syncytium-like structure and the release of human chorionic gonadotropin (β-hCG) as well as paracrine/endocrine activity through the stimulation of the H295R cells to secrete additional androgen precursors and complete the positive feedback loop (Fig. 1-14) (Thibeault et al., 2014).

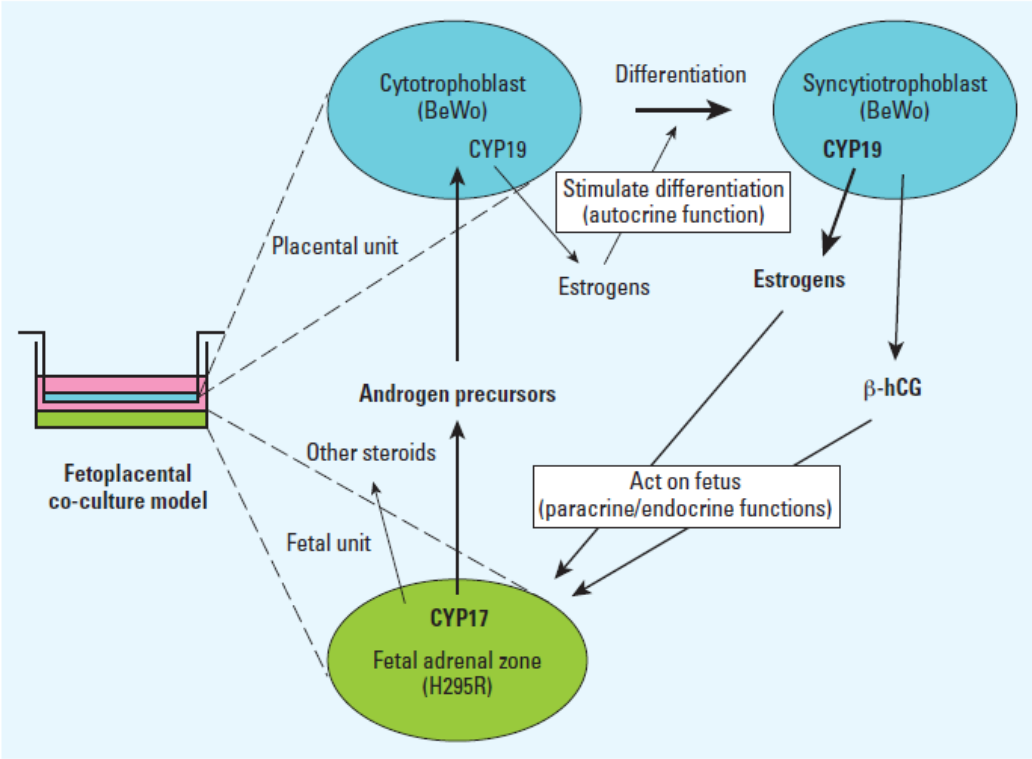

Figure 1-14: *Interactions in the feto-placental co-culture model*

(Thibeault et al., 2014)

1.6.2 Estrogen dependent breast cancer

1.6.2.1 Hs578t cell line

Hs578t (American Type Culture Collection, no. HTB-126) are non-ER expressing, aromatase-containing cancer (or cancer-associated) cells derived from a carcinosarcoma; a rare

stromal-like tumor. They are considered a “mesenchymal-like” or “stromal-like” breast cancer cell line because they have low to negligible expression of epithelial luminal markers (such as E-cadherin) and express high levels of mesenchymal markers (such as vimentin and N-cadherin) (Lacroix & Leclercq, 2004), and display a fibroblast-like phenotype in cell culture (Lacroix & Leclercq, 2004; Prat et al., 2010). Interestingly, despite possessing stromal or mesenchymal phenotype, Hs578t cells do not express the unique gene signatures (such as the HER2 and P-cadherin) found in ER-independent tumors, which marks them of particular interest to understand the interactions that take place in the microenvironment of and estrogen dependent breast cancer (Lacroix & Leclercq, 2004). Most importantly, Hs578t cells possess all breast cancer relevant CYP19 promoters (I.4, PII, I.3, I.7) which allows the ability to the study the effects that potentially endocrine-disrupting compounds may have on aromatase activity (Caron-Beaudoin et al., 2018).

1.6.2.2 T47D cell line

T47D (American Type Culture Collection (ATCC), Manassas, VA, USA) is a tumorigenic cell line isolated from a ductal carcinoma and is an ER-positive hormone-dependent breast cancer cell line. T47D cells are of epithelial origin, depend on estrogen for their proliferation and importantly express only negligible amounts of the aromatase enzyme; even less so than the classical ER-expressing cancerous epithelial cells MCF-7 (Aka & Lin, 2012). Therefore, they do not produce estrogens and are dependent on estrogens provided by the surrounding stroma in order to proliferate. They secrete various inflammatory factors required to condition the surrounding stroma into increasing its aromatase activity and producing more estrogens (Lacroix & Leclercq, 2004; Liubomirski et al., 2019). T47D cell line is preferred over the MCF-7 cell line as it expresses higher levels of ER and is more responsive to stimulation by estrogens, making it a more sensitive biosensor of estrogenic activity (Lacroix & Leclercq, 2004).

1.6.2.3 Estrogen-dependent breast cancer co-culture

In order to reflect *in vivo* conditions, whereby a positive feedback loop exists between the cancer cells and surrounding stroma region, in regards to estrogen production via aromatase enzyme, two cell lines were chosen: T47D and Hs578t cells to represent an estrogen-dependent

breast cancer. Hs578t cells possess the stromal characteristic of expressing aromatase via promoter I.4, the main driver of CYP19 regulation in healthy stromal cells, while also possessing the ability to undergo a promoter-switch through activation of promoter I.3, PII, and I.7 that enables breast cancer progression (Caron-Beaudoin et al., 2018). Furthermore, T47D cells do not possess aromatase activity and utilize estrogens required for their growth and proliferation that are provided from the surrounding stroma, in this case, the Hs578t cells. Taken together, the characteristics of both cell lines enable the study of an ER-dependent breast cancer microenvironment.

1.7 Hypothesis and Objectives

1.7.1 Research question

There has been a steady increase in the use of complementary and alternative medicine (CAM) over the past decade. Many Western countries report over a third of their population have used some form of CAM, whether for preventive reasons, adjuvant therapies, or as a form of replacement treatment in efforts to combat toxic side-effects associated with traditional therapies (Frass et al., 2012). Among CAMs, essential oils have seen their popularity drastically increase in the 21st century, mostly due to their widespread applications in the food, sanitation, cosmetic, and perfume industries. With growing use of essential oils, it is of utmost importance to uncover any potential biological actions that may encourage the development of new applications in human health but also to discourage practices that may harm human health. Certain essentials oils have already proven to have antibiotic and antiviral activities, with increasing research focused on their potential effectiveness in treating neurological disorders, inflammatory diseases, cardiovascular issues, menopausal symptoms, and cancer (Ali et al., 2015; Choi et al., 2014; Navarra et al., 2015a; Shinohara et al., 2017). Until now, however, very little information is available on the potential effects of essential oils (and their most active constituents) on uniquely human steroidogenic pathways, beyond possible estrogenicity at the receptor level (Choi et al., 2014; Patel, 2017; Shinohara et al., 2017; Simões et al., 2018). It is of particular interest to know whether an essential oil may have adverse effects on steroidogenesis in females, who are more frequent users of CAMs than men (Frass et al., 2012). Women who are most at risk include pregnant women and those who suffer from or are at elevated risk of developing estrogen-

dependent breast cancer as in both these situations steroidogenesis, especially estrogen biosynthesis, plays a crucial role.

1.7.2 Hypothesis

The complex essential oil mixture and/or its predominantly active compounds may interfere with key enzymes in the steroidogenic pathway in women during critical periods such as pregnancy or when predisposed to or suffering from hormone-dependent breast cancer.

1.7.3 Objectives

General objective: Evaluate the effects of common essential oils on steroidogenesis in two physiologically relevant human cellular co-culture systems; one already established, representing the steroidogenic interactions that occur in the feto-placental unit during pregnancy and the other, under development, representing the hormone-dependent breast cancer microenvironment.

Specific objectives:

1. Characterization of the effects of common essential oils and their respective active ingredients on steroidogenesis in a feto-placental co-culture model composed of adrenocortical H295R cells and placental trophoblast-like BeWo cells. We place a focus on measuring hormone production levels, changes to promoter-specific expression of *CYP19*, and aromatase catalytic activity to determine the signaling pathways involved in the response of the co-culture to essential oils.

2. Characterize the ability of essentials oils to interfere with steroidogenesis in a monoculture of Hs578t cells; these cells express aromatase via breast cancer-relevant promoters, which makes them a representative model of the mammary stromal cells surrounding the hormone-dependent breast tumor. Our goal is to assess changes to the promoter-specific expression of *CYP19* and the effects of essential oils. This objective is a prerequisite for the development of the human breast cancer co-culture model described in objective 3.

3. Develop an estrogen-dependent breast cancer co-culture system and define the key interactions between stromal-like breast cancer cell line Hs578t and tumor epithelial cell line T47D. We place a focus measuring hormone production levels, changes to the promoter-specific expression of *CYP19*, and aromatase catalytic activity. Once established, the effects of essential oils on promoter-specific aromatase expression and hormone-dependent breast cancer cell proliferation will be evaluated.

1.8 Importance of project

With the increasing popularity of complementary and alternative medicine and naturally-derived products, such as essential oils, in particular, it is important to have insights into their biological activity beyond anecdotal folk medicine claims. Women are the most predominant consumers of these traditional forms of therapy and it is imperative to know whether any potential beneficial or adverse effects are associated to these products. Since knowledge of the effects of essential oils on steroidogenesis was severally lacking, a focus was placed on this aspect of its potential biological role. At the same time, verifying that any observed steroidogenic activity is mediated by the most prevalent compound in the volatile essential oil mixtures. The essential oils used in this study were chosen because of their ubiquitous use in CAM and numerous other commercial applications. The essential oils were also chosen based on their chemical constituents, to evaluate whether terpene and/or terpenoid constituents are more effective than other classes. More specifically, essential oils were chosen to determine whether the size and complexity of the major terpene/terpenoid molecule present within the oil relates to its effects on steroidogenesis. The essential oils evaluated in this thesis belong to the monoterpene (limonene; sweet orange essential oil), sesquiterpene (β-caryophyllene; black pepper essential oil), oxygenated terpene (camphor; common sage essential oil) and phenylpropene class (Trans-anethole; fennel seed essential oil, estragole; basil essential oil). Due to the fact that many essential oil constituents utilize the isoprene unit as their functional building block and that steroid hormones are derived from polymerization of isoprene units; it was hypothesized that active compounds present within essential oils may mimic the effect of certain hormones and/or interfere with the biological activity of steroidogenic enzymes.

In order to demonstrate scientifically that naturally derived compounds such as essential oils exert their proclaimed (or novel) biological activities in humans, *in vitro* assays that allow cell-to-cell interactions, such as occurs *in vivo,* are required in order to obtain physiologically relevant information. The development and application of cellular models with improved physiological/toxicological relevance is a driving theme of this thesis. The steroidogenic processes that occur in pregnant women cannot be represented by single cell assays or rodent models as they do not mimic the cooperation within the different tissues of the feto-placental unit. The popularity of complementary and alternative medicines among pregnant women has grown significantly in recent years with herbal products being the most frequently reported (Chuang et al., 2006; Pelkonen et al., 2017). Despite lack of scientifically validated safety and efficacy data, many of these women choose to put their trust in the safety of these herbal remedies based on the fact that they are 'natural' and have been used by many ancient civilizations, such as Egypt, China and India (Ali et al., 2015). The safety of herbal products during early stages of pregnancy and fetal development are of particular concern as many products have been shown to cross the feto-placental barrier and may have potential adverse effects in the fetus as well as the mother, such as the essential oils of wormwood, rue, oak moss, and hyssop, and herbal extracts of the huanglian and dang gui (Chuang et al., 2006; Pelkonen et al., 2017; Tillett & Ames, 2010). Given the crucial role that steroid hormones, notably estradiol and estriol, play in maintaining a healthy pregnancy, it is important to determine whether commonly used herbal treatments impact the carefully tuned steroidogenic interactions that occur during pregnancy, to better understand the potential risks posed to mother and unborn child.

Estrogen-dependent breast cancer is a disease that is also reliant on steroidogenic interactions among different cell-types, in this case, within the mammary tumor microenvironment. To better represent the *in vivo* human breast tumor microenvironment, use of co-culture systems composed of at least two different cell types have either been proposed or established. However, the few breast cancer co-culture systems that have been developed have focused on the communication between epithelial breast tumor cells and healthy primary human mammary associated fibroblasts (Heneweer et al., 2005; Wang et al., 2015). In this study, a unique model was developed to study interactions between cancer cells within the human breast

cancer microenvironment. Specifically, an estrogen-dependent breast cancer co-culture system whereby the interactions between ER-expressing mammary cancer epithelial cells and aromatase containing, non-ER expressing, breast cancer-associated stromal cells can be investigated at the level of steroidogenesis. This co-culture system will enable studies on the potential impact that endocrine disruptors have on steroidogenesis that takes place between tumor cells of an estrogen-dependent breast cancer. It will also provide human-specific data and it will aid in determining breast cancer progression and potential therapeutic intervertion, because it can study steroidogenic interactions that do not occur in rodents, such as the promoter-specific regulation of the enzyme aromatase. Moreover, we utilized this model to obtain information on essential oils for which toxicities/endocrine disrupting effects are for the most part unknown.

In this thesis, we have evaluated the steroidogenic effects of essential oils in cellular co-culture models representing women who are pregnant or have estrogen-dependent breast cancer using a well-established feto-placental co-culture model, a representative cellular model of estrogen-dependent breast cancer (Hs578t cells), and a newly-designed estrogen-dependent breast-cancer co-culture model. These models were physiological-relevant since women are the predominant users of complementary and alternative medicines and very little information was available regarding the potential adverse effects at the level of steroidogenesis. Since the functional building blocks of steroid hormones and the components of essential oils are identical, it was hypothesized that essential oils may mimic endogenous steroids to interfere with steroidogenic activity. To support this hypothesis, levels of hormones in the steroidogenic pathways were analyzed, as well as, gene expression of key steroidogenic enzymes, to determine possible mechanistic action of essential oils on steroidogenic systems. This research could have significant impact by providing new insights into potential risks of the use of common essential oils during pregnancy and by providing a new tool for studying potential therapeutic interventions of estrogen-dependent breast cancer patients.

Chapter 2 : ESSENTIAL OILS DISRUPT STEROIDOGENESIS IN A FETO-PLACENTAL CO-CULTURE MODEL

Debbie Yancu* and Thomas Sanderson*

INRS - Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada

* Corresponding author at:
INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada
Laval, QC H7V 1B7, Canada.
E-mail addresses: debbie.yancu@iaf.inrs.ca (D. Yancu) and thomas.sanderson@iaf.inrs.ca (J.T. Sanderson)

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Author contributions:
Debbie Yancu developed the hypothesis and objectives, designed and performed the experiments, performed the statistical analyses and wrote the manuscript.
Prof. Thomas Sanderson obtained the research funding, supervised the project and participated in writing and revising the manuscript

2.1 Abstract

We determined whether 5 common essential oils (basil, fennel seed, orange, black pepper and sage) interfered with feto-placental steroidogenesis in a co-culture model composed of fetal-like adrenocortical (H295R) and placental trophoblast-like (BeWo) cells. After a 24h exposure, only basil and fennel seed oil significantly increased hormone concentrations of estradiol, estrone, dehydroepiandrosterone (DHEA), androstenedione, progesterone, and estriol. Basil and fennel seed oil were shown to significantly alter the expression of steroidogenic enzymes involved in cholesterol transport and steroid hormone biosynthesis, including *StAR*, *CYP11A1*, *HSD3B1 and -2*, *SULT2A1*, and *HSD17β1*, *-4*, and *-5*. Also, basil and fennel seed oil stimulated placental-specific promoter I.1 and pII-derived *CYP19* mRNA in BeWo and H295R cells, respectively as well as increased CYP19 enzyme activity. Our results indicate that further study is necessary to determine the potential risks of using basil and fennel seed oils during pregnancy considering their potential to disrupt steroidogenic enzyme activity and expression *in vitro.*

Keywords

Feto-placental unit	Essential oils
Steroidogenesis	Co-culture
Aromatase	Estrogen

2.2 Introduction

Essential oils are aromatic liquids isolated from the natural secretions produced by different plant tissues and stored within specialized secretory cells. They are commonly extracted via steam or hydro-distillation from the plant materials. The extraction yields are extremely low, vary depending on climate, soil composition, plant organ, age, and vegetative life cycle, and only 10% of plant species, termed aromatic plants, are able to produce essential oils; (Svoboda & Greenaway, 2003). The biological role of essential oils is to act as chemical signals through which the plant communicates with its environment to ensure proper development (Elshafie & Camele, 2017). These actions include inhibiting proximate growth of other plants and their subsequent germination as well as defensive roles against pathogenic microorganisms, herbivores and insects (Elshafie & Camele, 2017). Due to their biological roles and aromatic nature, essential oils have numerous applications, including use in the food, sanitation, cosmetic,

and perfume industries. Aromatherapy is another major area in which essential oils are used, although its effectiveness is controversial. Aromatherapy is a popular alternative approach to healing that employs essential oils with the goal of improving cognitive or physiologically health. It is not a new concept, with essential oils having been recognized since ancient civilizations for their medicinal qualities, such as their analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic properties (Bakkali et al., 2008; Tillett & Ames, 2010). The controversy lies within the numerous claims made about the effectiveness of aromatherapy in treating various conditions such as depression, indigestion, headache, insomnia, muscular pain, respiratory problems, skin conditions, swollen joints, labor pains, urinary tract disorders etc., most of which have not yet been scientifically validated. However, renewed public and scientific interest in applying natural products and discovering new applications for natural products to counter developing resistance to current synthetic medications is stimulating more research into the therapeutic effectiveness of essential oils and other traditional natural remedies (Herman & Herman, 2015; Yap et al., 2013).

Essentials oils are a complex mixture of volatile odorous compounds of which the majority are considered to be hydrocarbon terpenes and terpenoids characterized by low molecular weight and good bioavailability (El Asbahani et al., 2015). The building blocks of these compounds are isoprene units (2-methyl-1,3-butadiene) which are linked together. Despite the many constituents present within essential oils, sometimes up to 100 in differing concentrations, they are typically categorized by their major components found with the highest concentrations (20-70%) according to analysis using gas chromatography and mass spectrometry (GC/MS) (El Asbahani et al., 2015). It is generally believed that the most abundant compounds within the essential oil are responsible for its biological activity (El Asbahani et al., 2015).

Since women are the predominant consumers of alternative therapies (Chuang et al., 2006), including essential oils, it is imperative to know whether any potential beneficial or detrimental effects be attributed to these products. Pregnant women are at particular risk since proper development of the fetus relies on correct feto-placental steroidogenesis. An understanding of the effects of essentials oils on steroidogenesis is lacking and is therefore the focus of the current study. A recently established feto-placental co-culture system (Thibeault et al., 2014) that enables the *in vitro* study of steroidogenesis during human pregnancy is utilized in

this study to characterize the steroidogenic properties of five common essential oils: basil (Ocimum basilicum), fennel seed (Foeniculum vulgare), sweet orange (Citrus sinensis), black pepper (Piper nigrum), and common sage (Salvia officinalis). Due to the fact that essential oil constituents utilize the isoprene unit as their functional building block and that steroid hormones are derived from polymerization of isoprene units; it was hypothesized in this current study that active compounds present within essential oils may interfere with key enzymes in the feto-placental steroidogenic pathway.

2.3 Materials and methods

2.3.1 Treatments

Basil, fennel seed, sweet orange, common sage and black pepper essential oil were obtained from Rocky Mountain Oils (Orem, UT, USA). Importantly, all essential oils utilized were provided with a GC/MS data report listing all the contained molecules and their respective concentrations within the mixture (see supplementary material). Essential oils were dissolved in DMSO and all concentrations tested were adjusted in culture medium to obtain a maximum final DMSO concentration of 0.1%. Based on information from other studies with different essential oils (Navarra et al., 2015a; Zu et al., 2010); the concentrations used in these studies ranged from 0.00005% (v/v) to 0.5% (v/v). Our initial treatment concentrations were 0.00005, 0.005, 0.005, 0.05 and 0.5% with three replicates in each experiment.

2.3.2 Feto-placental co-culture

A well-established feto-placental co-culture system that enables the *in vitro* study of steroidogenesis during human pregnancy is used in this study with slight modifications (Thibeault et al., 2014). This model consists of two different cell lines in a transwell co-culture system that mimics the steroidogenic cooperation between the placental trophoblast, fetal adrenal zone, and the fetal liver that are essential for maintaining steroid hormone production throughout a healthy pregnancy. The fetal compartment (adrenal zone and liver) is represented by H295R human adrenocortical carcinoma cells (American Type Culture Collection, Manassas, VA, no. CRL-2128) and BeWo human placental choriocarcinoma cells (ATCC no. CCL-98) are used to represent the placental trophoblast compartment.

H295R cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) without phenol red containing 1.2 g/L sodium bicarbonate and 2 mg/L pyridoxine HCl (Gibco, Luzern, Switzerland). Medium was completed with 2.5% NuSerum (VWR International, Radnor, PA), 1% ITS + Premix (Fisher Scientific, Waltham, MA), and 1% penicillin/streptomycin (Gibco). BeWo cells were cultured in DMEM/F12 without phenol red containing 1.2 g/L sodium bicarbonate and 2 mg/L pyridoxine HCl. Medium was completed with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ), and 100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco). For hormone quantification and RNA extraction under monoculture and co-culture conditions, 7.5 x 10^5 H295R cells/2 ml recommended culture medium were seeded in 6-well plates and 3.5 x10^5 BeWo cells/1.5 ml in transwell inserts (polycarbonate membrane with 0.4μM pores, Corning Life Sciences, Corning, NY) in separate 6-well plates. After 24 h, original media was removed and replaced with co-culture medium based on the recommended H295R medium, but supplemented with 1% stripped FBS (Hyclone) and various concentration of essential oils. The co-culture system was then assembled as previously described (Thibeault et al., 2014). All experiments were performed using cells with passage numbers of 8 to 25. Exposures to essential oils were for 24 h in an incubator at 37°C with a humidified atmosphere containing 5% CO_2.

2.3.3 Cell viability

The toxicity of basil, fennel seed, sweet orange, sage, and black pepper essential oils to H295R and BeWo cells was determined using WST-1 cell viability reagent (Roche, Basel, Switzerland) which is based on the cleavage of a tetrazolium salt by the mitochondria in metabolically active cells. Each cell type was seeded separately in 96-well plates at a density of 10^4 cells/well in 200 µl of appropriate culture medium for 24 h. After 24 h, cells were exposed to fresh medium containing increasing concentrations of essential oils for another 24 h. Cells were then incubated for 1.5 h with 20 ul of WST-1 reagent and the formation of formazan was measured using the SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California) at a wavelength of 450 nm.

2.3.4 Hormone quantification

After a 24 h exposure to essential oils, co-culture media was collected. Hormone concentrations were determined by ELISA using assay kits obtained from DRG Diagnostics (Marburg, Germany) according to manufacturer's recommendations; kits included estradiol (EIA-2693), estrone (EIA-4174), progesterone (EIA-1561), androstenedione (EIA-3265), DHEA (EIA-3415) and estriol (EIA-3717).

2.3.5 RT-qPCR

Real-time quantitative PCR (RT-qPCR) was used to assess gene expression of steroidogenic enzymes. After 24 h of cell acclimatization following plating, cells were exposed for 24 h to the treatments. The DMSO vehicle (0.1%) was used as a negative control. For monoculture experiments, H295R cells were removed from the wells and BeWo cells from the inserts by washing the cells with PBS and then detaching them with trypsin. In co-culture experiments, BeWo cells in the inserts were isolated separately from the H295R cells in the wells below. RNeasy mini kits (Qiagen, Mississauga, ON) were used to isolate RNA, which was then stored at -80°C. RNA purity was assessed by determining the 260/280 nm absorbance ratio (~2.0) using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using 0.5 µg RNA with an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) and T3000 Thermocycler (Biometra, Göttingen, Germany); resultant cDNA was stored at -20°C. RT-qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with a CFX96 real-time PCR Detection System (Bio-Rad). Suitable reference genes for normalizing target gene expression were selected using geNorm software (Biogazelle, Zwijnaarde, Belgium) (Table 2.1). All validated primer pair sequences are listed in Table 2.1.

Table 2-1 Primer pair sequences used to determine gene expression of steroidogenic enzymes and reference genes

Steroidogenic Gene	Primer Pairs (5' → 3')	Reference:
CYP19 coding region (exon II-X)	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC Rv: TCACCAATAACAGTCTGGATTTCC	(Sanderson et al., 2000)
CYP19 I.1	Fw: GGATCTTCCAGACGTCGCGA Rv: CATGGCTTCAGGCACGATGC	(Klempan et al., 2011)
CYP19 PII	Fw: TCTGTCCCTTTGATTTCCACAG Rv: GCACGATGCTGGTGATGTTATA	(Heneweer et al., 2004)

CYP19 I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA RV: AGAGGGGGCAAT TTAGAGTCTGTT	(Wang et al., 2008)
CYP11A1	Fw: CTTCTTCGACCCGGAAAATTT Rv: CCGGAAGTAGGTGATGTTCTTGT	(Oskarsson et al., 2006)
CYP17	Fw: AGCCGCACACCAACTATCAG Rv: TCACCGATGCTGGAGTCAAC	(Kim et al., 2016)
HSD17B7	Fw: CTGGAATGGCTCCGGGCTTTG C Rv: CCTGCCCTCGGAGACGGCGTCG	(Shehu et al., 2011)
HSD17B1	Fw: GTCTTCCTCACCGCTTTGCGCGCC Rv: GCACTGCGCCCGGCCTCGTCCTC	(Takagi et al., 2017)
HSD17B4	Fw: TGCGGGATCACGGATGACTC Rv: GCCACCATTCTCCTCACAACTC	(von Krogh et al., 2010)
HSD17B5	Fw: GGGATCTCAACGAGACAAACG Rv: AAAGGACTGGGTCCTCCAAGA	(Xu et al., 2017)
HSD17B3	Fw: AACGCACCGGATGAAATCCAGAGC Rv: GCCTGGCTACCTGACCTTGGTGTT	(Qin & Rosenfield, 2000)
HSD17B2	Fw: CTGAGGAATTGCGAAGAACC Rv: AAGAAGCTCCCCATCAGTTG	(Casey et al., 1994)
SULT2A1	Fw: CCTCCAGCGGTGGCTACA Rv: AATCGTCCGACATGATGATGAC	(Oskarsson et al., 2006)
STAR	Fw: TTGCTTTATGGGCTCAAGAATG Rv: GGAGACCCTCTGAGATTCTGCTT	(Oskarsson et al., 2006)
HSD3B1	Fw: GGAGATCAGGGTCTTGGACA Rv: CAGGCTCTCTTCAGGAATGG	(Hogg et al., 2014)
HSD3B2	Fw: TGCCAGTCTTCATCTACACCAG Rv: TTCCAGAGGCTCTTCTTCGTG	(Kim et al., 2016)
SULT2A1	Fw: TCGTCATAAGGGATGAAGATGTAATAA Rv: TGCATCAGGCAGAGAATCTCA	(Shiraki et al., 2011)
CYP3A7	Fw: CTCTTTAAGAAAGCTGTGCCCC Rv: GGGTGGTGGAGATAGTCCTA	(Kondoh et al., 1999)
Reference gene:	**Primer Pairs (5' → 3')**	**Reference:**
UBC	Fw: ATTTGGGTCGCGGTTCTTG Rv: TGCCTTGACATTCTCGATGGT	(Vandesompele et al., 2002)
TBP	Fw: TGCACAGGAGCCAAGAGTGAA Rv: CACATCACAGCTCCCCACCA	(Tratwal et al., 2014)
RPLO	Fw: GGCGACCTGGAAGTCCAACT Rv: CCATCAGCACCACAGCCTTC	(Good et al., 2016)
RPII	Fw: GCACCACGTCCAATGACAT Rv: GTGCGGCTGCTTCCATAA	(Radonic et al., 2004)
PBGD	Fw: GGCAATGCGGCTGCAA Rv: GGGTACCCACGCGAATCAC	(Dolstra et al., 1999)

2.3.6 Aromatase catalytic activity

Cellular CYP19 activity was assessed using a tritiated water-release assay as previously described (Caron-Beaudoin et al., 2016; J. T. Sanderson et al., 2000; Thibeault et al., 2014). The amount of tritiated water released, as a measure of aromatase activity, was determined using a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Negative controls contained radioactive substrate but no cells. A positive control radioactive substrate was directly added to scintillation cocktail. Aromatase activity was expressed as a percent of control activity (DMSO). Formestane (4-hydroxyandrostenedione) (1 μM), a selective aromatase inhibitor, was used to verify the specificity of the assay for the enzymatic aromatization reaction.

2.3.7 Statistical analysis

All data were analyzed using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA). Results are presented as means with standard error (SEM) of three different experiments; per experiment each treatment was tested in triplicate. Statistically significant differences from control (*$p<0.05$; **$p<0.01$; ***$p<0.001$) were determined using one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test.

2.4 Results

2.4.1 The effect of essential oils on cell viability of H295R and BeWo cells

Basil, fennel seed, orange, sage, and black pepper essential oils were administered to H295R and BeWo cells for 24 h at concentrations ranging from 0.00005% to 0.5%. All essential oils resulted in a significant reduction in cell viability of 30% in H295R cells at a concentration of 0.05% as compared to cells receiving the vehicle control (DMSO) (Fig. 2-1A). Cell viability was further reduced in H295R cells at a concentration of 0.5% with basil essential leading the most toxic response and reducing viability by almost 80% (22.7 ± 0.6%) (Fig. 2-1A). Fennel seed, orange, sage, and black pepper essential oil significantly reduced cell viability by an average of 40% in H295R cells (63.2 ± 4.0%, 60.0 ± 2.1%, 49.0 ± 2.0%, and 71.3 ± 1.7%, respectively) (Fig. 2-1A). There was no change in the viability of H295R cells between the concentrations of 0.00005% to 0.005% basil, fennel seed, and orange essential oils, 0.00005% to 0.0005% black pepper essential oil, and 0.00005% sage essential oil (Fig. 2-1A).

In BeWo cells, all essential oils tested significantly reduced viability by an average of 30% at a concentration of 0.5% (Fig. 2-1B). A 0.05% concentration of basil and fennel seed essential oil did not affect viability of BeWo cells, but orange, black pepper and sage significantly reduced cell viability by an average of 20% (79.0 ± 2.2%, 72.8 ± 0.8%, and 86.6 ± 2.2%, respectively) (Fig 2-1B). A 0.005% concentration of black pepper and sage essential oil also proved toxic to BeWo cells, while no effect was detected for basil, fennel seed, and orange essential oil concentrations ranging from 0.0005% to 0.005% (Fig. 2-1B).

The observed viabilities for H295R and BeWo cells resulted in a concentration range of 0.00005% to 0.005% basil, fennel seed, and orange essential oil, 0.00005% to 0.0005% black pepper oil, and 0.00005% sage oil being chosen for use in following experiments.

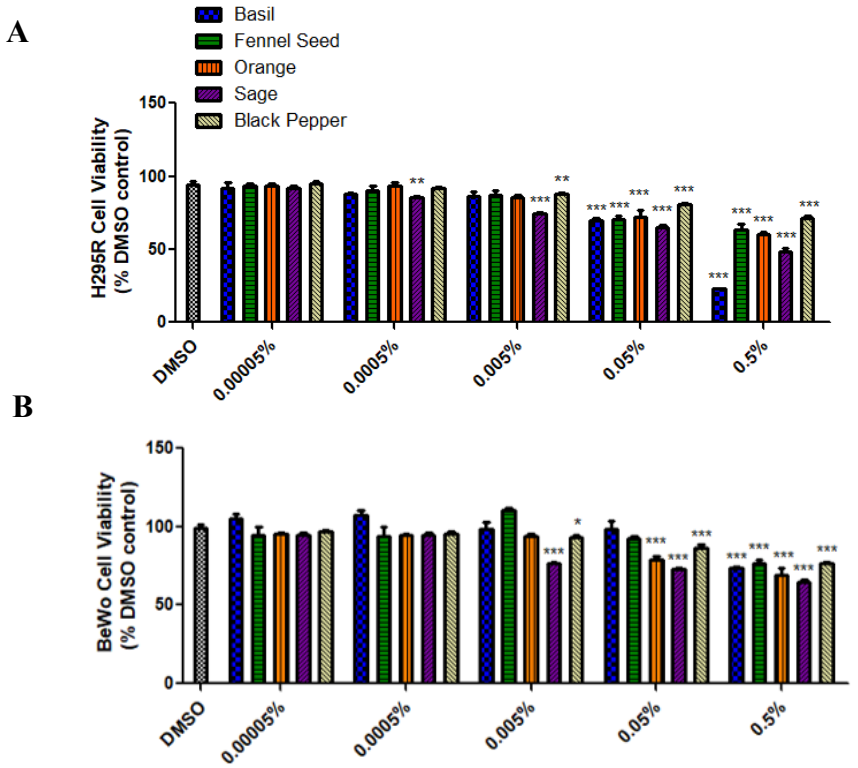


Figure 2-1: *Viability of H295R (A) and BeWo (B) cells exposed for 24 h to various concentrations of basil, fennel seed, orange, sage or black pepper essential oil as a percentage of DMSO.*

*Results are presented as the mean ± SEM (n=3). Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.*

2.4.2 The effects of essential oils on hormone production in the feto-placental co-culture system

Basil essential oil significantly increased DHEA levels in the co-culture (Fig. 2-2A) at concentrations of 0.0005% (3.8 ± 0.1 ng/ml), 0.001% (4.6 ± 0.1 ng/ml) and 0.005% (3.9 ± 0.3 ng/ml) compared to DMSO control (1.0 ± 0.1 ng/ml). Androstenedione levels were also significantly increased in co-culture by basil oil (Fig. 2-2B) at concentrations of 0.0005% (6.9 ± 0.3 ng/ml), 0.001% (5.5 ± 0.6 ng/ml) and 0.005% (5.4 ± 0.5 ng/ml) compared to vehicle control (1.7 ± 0.2 ng/ml). Basil essential oil significantly increased progesterone levels in co-culture (fig.

2-2C) at concentrations of 0.0005% (32.4 ± 2.4 ng/ml) and 0.0001% (28.8 ± 1.6 ng/ml) compared to DMSO control (3.6 ± 0.5 ng/ml). Estradiol and estrone levels were also significantly increased by basil oil in co-culture at concentrations of 0.0005%, 0.001% and 0.005% (estradiol: 737.7 ± 14.1 pg/ml, 373.2 ± 1.9 pg/ml and 361.5 ± 39.6 pg/ml; estrone: 201.0 ± 24.8 pg/ml, 166.8 ± 5.7 pg/ml, and 146.5 ± 12.1 pg/ml, respectively) compared to DMSO control (estradiol: 159.4 ± 8.7 pg/ml; estrone: 132.8 ± 7.6 pg/ml) (Fig. 2-2D,E). Basil oil significantly increased estriol levels compared to vehicle control (3.5 ± 0.6 ng/ml) in a concentration-dependent manner at 0.0005% (6.6 ± 0.3 ng/ml), 0.001% (8.8 ± 1.8 ng/ml) and 0.005% (9.0 ± 1.0 ng/ml) (Fig. 2-2F).

Exposure to fennel seed essential oil resulted in statistically significant increases of DHEA levels in co-culture at concentrations of 0.0005% (7.8 ± 0.6 ng/ml), 0.001% (6.7 ± 0.1 ng/ml) and 0.005% (6.5 ± 1.0 ng/ml) compared to control (1.0 ± 0.1 ng/ml) (Fig.2-3A). Androstenedione levels also significantly rose in co-culture in response to fennel seed oil at concentrations of 0.0005% (6.3 ± 0.2 ng/ml), 0.001% (6.1 ± 0.1 ng/ml) and 0.005% (4.9 ± 0.3 ng/ml) compared to DMSO control (1.7 ± 0.2 ng/ml) (Fig. 2-3B). Only fennel seed concentrations of 0.0005% and 0.001% significantly increased progesterone levels in co-culture (21.2 ± 1.0 ng/ml and 21.2 ± 1.4 ng/ml, respectively) compared to control levels (3.6 ± 0.5 ng/ml) (Fig. 2-3C). Estradiol and estrone levels were significantly increased in co-culture by fennel seed oil concentrations of 0.0005%, 0.001% and 0.005% (estradiol: 586.7 ± 28.1 pg/ml, 384.4 ± 4.1 pg/ml and 342.1 ± 16.0 pg/ml; estrone: 171.7 ± 1.8 pg/ml, 165.0 ± 7.0 pg/ml, and 158.2 ± 4.6 pg/ml, respectively) compared to DMSO control (estradiol: 159.4 ± 8.7 pg/ml; estrone: 132.8 ± 7.6 pg/ml) (Fig. 2-3D,E). Fennel seed oil significantly increased estriol levels in co-culture at concentrations of 0.001% and 0.005% (8.5 ± 1.1 ng/ml and 7.0 ± 0.9 ng/ml) compared to control (3.5 ± 0.6 ng/ml) (Fig. 2-3F).

No significant changes in hormone concentrations were detected after treatments with orange, black pepper or sage essential oil as well as with basil and fennel seed oil concentrations of 0.00005% and 0.0001% (data not shown); for this reason, experiments continued only with basil and fennel seed essential oil with concentration ranging from 0.0005% to 0.005%.

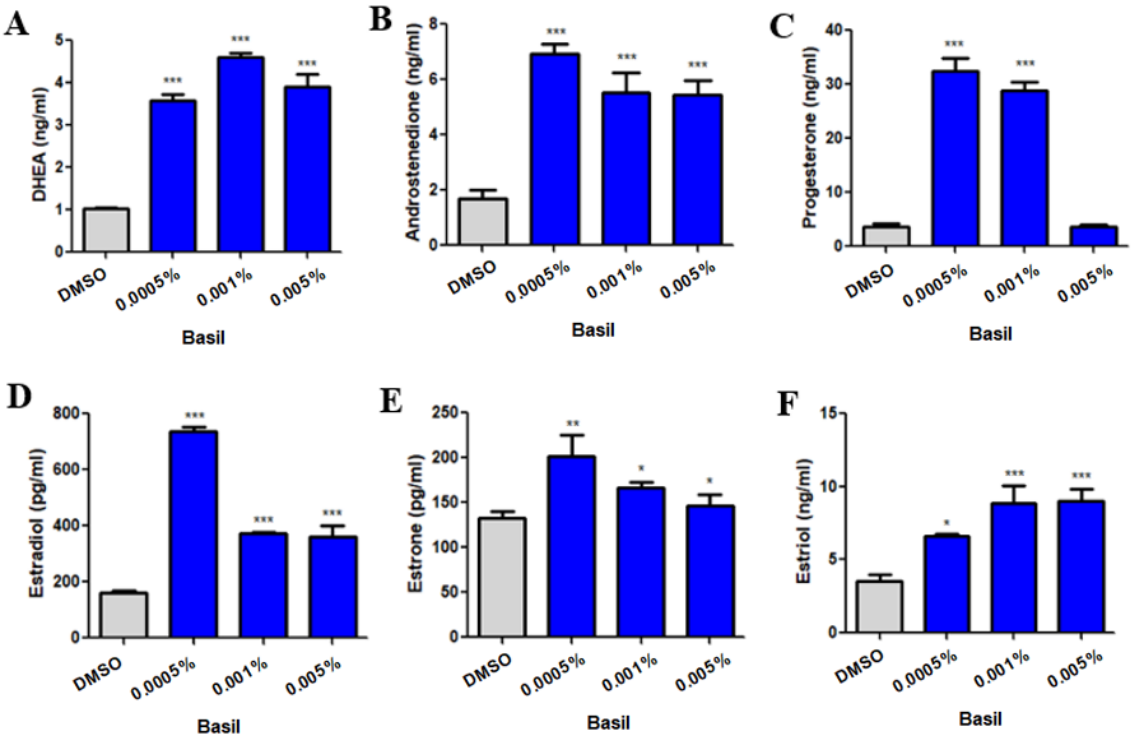


Figure 2-2: *DHEA (A), androstenedione (B), progesterone (C), estradiol (D), estrone (E) and estriol (F) production by the feto-placental (H295R/BeWo) co-culture after a 24-h treatment with basil essential oil.*

*Results are presented as the mean ± SEM (n=3). Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.*

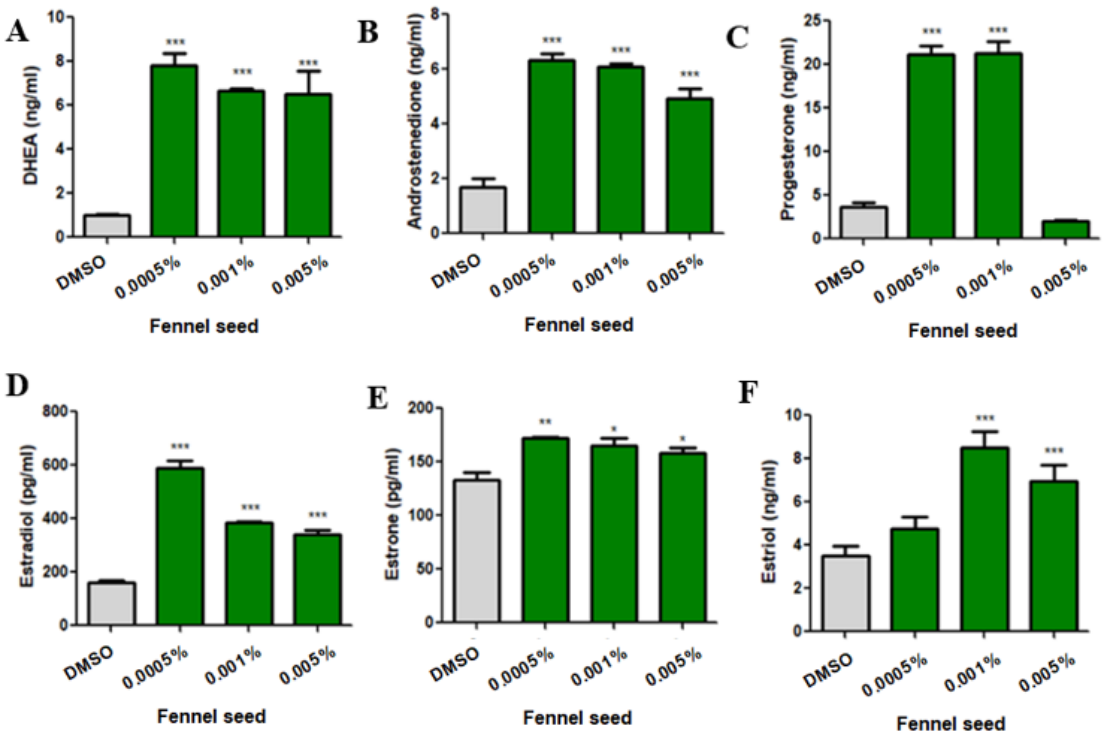


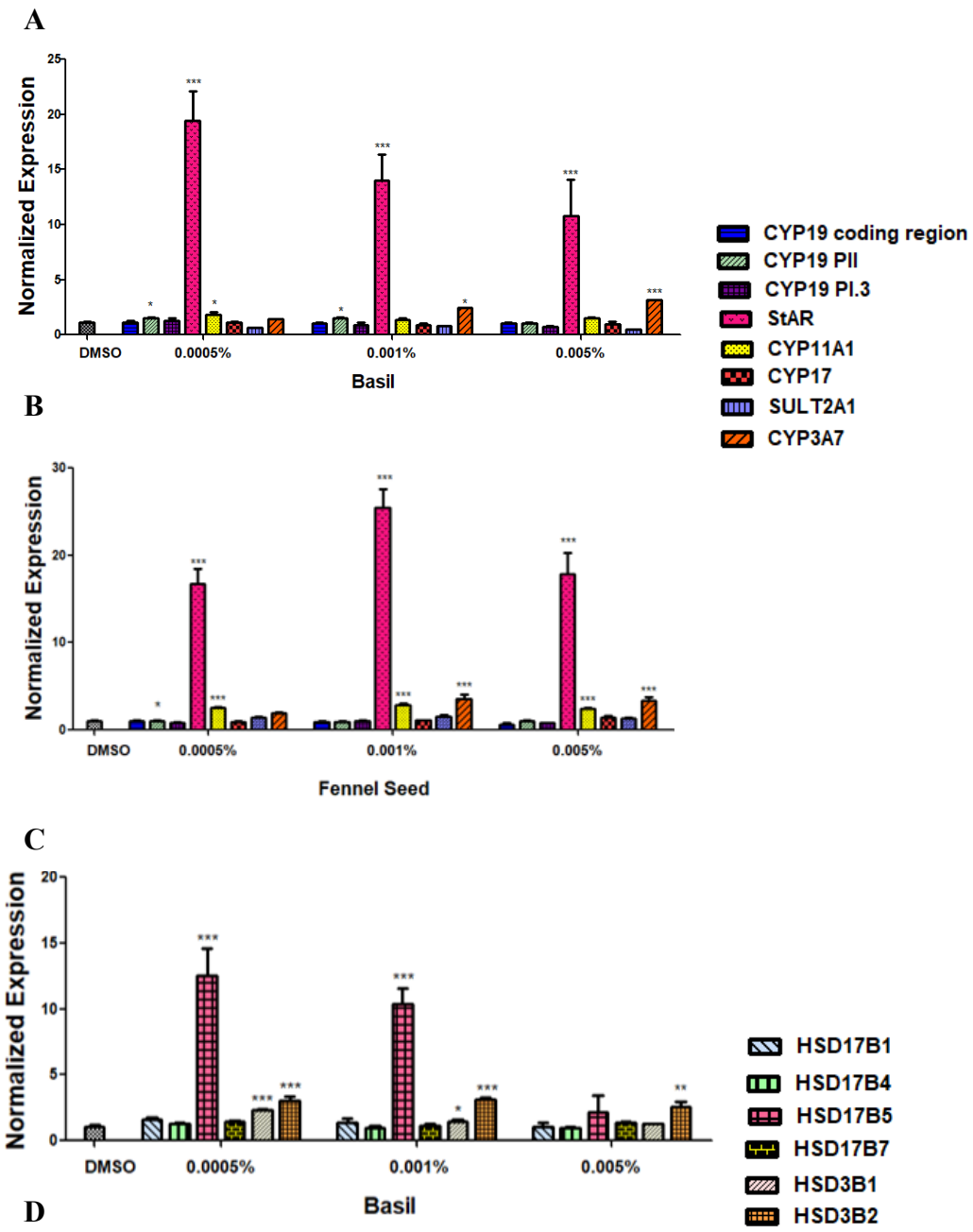
Figure 2-3: *DHEA (A), androstenedione (B), progesterone (C), estradiol (D), estrone (E) and estriol (F) production by the feto-placental (H295R/BeWo) co-culture after a 24-h treatment with fennel seed essential oil.*

*Results are presented as the mean ± SEM (n=3). Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.*

2.4.3 The effects of basil and fennel seed essential oils on the expression of steroidogenic enzymes in H295R cells in monoculture vs. co-culture conditions

In H295R cells in monoculture, *StAR* gene expression was not changed after treatment with basil or fennel seed essential oil, but its expression was significantly increased in co-culture with BeWo cells at all concentrations evaluated (Fig. 2-4E,F). We also detected significant increases in *CYP3A7* gene expression after exposure to 0.0001% and 0.005% basil or fennel seed essential oil in co-culture (fig 2-4A,B). In the co-culture, *CYP11A1* gene expression was increased significantly at 0.0005% basil oil and at all concentrations studied of fennel seed oil (Fig. 2-4A,B). Focusing on *CYP19*, we detected significant stimulation of promoter-specific *CYP19 PII* expression by both basil and fennel seed essential oil, with the lowest concentrations proving most effective. In monoculture, we detected stimulation of promoter I.3-mediated gene

expression, an effect not observed in co-culture in response to either essential oil (Fig. 2-4E,F). In regards to hydroxysteroid dehydrogenase enzymes (HSD), we detected significant increases in *HSD17B5* gene expression under co-culture conditions in response to basil and fennel seed oil, while no change was noted under monoculture conditions (Fig. 2-4E,F). Gene expression of *3β-HSD2* also rose significantly in response to both basil and fennel seed oil treatment (Fig. 2-4C,D). For gene expression results that were similar between H295R cells in monoculture and co-culture, only the latter are shown.


A
Normalized Expression
25
20
15
10
5
0
DMSO
0.0005%
0.001%
0.005%
Basil
CYP19 coding region
CYP19 PII
CYP19 PI.3
StAR
CYP11A1
CYP17
SULT2A1
CYP3A7
B
Normalized Expression
30
20
10
0
DMSO
0.0005%
0.001%
0.005%
Fennel Seed
C
Normalized Expression
20
15
10
5
0
DMSO
0.0005%
0.001%
0.005%
Basil
HSD17B1
HSD17B4
HSD17B5
HSD17B7
HSD3B1
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Normalized Expression
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Fennel Seed

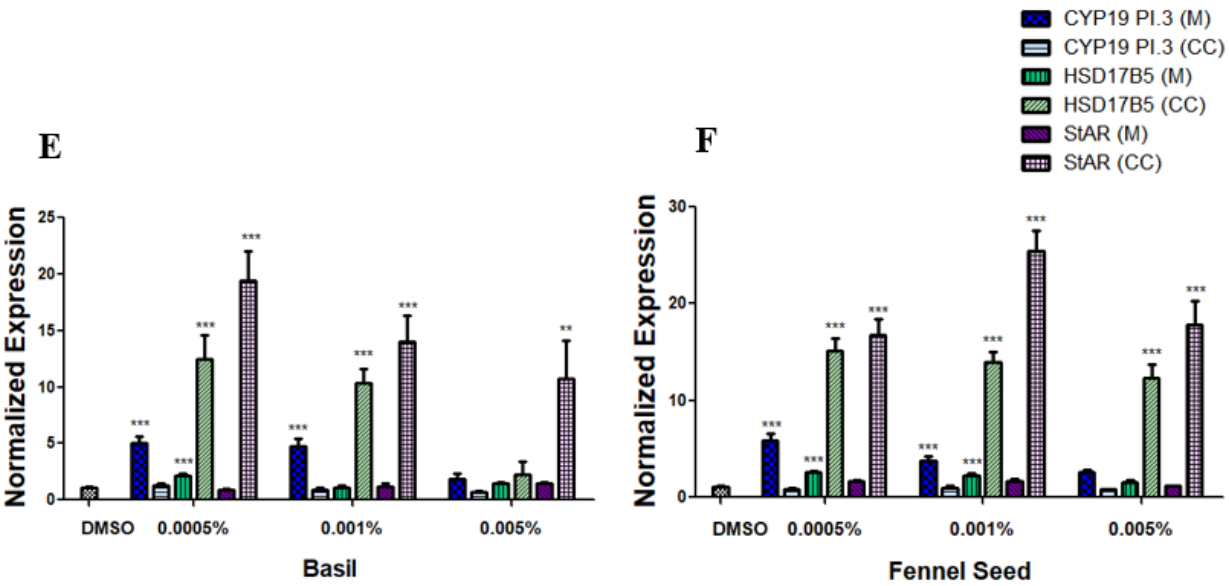
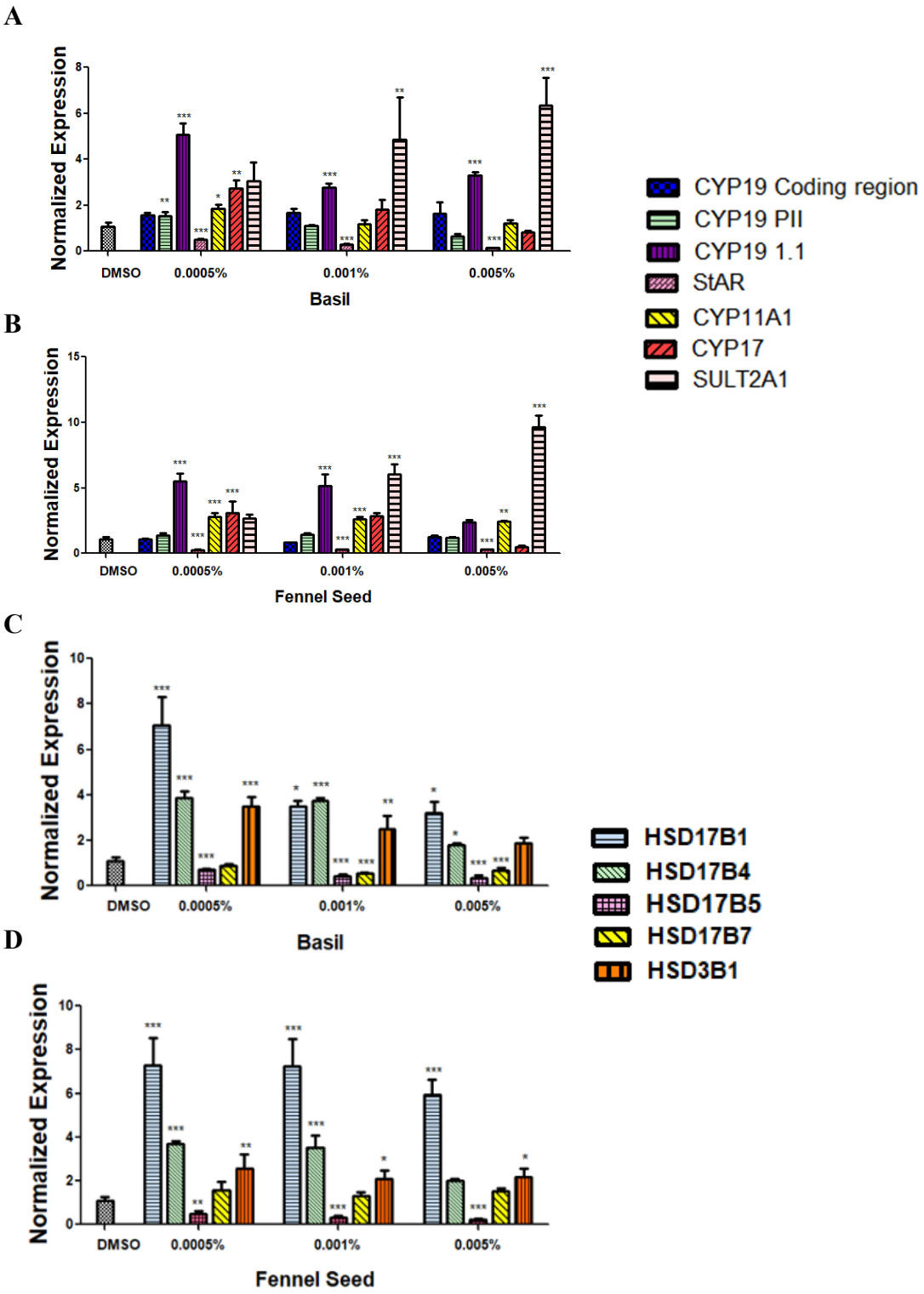


Figure 2-4: *The relative expression of genes involved in feto-placental steroidogenesis in **H295R** cells in co-culture after a 24-h treatment with basil and fennel seed essential oils.*

*CYP450 enzymes, sulfotransferase SULT2A and StAR (A,B) and HSD enzymes (C,D). Significant differences in gene expression between H295R cells in monoculture vs. co-culture after exposure to basil (E) or fennel seed essential oil (F). Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.*
***Note**: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

2.4.4 The effects of basil and fennel seed essential oils on the expression of steroidogenic enzymes in BeWo cells in monoculture vs. co-culture conditions

In BeWo cells exposed to basil or fennel seed essential oil under co-culture conditions, there was stimulation of promoter I.1-mediated *CYP19* gene expression (Fig 2-5A,B). We also detected a concentation-dependent increase in the sulfotransferase *SULT2A1* gene expression in co-culture, while no significant change was noted in monoculture (Fig. 2-5A,B; 2-5E,F). Basil and fennel seed oil also significantly increased expression of *CYP11A1* and *CYP17*, especially at the lowest concentrations studied (Fig. 2-5A,B). *StAR* gene expression rose significantly in BeWo cells in monoculture while significant decreases were noted in co-culture (Fig. 2-5A,B; 2-5E,F). *HSD17β1*, *HSD17β4*, and *HSD3B1* gene expression was significantly increased, whereas *HSD17β5* expression was significantly decreased (Fig. 2-5C,D). For gene expression results that were similar between BeWo cells in monoculture and co-culture, only the latter are shown.


A
Normalized Expression
DMSO
0.0005%
0.001%
0.005%
Basil
CYP19 Coding region
CYP19 PII
CYP19 1.1
StAR
CYP11A1
CYP17
SULT2A1
B
Normalized Expression
DMSO
0.0005%
0.001%
0.005%
Fennel Seed
C
Normalized Expression
DMSO
0.0005%
0.001%
0.005%
Basil
HSD17B1
HSD17B4
HSD17B5
HSD17B7
HSD3B1
D
Normalized Expression
DMSO
0.0005%
0.001%
0.005%
Fennel Seed

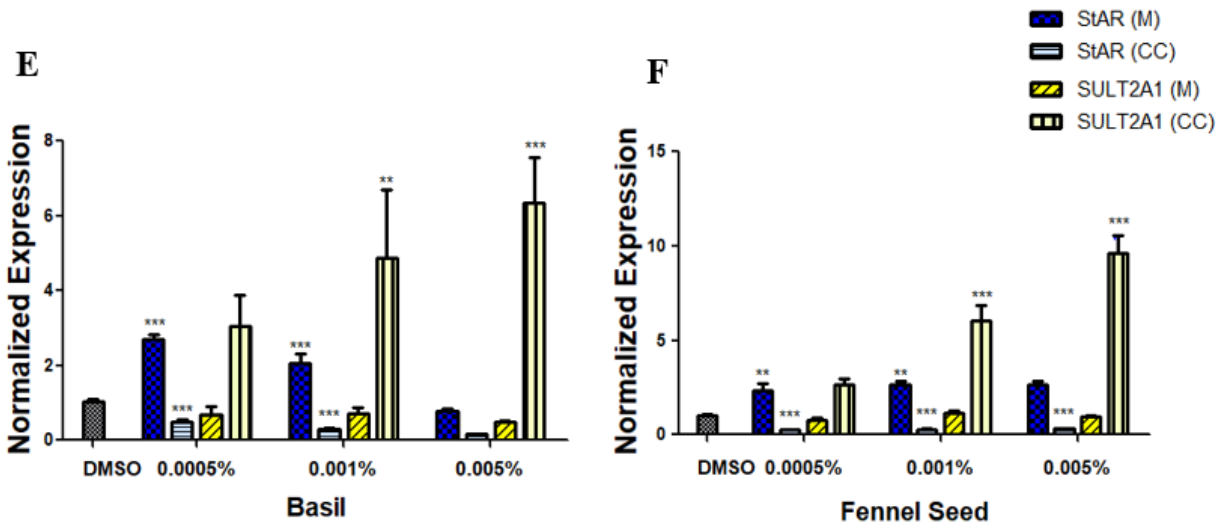


Figure 2-5*: The relative expression of genes involved in feto-placental steroidogenesis in* ***BeWo*** *cells in co-culture after a 24 h treatment with basil or fennel seed essential oils.*

*CYP450 enzymes, sulfotransferase SULT2A1 and StAR (A,B), and HSD enzymes (C,D). Significant differences in gene expression between BeWo cells in monoculture vs. co-culture after exposure to basil (E) or fennel seed essential oil (F). Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.* ***Note****: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

2.4.5 The effects of basil and fennel seed essential oils on aromatase catalytic activity of H295R and BeWo cells in co-culture conditions

Basil and fennel seed essential oil treatment significantly increased CYP19 activity in H295R cells in co-culture at concentrations of 0.0005% and 0.001% (Fig. 2-6A,B). In BeWo cells in co-culture, CYP19 activity was significantly induced by both basil and fennel seed essential oil at concentrations of 0.0005% and 0.001% (Fig. 2-6A,B).

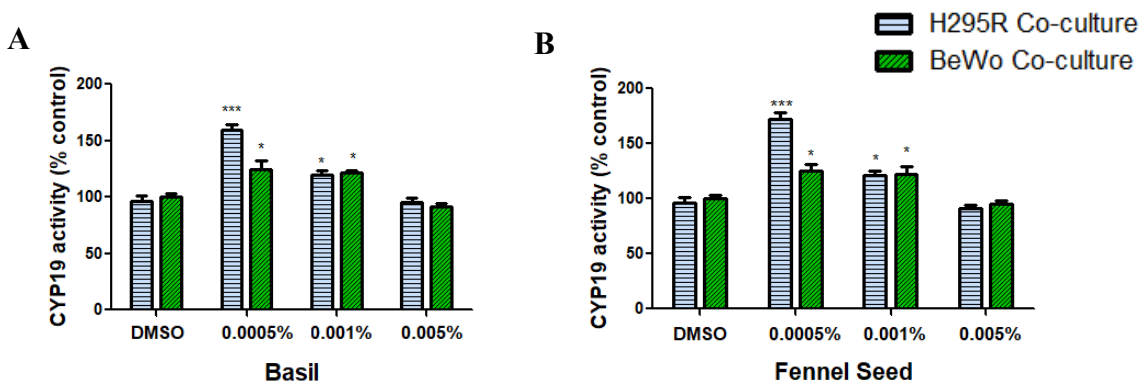


Figure 2-6: *Relative CYP19 activity in H295R and BeWo cells in co-culture after a 24 h exposure to basil (A) or fennel seed (B) essential oils and expressed as a percentage of DMSO.*

*Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *p < 0.05; **p < 0.01; ***p < 0.001.*

2.5 Discussion

In feto-placental steroidogenesis, communication among several areas is required. The finely tuned cooperation between the placental trophoblast, fetal adrenal zone and the fetal liver is essential for maintaining appropriate steroid hormone production throughout pregnancy (Fig. 7). In this study, we used a recently established co-culture system (Thibeault et al., 2014) that combines BeWo cells with H295R cells to mimic *in vitro* the feto-placental steroidogenic activity that occurs *in vivo*. Using this feto-placental co-culture model, we determined whether commonly used essential oils impact the carefully tuned steroidogenic interactions that occur during pregnancy, to better understand the potential risks posed to mother and unborn child. Studies have shown that certain essential oils can alleviate pregnancy symptoms, such as nausea and vomiting (Joulaeerad et al., 2018), stress (Igarashi, 2013) and lower back pain (Shirazi et al., 2017), among other conditions. Recommended human doses for essential oils vary between 1-5% depending on the absorption method according to pharmacopeias (Tisserand & Balacs, 2014). However, it is currently unknown what concentration actually reaches circulation and the targeted tissue due to the various application forms available for its use. In this study, concentrations chosen for evaluation were scaled back 10 to 100-fold from cytotoxic ranges of different essential oils studied in lung, prostate, and breast cancer cell lines (Zu et al., 2010).

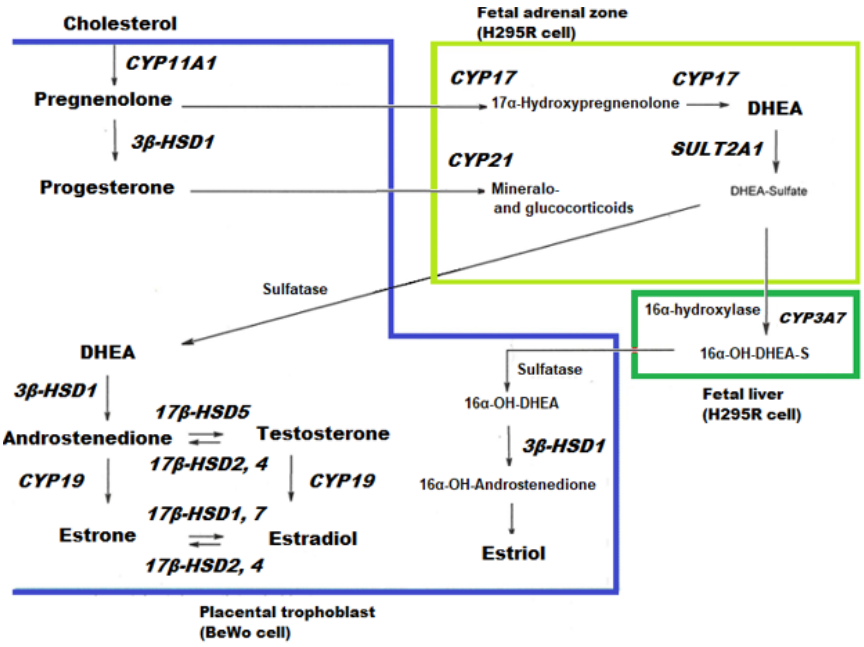


Figure 2-7: *Feto-placental steroidogenesis in the co-culture model using H295R and BeWo cells*

Image adapted from: Sanderson (2009)

2.5.1 Possible role of progesterone in StAR gene expression: monoculture vs co-culture

A critical step in steroidogenesis is the movement of cholesterol, derived either from low density lipoproteins (LDLs) in the endosomes or from *de novo* synthesis, to the mitochondria. Once free cholesterol enters the mitochondria, steroidogenic acute regulatory protein (StAR), a transport protein, transfers cholesterol to the inner membrane, which contains the enzyme CYP11A1. In this rate-limiting step of steroidogenesis (Oskarsson et al., 2006), CYP11A1 cleaves the side-chain of cholesterol to form pregnenolone, the precursor for all steroid enzymes (Miller & Auchus, 2011; Oskarsson et al., 2006). In H295R cells in monoculture, neither basil nor fennel seed essential oil had a significant effect on *StAR* mRNA levels. However, a significant increase in *StAR* expression by both essential oils was observed in H295R cells isolated from co-culture. Upregulation of *StAR* has been associated with increased progesterone production in mouse ovarian granulosa cells, and in mouse leydig cells progesterone has been shown to regulate *StAR* via the progesterone receptor (PR) (Dou et al., 2016; Schwarzenbach et al., 2003). Taken together, this suggests a regulatory role for progesterone in StAR expression in steroidogenic cell lines via PRs. Given that H295R cells possess PRs (de Cremoux et al., 2008) and we detected a rise in progesterone levels in response to basil and fennel seed oil in co-

culture, we suggest that basil and fennel seed oil upregulate *StAR* gene expression via increased PR activation by progesterone. This effect is unique to the the feto-placental co-culture as H295R cells in monoculture produce low amounts of progesterone because this hormone is rapidly converted to weak androgens, glucocorticoids and mineralocorticoids. In contrast, we observed significant decreases in *StAR* gene expression in BeWo cells exposed to basil and fennel seed oil in co-culture, yet significant increases in monoculture. Interestingly, Sugawara and coworkers initially reported that the *StAR* gene is not expressed in the placenta and BeWo cells, due to lack of promoter activity (Sugawara et al., 1997). Our results contradict this. However, it is currently believed that in BeWo cells and placental cells in general, a StAR-like protein MLN64 is expressed and possesses homologous C-terminal domain (Tuckey et al., 2004; Watari et al., 1997). Studies on intact human placental mitochondria have shown that MLN64 not only participates in placental steroidogenesis, but also increases progesterone synthesis (Uribe et al., 2003; Zhang et al., 2002). In our study, progesterone levels rose considerably in co-culture, but more research is needed to determine whether it is triggered by the combined role played by StAR in H295R cells and MLN64 in BeWo cells, especially considering the fact that progesterone levels returned to basal levels at a concentration of 0.005%, without associated changes to *StAR* expression. Moreover, StAR expression in BeWo cells being not fully understood is another area being explored in our future work.

2.5.2 Role of CYP17 and 3β-HSD enzymes in the feto-placental co-culture

CYP17 is responsible for the 17α-hydroxylase and 17,20-lyase activity that converts pregnenolone to dehydroepiandrosterone (DHEA), considered an endogenous precursor to more potent steroidogenic hormones, while 3β-HSD1, or its isoform 3β-HSD2, converts pregnenolone to progesterone. 3β-HSD1/2 also converts DHEA into androstenedione, considered a substrate for steroidogenesis of both male and female sex hormones (Miller & Auchus, 2011). In our study, concentrations between 0.0005%-0.005% basil and fennel seed essential oil significantly increased DHEA levels produced by H295R cells in monoculture (data not shown) as well as in the feto-placental co-culture. Exposure to basil or fennel seed oil induced *CYP17* mRNA expression in BeWo cells in monoculture and in co-culture, although levels remain very low. It is important to note that despite the increased gene expression, CYP17 protein is not produced in BeWo cells (Uhlen et al., 2015), which renders BeWo cells incapable of de novo synthesis of

androgens and estrogens. A profile of the transcript and protein levels of steroidogenic enzymes has also reported limited to no presence of CYP17 protein in the placenta (Pezzi et al., 2003). Consistent with this, we did not detect DHEA or androstenedione production by BeWo cells in monoculture exposed to either basil or fennel seed essential oil (data not shown)

Regulation of human 3β-HSD enzymes is complex, involving multiple factors and receptors; even dietary compounds have been shown to influence their expression in various organs (Rasmussen et al., 2013). Both *3β-HSD1* and *3β-HSD2* are expressed in H295R cells (Huang et al., 2011; Xu et al., 2006), whereas *3β-HSD1* is the only isoform expressed in the placenta (Simard et al., 2005). Upon exposure of the co-culture to basil and fennel seed oils, we detected a significant rise in *3β-HSD1* and *3β-HSD2* expression in BeWo and H295R cells, respectively. Although it is still uncertain how 3β-HSD enzymes are regulated, the expression of *3β-HSD1/2* is upregulated by cAMP in fetal rat ovaries (Picon et al., 1988). With reports that the major compounds found in basil and fennel seed essential oils, estragole and trans-anethole, respectively, are able to significantly increase intracellular cAMP levels in rat smooth muscle cells and in human embryonic kidney cells (Enan, 2005; Henrique Bezerra Cabral et al., 2014), it is possible that the observed increase in *3β-HSD1/2* is mediated by increased cAMP levels in our fetal-placental model. Future research will determine the effects of the essential oils or their active compounds on cAMP levels and downstream events in our feto-placental co-culture system

2.5.3 The effect of essential oils on HSD17B expression and estrogen levels

Among other substrates, 3β-HSD1/2 is responsible for the conversion of DHEA to androstenedione. Androstenedione is further converted by 17β-HSD enzymes and CYP19, which together are the key catalysts in forming active sex steroids, such as testosterone and estradiol. Twelve human 17β-HSD enzymes have been characterized to date, and they have oxidative or reductive capabilities, thereby playing a role in activating or inactivating sex steroid hormones (Luu-The, 2001). In the present study, we evaluated changes in gene expression of 17β-HSD enzymes relevant to our feto-placental co-culture, namely *HSD17B1*, *HSD17B2*, *HSD17B3*, *HSD17B4*, *HSD17B5*, and *HSD17B7*. 17β-HSD3 and 17β-HSD5 preferentially use androstenedione as a substrate for testosterone biosynthesis. *HSD17B5* is widely expressed in

steroidogenic and non-steroidogenic tissues (Qin et al., 2006), whereas *HSD17β3* is expressed mainly in the testis, where it is essential for proper male sexual differentiation and development (Mendonca et al., 1999). Consistent with this, we did not detect any *HSD17B3* mRNA in BeWo or H295R cells either in monoculture or co-culture. On the other hand, basil oil increased and fennel seed oil decreased *HSD17B5* mRNA expression in H295R and BeWo cells in co-culture. In our study, testosterone was not detected in the feto-placental co-culture and we suggest that an increase in *HSD17B5* expression is not biologically significant given that are system favored estrogen production.

In our study, basil and fennel seed essential oils significantly increased levels of estrone and estradiol in the co-culture system. Estradiol is formed by the reduction of estrone (generated by aromatization of androstenedione) which is catalyzed by 17β-HSD1, and to a lesser extent 17β-HSD7. 17β-HSD1 is highly expressed in placenta and BeWo cells (Luu-The, 2001; Miettinen et al., 1996), and in H295R cells *17HSDB1* and *17HSDB4* are expressed at about equal levels (Gracia et al., 2006; Hilscherova et al., 2004; Kim et al., 2016; von Krogh et al., 2010). In BeWo cells, we detected significant increases in *HSD17B1* and *HSD17B7* expression in response to basil and fennel seed oil. 17β-HSD1 has been shown to be overexpressed in estrogen-dependent breast cancer and to contribute to breast cancer cell growth and tumor progression (Aka et al., 2012; Ayan et al., 2012). The ability of basil and fennel seed essential oils to stimulate *HSD17B1* expression and increase estradiol levels *in vitro* raises concerns for its use not only by pregnant women, but in women suffering or predisposed to hormone-dependent breast cancer. More work is needed to determine the *in vivo* implications of our findings.

17β-HSD4 is an oxidative enzyme that catalyzes the conversion of estradiol to estrone (Kim et al., 2016), and it also plays a role in converting testosterone to androstenedione (Poirier, 2010). 17β-HSD2 preferentially converts estradiol to estrone, but also testosterone to androstenedione. *HSD17B2* mRNA has not been found in adrenal tissues (Luu-The, 2001), which supports our lack of detection in H295R cells. Although BeWo cells have been shown to possess slight *HSD17B2* expression, it is not found at the protein level (Miettinen et al., 1996). In our study, we did not detect any *HSD17B2* mRNA in BeWo cells in co-culture, but we did see

significant rises in *HSD17B4* which can explain the elevated levels of estrone (and lack of detectable testosterone) we detected in our co-culture in response to basil and fennel seed oil.

2.5.4 Promoter-specific expression of aromatase

CYP19 is responsible for the conversion of androstenedione to estrone, and testosterone to estradiol. Alternate promoter regions, regulated in a tissue-specific manner, are responsible for the expression of the *CYP19* gene (Simpson et al., 2002). In this study, we looked at the effects that essential oils have on the activity of promoters known to be activated (PII, I.3 and I.7) or inactivated (I.4) in estrogen-dependent breast cancer, those that play a role in feto-placental steroidogenesis (I.1 and PII), and expression changes related to the promoter-indistinct *CYP19* coding region. In H295R cells, consistent with previous reports (Caron-Beaudoin et al., 2016; Watanabe & Nakajin, 2004), we were not able to detect *CYP19* transcript derived from the placenta-specific promoter I.1, nor I.4 and I.7, which are typically localized to adipose (I.4), stromal (I.4), and endothelial (I.7) tissues. At the lowest concentrations, basil and fennel seed oil significantly increased PII-promoter derived *CYP19* expression in H295R cells as well as I.1 promoter-derived *CYP19* expression in BeWo cells. An increase in mRNA levels is not always associated with an increase in enzymatic activity, so we confirmed that basil and fennel seed oil also significantly increased CYP19 catalytic activity in each cell type in the co-culture model. Important to note, basil and fennel seed essential oil produce a non-monotonic effect on *CYP19* expression and catalytic activity. It has been shown that endocrine-disrupting compounds in low doses can influence the response of an organ/system to endogenous hormones or even work synergistically with other chemicals and natural hormones to create an additive response (Vandenberg et al., 2012). Moreover, it has been recently reported that neonicotinoids thiaclopid and thiamethoxam also possesses this non-monotonic effect in H295R cells (Caron-Beaudoin et al., 2016).

2.5.5 Estriol production in the feto-placental co-culture

During pregnancy, estriol is a unique estrogen that is produced by the placenta from a fetal androgen precursor, 16α-DHEA-sulfate, which is produced by the fetal liver (Gerhard et al., 1986; Levitz & Young, 1977). Fetal hepatic 16α-hydroxylation is mediated by the cytochrome

P450 enzyme CYP3A7. The produced 16α-DHEA-sulfate enters the placenta where it is deconjugated by sulfatases, then converted by 3β-HSD1 and CYP19 to produce estriol, which directly enters maternal circulation (Falah et al., 2015). Estriol is a marker of fetal well-being (Shenhav et al., 2003) as it is critical for fetal development by regulating utero-placental blood flow and placental vascularization (Falah et al., 2015) among numerous less well-understood functions. H295R and BeWo cells produce low amounts of estriol in monoculture, whereas in co-culture physiologically relevant levels are produced (Thibeault et al., 2014). This is consistent with our findings that significant amounts of estriol are only produced when H295R and BeWo cells are placed in co-culture. In this study, basil and fennel seed essential oil significantly increased estriol levels in the feto-placental co-culture model. This effect is in contrast to majority of the non-monotonic effects that basil and fennel seed essential oil had on hormone production levels of DHEA, androstenedione, progesterone, estrone, and estradiol. Presumably the dose-dependent increase in estriol levels in co-culture is associated to the dose-dependent increase of *CYP3A7* detected in H295R cells in co-culture. Both H295R (Oskarsson et al., 2006) and BeWo cells have previously been found to express *SULT2A1* mRNA, although there are conflicting reports as to whether BeWo cells express the SULT2A1 protein (Mitra & Audus, 2009; Stanley et al., 2001). It is believed that *SULT2A1* mRNA levels correlate with that of its isoform SULT2B1b, which is expressed at the protein level and considered to be predominantly responsible for DHEA sulfation activity in BeWo cells (Mitra & Audus, 2009).

2.6 Conclusion

We determined whether 5 common essential oils (basil, fennel seed, orange, black pepper and sage) interfered with feto-placental steroidogenesis in a co-culture model composed of fetal-like adrenocortical (H295R) and trophoblast-like (BeWo) cells. After a 24-h exposure of the co-culture to essential oil concentrations ranging from 0.0005% to 0.005%, only basil and fennel seed oil significantly increased hormone concentrations and expression of steroidogenic genes involved in cholesterol transport and steroid hormone biosynthesis. We also detected significant changes to the promoter-specific expression of the enzyme aromatase and its catalytic activity. Our results indicate that further study is necessary to determine any potential risks of using basil and fennel seed essential oil during pregnancy given their potential to disrupt steroidogenic enzyme activity and gene expression *in vitro.* At present, investigations are aimed at determining

whether the major chemical constituents of basil and fennel seed essential oil, estragole and trans-anethole, respectively, are mediating the observed effects on steroidogenesis and to further delineate the molecular mechanisms involved.

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2.8 Conflicts of interest

The authors declare to have no conflicts of interest.

2.9 References

Aka, J. A., Zerradi, M., Houle, F., Huot, J., & Lin, S.-X. (2012). 17beta-hydroxysteroid dehydrogenase type 1 modulates breast cancer protein profile and impacts cell migration. Breast Cancer Res, 14(3), R92-R92. doi:10.1186/bcr3207

Ayan, D., Maltais, R., Roy, J., & Poirier, D. (2012). A new nonestrogenic steroidal inhibitor of 17beta-hydroxysteroid dehydrogenase type I blocks the estrogen-dependent breast cancer tumor growth induced by estrone. Mol Cancer Ther, 11(10), 2096-2104. doi:10.1158/1535-7163.mct-12-0299

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils--a review. Food Chem Toxicol, 46(2), 446-475. doi:10.1016/j.fct.2007.09.106

Caron-Beaudoin, E., Denison, M. S., & Sanderson, J. T. (2016). Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. Toxicol Sci, 149(1), 134-144. doi:10.1093/toxsci/kfv220

Casey, M. L., MacDonald, P. C., & Andersson, S. (1994). 17 beta-Hydroxysteroid dehydrogenase type 2: chromosomal assignment and progestin regulation of gene expression in human endometrium. J Clin Invest, 94(5), 2135-2141. doi:10.1172/jci117569

Chuang, C. H., Doyle, P., Wang, J. D., Chang, P. J., Lai, J. N., & Chen, P. C. (2006). Herbal medicines used during the first trimester and major congenital malformations: an analysis of data from a pregnancy cohort study. Drug Saf, 29(6), 537-548.

de Cremoux, P., Rosenberg, D., Goussard, J., Bremont-Weil, C., Tissier, F., Tran-Perennou, C., .

. . Raffin-Sanson, M. L. (2008). Expression of progesterone and estradiol receptors in normal adrenal cortex, adrenocortical tumors, and primary pigmented nodular adrenocortical disease. Endocr Relat Cancer, 15(2), 465-474. doi:10.1677/erc-07-0081
Dolstra, H., Fredrix, H., Maas, F., Coulie, P. G., Brasseur, F., Mensink, E., . . . van de Wiel-van Kemenade, E. (1999). A Human Minor Histocompatibility Antigen Specific for B Cell Acute Lymphoblastic Leukemia. The Journal of Experimental Medicine, 189(2), 301-308. doi:10.1084/jem.189.2.301
Dou, Y.-D., Zhao, H., Huang, T., Zhao, S.-G., Liu, X.-M., Yu, X.-C., . . . Chen, Z.-J. (2016). STMN1 Promotes Progesterone Production Via StAR Up-regulation in Mouse Granulosa Cells. Scientific Reports, 6, 26691. doi:10.1038/srep26691 https://www.nature.com/articles/srep26691#supplementary-information
El Asbahani, A., Miladi, K., Badri, W., Sala, M., Ait Addi, E. H., Casabianca, H., . . . Elaissari, A. (2015). Essential oils: from extraction to encapsulation. Int J Pharm, 483(1-2), 220-243. doi:10.1016/j.ijpharm.2014.12.069
Elshafie, H. S., & Camele, I. (2017). An Overview of the Biological Effects of Some Mediterranean Essential Oils on Human Health. Biomed Res Int, 2017, 9268468. doi:10.1155/2017/9268468
Enan, E. E. (2005). Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils. Arch Insect Biochem Physiol, 59(3), 161-171. doi:10.1002/arch.20076
Falah, N., Torday, J., Quinney, S., & Haas, D. (2015). Estriol review: Clinical applications and potential biomedical importance (Vol. 1).
Gerhard, I., Fitzer, C., Klinga, K., Rahman, N., & Runnebaum, B. (1986). Estrogen screening in evaluation of fetal outcome and infant's development. J Perinat Med, 14(5), 279-291.
Good, M., Sodhi, C. P., Yamaguchi, Y., Jia, H., Lu, P., Fulton, W. B., . . . Hackam, D. J. (2016). The human milk oligosaccharide 2'-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. Br J Nutr, 116(7), 1175-1187. doi:10.1017/s0007114516002944
Gracia, T., Hilscherova, K., Jones, P. D., Newsted, J. L., Zhang, X., Hecker, M., . . . Giesy, J. P. (2006). The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol Environ Saf, 65(3), 293-305. doi:10.1016/j.ecoenv.2006.06.012
Heneweer, M., van den Berg, M., & Sanderson, J. T. (2004). A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett, 146(2), 183-194.
Henrique Bezerra Cabral, P., Campos, R., Fonteles, M., Ferreira Santos, C., Leal-Cardoso, J., & Nascimento, N. (2014). Effects of the essential oil of Croton zehntneri and its major components, anethole and estragole, on the rat corpora cavernosa (Vol. 112).
Herman, A., & Herman, A. P. (2015). Essential oils and their constituents as skin penetration enhancer for transdermal drug delivery: a review. J Pharm Pharmacol, 67(4), 473-485. doi:10.1111/jphp.12334
Hilscherova, K., Jones, P. D., Gracia, T., Newsted, J. L., Zhang, X., Sanderson, J. T., . . . Giesy, J. P. (2004). Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol Sci, 81(1), 78-89.
Hogg, K., Robinson, W. P., & Beristain, A. G. (2014). Activation of endocrine-related gene

expression in placental choriocarcinoma cell lines following DNA methylation knock-down. Mol Hum Reprod, 20(7), 677-689. doi:10.1093/molehr/gau020
Huang, C., Wu, S., Zhang, X., Chang, H., Zhao, Y., Giesy, J. P., & Hu, J. (2011). Modulation of estrogen synthesis through activation of protein kinase A in H295R cells by extracts of estuary sediments. Environ Toxicol Chem, 30(12), 2793-2801. doi:10.1002/etc.688
Kim, M., Park, Y. J., Ahn, H., Moon, B., Chung, K. H., & Oh, S. M. (2016). The effects of the standardized extracts of Ginkgo biloba on steroidogenesis pathways and aromatase activity in H295R human adrenocortical carcinoma cells. Environ Health Toxicol, 31, e2016010. doi:10.5620/eht.e2016010
Klempan, T., Hudon-Thibeault, A. A., Oufkir, T., Vaillancourt, C., & Sanderson, J. T. (2011). Stimulation of serotonergic 5-HT2A receptor signaling increases placental aromatase (CYP19) activity and expression in BeWo and JEG-3 human choriocarcinoma cells. Placenta, 32(9), 651-656. doi:10.1016/j.placenta.2011.06.003
Kondoh, N., Wakatsuki, T., Ryo, A., Hada, A., Aihara, T., Horiuchi, S., . . . Yamamoto, M. (1999). Identification and characterization of genes associated with human hepatocellular carcinogenesis. Cancer Res, 59(19), 4990-4996.
Levitz, M., & Young, B. K. (1977). Estrogens in pregnancy. Vitam Horm, 35, 109-147.
Luu-The, V. (2001). Analysis and characteristics of multiple types of human 17β-hydroxysteroid dehydrogenase (Vol. 76).
Navarra, M., Mannucci, C., Delbo, M., & Calapai, G. (2015). Citrus bergamia essential oil: from basic research to clinical application. *Front Pharmacol, 6*, 36. doi:10.3389/fphar.2015.00036
Mendonca, B. B., Arnhold, I. J., Bloise, W., Andersson, S., Russell, D. W., & Wilson, J. D. (1999). 17Beta-hydroxysteroid dehydrogenase 3 deficiency in women. J Clin Endocrinol Metab, 84(2), 802-804. doi:10.1210/jcem.84.2.5477
Miettinen, M. M., Mustonen, M. V., Poutanen, M. H., Isomaa, V. V., & Vihko, R. K. (1996). Human 17 beta-hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression. Biochemical Journal, 314(Pt 3), 839-845.
Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev, 32(1), 81-151. doi:10.1210/er.2010-0013
Mitra, P., & Audus, K. L. (2009). Expression and functional activities of selected sulfotransferase isoforms in BeWo cells and primary cytotrophoblast cells. Biochem Pharmacol, 78(12), 1475-1482. doi:10.1016/j.bcp.2009.07.013
Oskarsson, A., Ulleras, E., Plant, K. E., Hinson, J. P., & Goldfarb, P. S. (2006). Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. J Appl Toxicol, 26(6), 484-492. doi:10.1002/jat.1166
Pezzi, V., Mathis, J. M., Rainey, W. E., & Carr, B. R. (2003). Profiling transcript levels for steroidogenic enzymes in fetal tissues. The Journal of Steroid Biochemistry and Molecular Biology, 87(2), 181-189. doi:https://doi.org/10.1016/j.jsbmb.2003.07.006
Picon, R., Darmoul, D., Rouiller, V., & Duranteau, L. (1988). Activity of 3 beta-hydroxysteroid dehydrogenase/isomerase in the fetal rat ovary. J Steroid Biochem, 31(5), 839-843.
Poirier, D. (2010). 17β-Hydroxysteroid dehydrogenase inhibitors: a patent review. Expert Opinion on Therapeutic Patents, 20(9), 1123-1145. doi:10.1517/13543776.2010.505604
Qin, K., Ehrmann, D. A., Cox, N., Refetoff, S., & Rosenfield, R. L. (2006). Identification of a

functional polymorphism of the human type 5 17beta-hydroxysteroid dehydrogenase g ene associated with polycystic ovary syndrome. J Clin Endocrinol Metab, 91(1), 270-276. doi:10.1210/jc.2005-2012

Qin, K. N., & Rosenfield, R. L. (2000). Expression of 17 beta-hydroxysteroid dehydrogenase type 5 in human ovary: a pilot study. J Soc Gynecol Investig, 7(1), 61-64.

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun, 313(4), 856-862.

Rasmussen, M. K., Ekstrand, B., & Zamaratskaia, G. (2013). Regulation of 3β-Hydroxysteroid Dehydrogenase/Δ(5)-Δ(4) Isomerase: A Review. International Journal of Molecular Sciences, 14(9), 17926-17942. doi:10.3390/ijms140917926

Sanderson, J. T. (2009). Placental and Fetal Steroidogenesis. In C. Vaillancourt & J. Lafond (Eds.), Human Embryogenesis: Methods and Protocols (pp. 127-136). Totowa, NJ: Humana Press.

Sanderson, J. T., Seinen, W., Giesy, J. P., & van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci, 54(1), 121-127.

Schwarzenbach, H., Manna, P. R., Stocco, D. M., Chakrabarti, G., & Mukhopadhyay, A. K. (2003). Stimulatory effect of progesterone on the expression of steroidogenic acute regulatory protein in MA-10 Leydig cells. Biol Reprod, 68(3), 1054-1063.

Shehu, A., Albarracin, C., Devi, Y. S., Luther, K., Halperin, J., Le, J., . . . Gibori, G. (2011). The Stimulation of HSD17B7 Expression by Estradiol Provides a Powerful Feed-Forward Mechanism for Estradiol Biosynthesis in Breast Cancer Cells. Molecular Endocrinology, 25(5), 754-766. doi:10.1210/me.2010-0261

Shenhav, S., Gemer, O., Volodarsky, M., Zohav, E., & Segal, S. (2003). Midtrimester triple test levels in women with severe preeclampsia and HELLP syndrome. Acta Obstet Gynecol Scand, 82(10), 912-915.

Shiraki, N., Yamazoe, T., Qin, Z., Ohgomori, K., Mochitate, K., Kume, K., & Kume, S. (2011). Efficient Differentiation of Embryonic Stem Cells into Hepatic Cells In Vitro Using a Feeder-Free Basement Membrane Substratum. PLOS ONE, 6(8), e24228. doi:10.1371/journal.pone.0024228

Simard, J., Ricketts, M.-L., Gingras, S. b., Soucy, P., Feltus, F. A., & Melner, M. H. (2005). Molecular Biology of the 3β-Hydroxysteroid Dehydrogenase/Δ5-Δ4 Isomerase Gene Family. Endocrine Reviews, 26(4), 525-582. doi:10.1210/er.2002-0050

Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., . . . Jones, M. (2002). Aromatase-a brief overview. Annu Rev Physiol, 64.

Stanley, E. L., Hume, R., Visser, T. J., & Coughtrie, M. W. (2001). Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development. J Clin Endocrinol Metab, 86(12), 5944-5955. doi:10.1210/jcem.86.12.8081

Sugawara, T., Kiriakidou, M., McAllister, J. M., Holt, J. A., Arakane, F., & Strauss, J. F. (1997). Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: A central role for steroidogenic factor 1. Steroids, 62(1), 5-9. doi:http://dx.doi.org/10.1016/S0039-128X(96)00152-3

Svoboda, K. P., & Greenaway, R. I. (2003). Lemon scented plants. International Journal of Aromatherapy, 13(1), 23-32. doi:http://dx.doi.org/10.1016/S0962-4562(03)00048-1

Takagi, S., Naito, M., Kawai, S., Okada, R., Nagata, C., Hosono, S., . . . Wakai, K. (2017). Macronutrient intakes and serum oestrogen, and interaction with polymorphisms in CYP19A1 and HSD17B1 genes: a cross-sectional study in postmenopausal Japanese women. British Journal of Nutrition, 118(6), 463-472. doi:10.1017/S0007114517002239

Thibeault, A. A., Deroy, K., Vaillancourt, C., & Sanderson, J. T. (2014). A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals. Environ Health Perspect, 122(4), 371-377. doi:10.1289/ehp.1307518

Tillett, J., & Ames, D. (2010). The uses of aromatherapy in women's health. J Perinat Neonatal Nurs, 24(3), 238-245. doi:10.1097/JPN.0b013e3181ece75d

Tratwal, J., Follin, B., Ekblond, A., Kastrup, J., & Haack-Sørensen, M. (2014). Identification of a common reference gene pair for qPCR in human mesenchymal stromal cells from different tissue sources treated with VEGF. BMC Molecular Biology, 15(1), 11. doi:10.1186/1471-2199-15-11

Tuckey, R. C., Bose, H. S., Czerwionka, I., & Miller, W. L. (2004). Molten Globule Structure and Steroidogenic Activity of N-218 MLN64 in Human Placental Mitochondria. Endocrinology, 145(4), 1700-1707. doi:10.1210/en.2003-1034

Uribe, A., Strauss, I. I. I. J. F., & Martínez, F. (2003). Contact sites from human placental mitochondria: Characterization and role in progesterone synthesis. Archives of Biochemistry and Biophysics, 413(2), 172-181. doi:https://doi.org/10.1016/S0003-9861(03)00097-3

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, 3(7), Research0034.

von Krogh, K., Harjen, H., Almas, C., Zimmer, K. E., Dahl, E., Olsaker, I., . . . Verhaegen, S. (2010). The effect of valproate and levetiracetam on steroidogenesis in forskolin-stimulated H295R cells. Epilepsia, 51(11), 2280-2288. doi:10.1111/j.1528-1167.2010.02702.x

Wang, Y., Gho, W. M., Chan, F. L., Chen, S., & Leung, L. K. (2008). The red clover (trifolium pretense) isoflavone biochenin A inhibits aromatase activity and expression. Brit J Nutr, 99.

Watanabe, M., & Nakajin, S. (2004). Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. J Endocrinol, 180(1), 125-133.

Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., . . . Strauss, J. F., 3rd. (1997). MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. Proc Natl Acad Sci U S A, 94(16), 8462-8467.

Xu, D., Aka, J. A., Wang, R., & Lin, S. X. (2017). 17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation. J Steroid Biochem Mol Biol, 171, 270-280. doi:10.1016/j.jsbmb.2017.04.009

Xu, Y., Yu, R. M., Zhang, X., Murphy, M. B., Giesy, J. P., Lam, M. H., . . . Yu, H. (2006). Effects of PCBs and MeSO2-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. Chemosphere, 63(5), 772-784. doi:10.1016/j.chemosphere.2005.08.013

Yap, P. S., Lim, S. H., Hu, C. P., & Yiap, B. C. (2013). Combination of essential oils and antibiotics reduce antibiotic resistance in plasmid-conferred multidrug resistant bacteria. Phytomedicine, 20(8-9), 710-713. doi:10.1016/j.phymed.2013.02.013

Zhang, M., Liu, P., Dwyer, N. K., Christenson, L. K., Fujimoto, T., Martinez, F., . . . Strauss, J. F., 3rd. (2002). MLN64 mediates mobilization of lysosomal cholesterol to steroidogenic mitochondria. J Biol Chem, 277(36), 33300-33310. doi:10.1074/jbc.M200003200

Zu, Y., et al., Activities of ten essential oils towards Propionibacterium acnes and PC-3, A-549 and MCF-7 cancer cells. Molecules, 2010. 15(5): p. 3200-10

Chapter 3 : EVALUATING THE EFFECTS ON STEROIDOGENESIS OF ESTRAGOLE AND TRANS-ANETHOLE IN A FETO-PLACENTAL CO-CULTURE MODEL

Debbie Yancu*, Cathy Vaillancourt and J. Thomas Sanderson*

INRS - Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada

* Corresponding author at:
INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada
Laval, QC H7V 1B7, Canada.
E-mail addresses: debbie.yancu@iaf.inrs.ca (D. Yancu) and thomas.sanderson@iaf.inrs.ca (J.T. Sanderson)

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Author contributions:
Debbie Yancu developed the hypothesis and objectives, designed and performed the experiments, performed the statistical analyses and wrote the manuscript.
Prof. Cathy Vaillancourt participated in revising the manuscript.
Prof. Thomas Sanderson obtained the research funding, supervised the project and participated in writing and revising the manuscript.

3.1 Abstract

In this study, we determined whether estragole and its isomer trans-anethole interfered with feto-placental steroidogenesis in a human co-culture model composed of fetal-like adrenocortical (H295R) and placental trophoblast-like (BeWo) cells. Estragole and trans-anethole are considered the biologically active compounds within basil and fennel seed essential oils, respectively. After a 24h exposure of the co-culture to 2.5, 5.2 and 25 μM estragole or trans-anethole, hormone concentrations of estradiol, estrone, dehydroepiandrosterone, androstenedione, progesterone and estriol were significantly increased. Using RT-qPCR, estragole and trans-anethole were shown to significantly alter the expression of several key steroidogenic enzymes, such as those involved in cholesterol transport and steroid hormone biosynthesis, including *StAR*, *CYP11A1*, *HSD3B1/2*, *SULT2A1*, and *HSD17B1*, *-4*, and *-5*. Furthermore, we provided mechanistic insight into the ability of estragole and trans-anethole to stimulate promoter-specific expression of *CYP19* through activation of the PKA pathway in H295R cells and the PKC pathway in BeWo cells, in both cases associated with increased cAMP levels. Moreover, we show new evidence suggesting a role for progesterone in regulating steroid hormone biosynthesis through regulation of the *StAR* gene.

Keywords

Feto-placental unit	Estragole
Steroidogenesis	Trans-anethole
Aromatase	Co-culture

3.2 Introduction

Essential oils are aromatic liquids isolated from the natural secretions synthesized by different plant organs and stored within specialized secretory cells (El Asbahani et al., 2015). They are commonly extracted via steam or hydro-distillation from the plant materials. The extraction yields are extremely low, vary dependent on climate, soil composition, plant organ, age, and vegetative life cycle, and only 10% of plant species (termed aromatic plants) are able to produce essential oils (Svoboda & Greenaway, 2003).

Essentials oils are composed of a complex mixture of volatile odorous compounds with good bioavailability, characterized by low molecular weight, and of which the majority are considered hydrocarbon terpenes and terpenoids. Despite the many constituents present within essential oils, sometimes up to 100 at various concentrations, they are typically categorized by their major components found at the highest concentrations (20-70%) according to analysis using gas chromatography and mass spectrometry (GC/MS) (El Asbahani et al., 2015). It is generally believed that the most abundant compounds within the essential oil are responsible for its biological activity (Bakkali et al., 2008; Bayala et al., 2014).

In this study, we chose estragole and trans-anethole, the most abundant compounds within basil and fennel seed essential oils, respectively, to confirm our hypothesis that the steroidogenic effects exhibited by the oils are mediated by their predominant constituents. Using GC/MS data reports supplied by our essential oil manufacturer, we were able to quantify the amount of estragole and trans-anethole present within the mixtures of basil and fennel seed oils (74.61% and 76.53%, respectively) and individually assess those concentrations in a fetal-placental co-culture model. Our main objective was to characterize the potential disruption of feto-placental steroidogenesis by estragole and trans-anethole. We focused on steroid hormone production, promoter-specific expression of *CYP19*, and aromatase catalytic activity with the goal of further delineating the signaling pathways involved in the steroidogenic response of the feto-placental co-culture to essential oils.

3.3 Materials and Methods

3.3.1. Treatments

Active compounds of basil and fennel seed essential oils: estragole and trans-anethole, respectively, were obtained from Sigma-Aldrich (St-Louis, MO). Cells were exposed for 24 h to concentrations of 2.5, 5.2 and 25 μM estragole or trans-anethole (representing the concentrations found in 0.0005%, 0.001% and 0.005% basil and fennel seed oils, respectively, from Rocky Mountain Essential Oils (Orem, UT) with or without the presence of H89 (Sigma), selective PKA inhibitor, or chelerythrine chloride (Sigma), selective PKC inhibitor, and mifepristone, progesterone receptor antagonist (Thermo Fisher Scientific, Waltham, MA). Importantly, kinase

inhibitors were administered to cells 4 h prior to exposure to estragole and trans-anethole. All compounds were dissolved in DMSO and final DMSO concentrations in co-culture medium never exceeded 0.1% for single treatments and 0.2% for co-treatments.

3.3.2. Cell of the co-culture model

H295R human adrenocortical carcinoma cells (American Type Culture Collection (ATCC), Manassas, VA, no. CRL-2128) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) without phenol red containing 1.2g/L sodium bicarbonate and 2mg/L pyridoxine HCl (Gibco, Luzern, Switzerland). Medium was completed with 2.5% NuSerum (VWR International, Radnor, Pennsylvania), 1% ITS + Premix (Thermo Fisher Scientific), and 100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco). BeWo human placental choriocarcinoma cells (ATCC no. CCL-98) were cultured in DMEM/F12 without phenol red containing 1.2g/L sodium bicarbonate and 2mg/L pyridoxine HCl. Medium was completed with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ), and100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco). Co-culture set-up was as previously described (Thibeault et al., 2014), except for the replacement of 24-well culture plates with 6-well plates. For hormone quantification and RNA extraction under co-culture conditions, 7.5 x 10^5 H295R cells were seeded in 2 ml per well in 6-well plates and 3.5 x10^5 BeWo cells were seeded in 1.5 ml in transwell inserts (polycarbonate membrane with 0.4µM pores, Corning Life Sciences, Corning, NY). All Experiments were performed using cells of 8-25 passage.

3.3.3. Cell viability

The toxicity of estragole and trans-anethole to H295R and BeWo cells was determined using WST-1 cell viability reagent (Roche, Basel, Switzerland) which is based on the cleavage of a tetrazolium salt by the mitochondria in metabolically active cells. Each cell type was seeded separately in 96-well plates at a density of 104 cells/well with 200 µl of appropriate culture medium. After 24 h, cells were exposed to fresh medium containing increasing concentrations of estragole or trans-anethole for another 24 h. Cells were then incubated for 1.5 h with 20 µl of WST-1 reagent and the formation of formazan was measured using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California) at a wavelength of 450 nm.

3.3.4. Steroid hormone quantification

After a 24-h exposure to estragole or trans-anethole, co-culture medium was collected. Hormone concentrations were determined by ELISA using assay kits obtained from DRG Diagnostics (Marburg, Germany) according to manufacturer's recommendations; kits included estradiol (DRG Diagnostics EIA-2693), estrone (DRG Diagnostics EIA-4174), progesterone (DRG Diagnostics EIA-1561), androstenedione (DRG Diagnostics EIA-3265), dehydroepiandrosterone (DHEA) (DRG Diagnostics EIA-3415) and estriol (EIA-3717).

3.3.5. RT-qPCR

Real-time quantitative PCR (RT-qPCR) was used to assess gene expression of steroidogenic enzymes. After 24 h of cell acclimatization following plating, cells were exposed for 24 h to the treatments. DMSO (0.1%) was used as a vehicle control. BeWo cells in the inserts were isolated separately from the H295R cells in the wells below. RNeasy mini kits (Qiagen, Mississauga, ON, Canada) were used to isolate RNA, which was then stored at -80°C. RNA purity was assessed by determining the 260/280 nm absorbance ratio (~2.0) using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using 0.5 µg RNA with an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) and T3000 Thermocycler (Biometra, Göttingen, Germany); resultant cDNA was stored at -20°C. RT-qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with a CFX96 real-time PCR Detection System (Bio-Rad). Suitable reference genes for normalizing target gene expression were selected using geNorm software (Biogazelle, Zwijnaarde, Belgium). All validated primer pair sequences are listed in Table 3.1.

Table 3-1 Primer pair sequences used to determine gene expression of steroidogenic enzymes

Steroidogenic Gene	Primer Pairs (5' → 3')	Reference:
CYP19 coding region (exon II-X)	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC Rv: TCACCAATAACAGTCTGGATTTCC	(Sanderson et al., 2000)
CYP19 I.1	Fw: GGATCTTCCAGACGTCGCGA Rv: CATGGCTTCAGGCACGATGC	(Klempan et al., 2011)
CYP19 PII	Fw: TCTGTCCCTTTGATTTCCACAG Rv: GCACGATGCTGGTGATGTTATA	(Heneweer et al., 2004)
CYP19 I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA RV: AGAGGGGGCAAT TTAGAGTCTGTT	(Wang et al., 2008)
CYP11A1	Fw: CTTCTTCGACCCGGAAAATTT Rv: CCGGAAGTAGGTGATGTTCTTGT	(Oskarsson et al., 2006)

CYP17	Fw: AGCCGCACACCAACTATCAG Rv: TCACCGATGCTGGAGTCAAC	(Kim et al., 2016)
HSD17B7	Fw: CTGGAATGGCTCCGGGCTTTG C Rv: CCTGCCCTCGGAGACGGCGTCG	(Shehu et al., 2011)
HSD17B1	Fw: GTCTTCCTCACCGCTTTGCGCGCC Rv: GCACTGCGCCCGGCCTCGTCCTC	(Takagi et al., 2017)
HSD17B4	Fw: TGCGGGATCACGGATGACTC Rv: GCCACCATTCTCCTCACAACTC	(von Krogh et al., 2010)
HSD17B5	Fw: GGGATCTCAACGAGACAAACG Rv: AAAGGACTGGGTCCTCCAAGA	(Xu et al., 2017)
HSD17B3	Fw: AACGCACCGGATGAAATCCAGAGC Rv: GCCTGGCTACCTGACCTTGGTGTT	(Qin & Rosenfield, 2000)
HSD17B2	Fw: CTGAGGAATTGCGAAGAACC Rv: AAGAAGCTCCCCATCAGTTG	(Casey et al., 1994)
SULT2A1	Fw: CCTCCAGCGGTGGCTACA Rv: AATCGTCCGACATGATGATGAC	(Oskarsson et al., 2006)
STAR	Fw: TTGCTTTATGGGCTCAAGAATG Rv: GGAGACCCTCTGAGATTCTGCTT	(Oskarsson et al., 2006)
HSD3B1	Fw: GGAGATCAGGGTCTTGGACA Rv: CAGGCTCTCTTCAGGAATGG	(Hogg et al., 2014)
HSD3B2	Fw: TGCCAGTCTTCATCTACACCAG Rv: TTCCAGAGGCTCTTCTTCGTG	(Kim et al., 2016)
SULT2A1	Fw: TCGTCATAAGGGATGAAGATGTAATAA Rv: TGCATCAGGCAGAGAATCTCA	(Shiraki et al., 2011)
CYP3A7	Fw: CTCTTTAAGAAAGCTGTGCCCC Rv: GGGTGGTGGAGATAGTCCTA	(Kondoh et al., 1999)
Reference gene:	**Primer Pairs (5' → 3')**	**Reference:**
UBC	Fw: ATTTGGGTCGCGGTTCTTG Rv: TGCCTTGACATTCTCGATGGT	(Vandesompele et al., 2002)
TBP	Fw: TGCACAGGAGCCAAGAGTGAA Rv: CACATCACAGCTCCCCACCA	(Tratwal et al., 2014)
RPLO	Fw: GGCGACCTGGAAGTCCAACT Rv: CCATCAGCACCACAGCCTTC	(Good et al., 2016)
RPII	Fw: GCACCACGTCCAATGACAT Rv: GTGCGGCTGCTTCCATAA	(Radonic et al., 2004)
PBGD	Fw: GGCAATGCGGCTGCAA Rv: GGGTACCCACGCGAATCAC	(Dolstra et al., 1999)

3.3.6. Aromatase catalytic activity

Cellular CYP19 activity was assessed using a tritiated water-release assay as previously described (Lephart & Simpson, 1991; Sanderson et al., 2000). The amount of tritiated water released, as a measure of aromatase activity, was determined using a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Negative controls contained radioactive substrate but no cells. A positive control radioactive substrate was directly added to scintillation cocktail. Aromatase activity was expressed as a percent of control activity (DMSO). Formestane (4-hydroxyandrostenedione) (1 μM), a selective aromatase inhibitor, was used to verify the specificity of the assay for the enzymatic aromatization reaction.

3.3.7. Kinase activities and cAMP

Activation of PKA, PKC, and cAMP levels were evaluated using a PKA activity assay kit, PKC activity assay kit, and cAMP direct immunoassay kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions. The co-culture was exposed to estragole or trans-anethole, with or without PKA inhibitor H89 (Sigma) or PKC inhibitor chelerythrine chloride (Sigma), for 24 h. Cells were then lysed in the culture plates and the supernatants were collected after 15 min of centrifugation at 15,000g. The samples were stored at -80°C prior to analysis of kinase activities or cAMP levels.

3.3.8. Statistical analysis

All data were analyzed using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA). Results are presented as means with standard errors (SEM) of three different experiments; per experiment each treatment was tested in triplicate. Statistically significant differences from control (*$p<0.05$; **$p<0.01$; ***$p<0.001$) were determined using one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test. We note that individual gene expression levels determined by RT-qPCR are relative to their own basal (DMSO) level of expression, therefore expression levels cannot be compared among genes or among promoter-specific CYP19 transcripts.

3.4 Results

3.4.1. The effects of estragole and trans-anethole on cell viability of H295R cells and BeWo cells

A 24-h exposure to estragole or trans-anethole did not significantly affect H295R (Fig. 3-1A) or BeWo cell (Fig. 3-1B) viability at concentrations up to 25 μM.

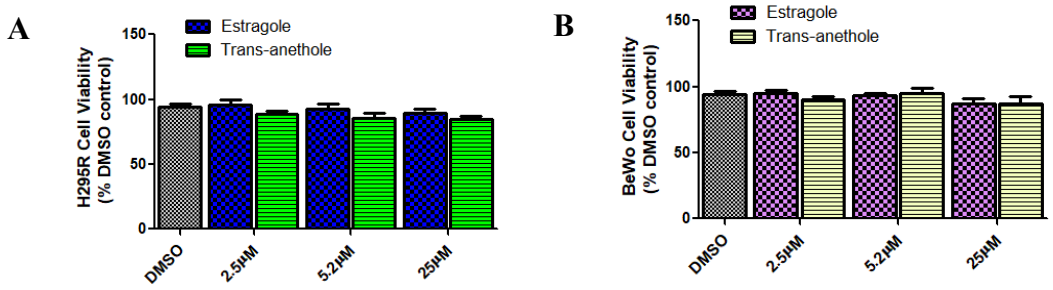

Figure 3-1: *Viability (mean ± SEM) of (A) H295R and (B) BeWo cells exposed for 24h to concentrations of 2.5, 5.2 and 25 µM estragole or trans-anethole as a percentage of DMSO (0.1%) vehicle control.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.

3.4.2. The effects of estragole and trans-anethole on hormone concentrations

Estragole and trans-anethole increased progesterone levels in co-culture by a statistically significant margin (Fig. 3-2A) at concentrations of 2.5 µM (33.2 ± 4.4 ng/ml and 32.3 + 2.4 ng/ml, respectively) and 5.2 µM (20.0 ± 3.9 ng/ml and 17.8 ± 0.9 ng/ml, respectively) compared to vehicle control, DMSO control (5.8 ± 0.5 ng/ml). There was no statistically significant change in progesterone levels at a 25 µM concentration of estragole or trans-anethole.

Estragole and trans-anethole significantly increased DHEA levels in co-culture at 2.5 µM (2.9 ± 0.4 ng/ml and 5.4 ± 0.2 ng/ml, respectively), 5.2 µM (3.6 ± 0.3 ng/ml and 5.0 ± 0.4 ng/ml, respectively) and 25 µM (3.4 ± 0.5 ng/ml and 5.1 ± 0.6 ng/ml, respectively) compared to DMSO control (1.0 ± 0.1 ng/ml) (Fig. 3-2B).

Androstenedione levels also increased significantly at estragole and trans-anethole concentrations of 2.5 µM (7.8 ± 0.4 ng/ml and 7.7 ± 0.1 ng/ml, respectively), 5.2 µM (5.8 ± 0.7 ng/ml and 6.6 ± 0.3 ng/ml, respectively) and 25 µM (4.4 ± 0.3 ng/ml and 4.7 ± 0.8 ng.ml, respectively) compared to DMSO control (1.2 ± 0.1 ng/ml) (Fig. 3-2C).

Estradiol levels in co-culture rose significantly after treatment with estragole and trans-anethole at concentrations of 2.5 µM (472.0 ± 30.2 pg/ml and 397.3 ± 32.2 pg/ml, respectively), 5.2 µM (482. 6 ± 30.5 pg/ml and 367.8 ± 16.2 pg/ml, respectively) and 25 µM (470.0 ± 11.4

pg/ml, and 290.5 ± 17.5 pg/ml, respectively) compared to vehicle control (91.8 ± 2.0 pg/ml) (Fig. 3-2D).

Estragole at concentrations of 2.5, 5.2 and 25 μM significantly increased levels of estrone in co-culture (184.2 ± 2.5 pg/ml, 173.4 ± 6.9 pg/ml and 165.1 ± 4.5 pg/ml, respectively) compared to DMSO control (134.8 ± 5.8 pg/ml) (Fig. 2E). However, only concentrations of 2.5 and 5.2 μM trans-anethole significantly increased estrone levels (171.8 ± 8.1 pg/ml and 171.0 ± 2.7 pg/ml, respectively) (Fig. 3-2E).

Finally, we also detected significant increases in estriol levels after treatment with 5.2 μM and 25 μM estragole (8.4 ± 0.8 ng/ml and 9.8 ± 0.4 ng/ml, respectively) compared to vehicle control (4.0 ± 0.5 ng/ml) (Fig. 3-2F). Each concentration of trans-anethole significantly increased estriol levels (7.1 ± 0.6 ng/ml at 2.5 μM, 9.8 ± 0.9 ng/ml at 5.2 μM and 8.6 ± 0.8 ng/ml at 25 μM) (Fig. 3-2F) compared to DMSO vehicle control.

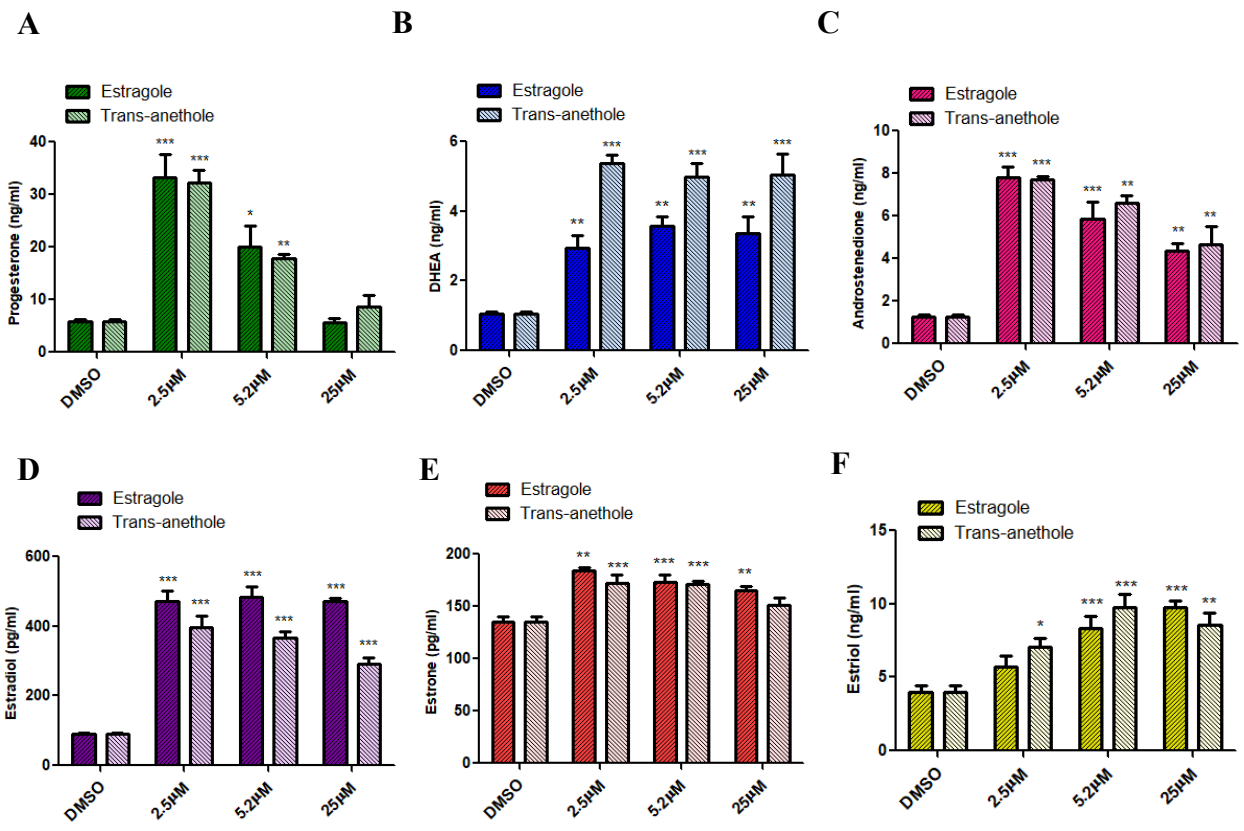

Figure 3-2 *: (A) Progesterone, (B) DHEA, (C) androstenedione, (D) estradiol, (E) estrone and (F) estriol production (mean ± SEM) by the feto-placental (H295R/BeWo) co-culture after a 24h exposure to estragole or trans-anethole.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
$*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

3.4.3. The effects of estragole and trans-anethole on the expression of steroidogenic enzymes in H295R cells in co-culture

In H295R cells (representing the fetal compartment of the feto-placental steroidogenic unit), a 2-fold increase in PII-derived *CYP19* mRNA expression levels was observed at an estragole concentration of 2.5 and 5.2 µM, and at a concentration of 2.5 µM trans-anethole, compared to DMSO control (Fig. 3-3A,B). *StAR* gene expression rose 5- to 13-fold compared to DMSO at estragole concentrations between 2.5 and 25 µM, with the lower concentrations producing the greater induction producing an inverted concentration-response curve (Fig. 3-3A). Similarly, trans-anethole significantly increased *StAR* gene expression levels by 2- to 8-fold in

the 2.5 to 25 μM range with the greatest induction at the lowest concentration (Fig. 3-3B). We also detected significant increases in the mRNA levels of *SULT2A1* stimulated by estragole and *CYP3A7* stimulated by both estragole and trans-anethole with greatest increases at 25 μM estragole and trans-anethole (Fig. 3-3A,B).

HSD17B5 mRNA levels rose between 4- and 17-fold at estragole concentrations in the 2.5 to 25 μM concentration range, whereas trans-anethole significantly induced *HSD17B5* mRNA levels by 6- and 10-fold at 2.5 and 5.2μM, respectively (Fig. 3-3C,D). In both cases, the lower concentrations of the compounds produced the greater induction. Estragole significantly increased mRNA levels of *HSD17B1* and *HSD17B7* in H295R cells (2-fold at 2.5μM; 1.5-fold at 25μM), whereas both estragole and trans-anethole increased *HSD3B1* and *HSD3B2* expression (Fig. 3-3C,D).

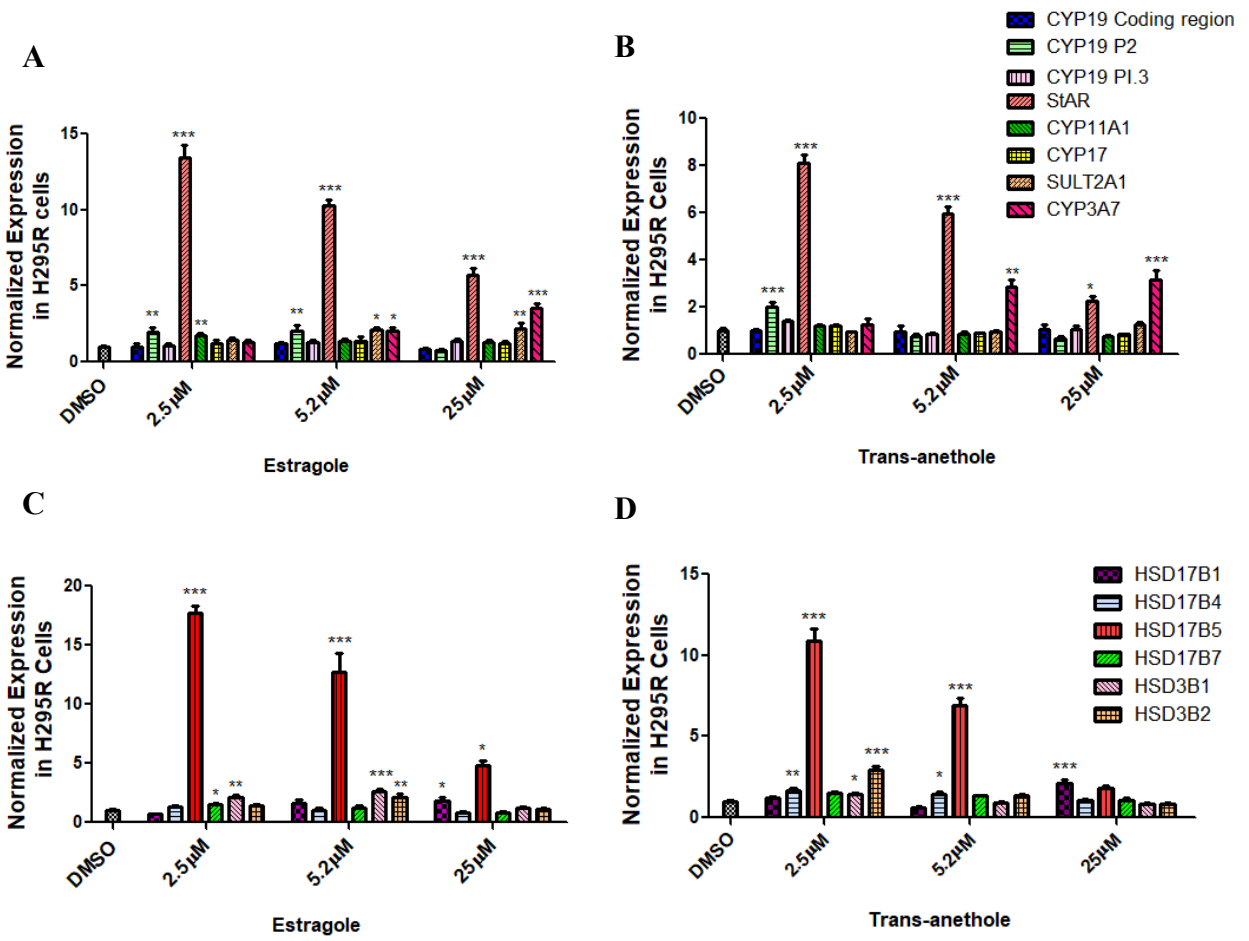

Figure 3-3: *The relative expression (mean ± SEM) of genes involved in feto-placental steroidogenesis in H295R cells in co-culture after a 24 h exposure to estragole or trans-anethole. CYP450 enzymes, sulfotransferase SULT2A1 and StAR (A,B), and HSD enzymes (C,D).*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
$*p < 0.05$; $**p < 0.01$; $***p < 0.001$.
Note: *Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

3.4.4. The effects of estragole and trans-anethole on the expression of steroidogenic enzymes in BeWo cells in co-culture

In BeWo cells, a 1.8-fold induction of I.1 promoter-derived *CYP19* mRNA expression was seen at estragole concentrations in the 2.5 and 25 µM range (Fig 3-4A), and a similar level of induction by 2.5 and 5.2µM trans-anethole (Fig. 3-4B). However, while estragole produced a

consistent effect on the expression of I.1-derived *CYP19* transcripts, trans-anethole showed a reoccurring inverse concentration-response relationship.

CYP11A1 mRNA levels rose by 2.8-fold at 2.5 μM, 2-fold at 5.2 μM and 2.4-fold at 25 μM estragole (Fig. 3-4A). Trans-anethole increased *CYP11A1* expression by 3.5-fold at 2.5 μM and 3-fold at 5.2μM (Fig. 3-4B). MLN64 expression decreased by approximately 40-50% of DMSO control values at all concentrations evaluated of estragole and trans-anethole (Fig. 3-4A-B).

Estragole and trans-anethole at concentrations of 2.5 and 5.2μM increased *HSD17β1* mRNA levels by about 2.8 to 3.4-fold (Fig. 3-4C,D). Estragole and trans-anethole at concentrations between 2.5 and 25 μM significantly decreased *HSD17B5* mRNA levels by 50 % of DMSO control levels (Fig. 3-4C, D). Estragole at 2.5 μM increased *HSD17B7* levels by 2.4-fold and trans-anethole increased its levels by 4.2 and 4.9-fold, respectively at 2.5 and 5.2 μM. (Fig. 3-4C,D). Estragole increased *HSD3B1* expression levels by 1.6-fold at 2.5 μM and 1.5-fold at 5.2 μM, whereas trans-anethole increased its expression by 1.5-fold at 2.5 μM (Fig. 3-4C,D).

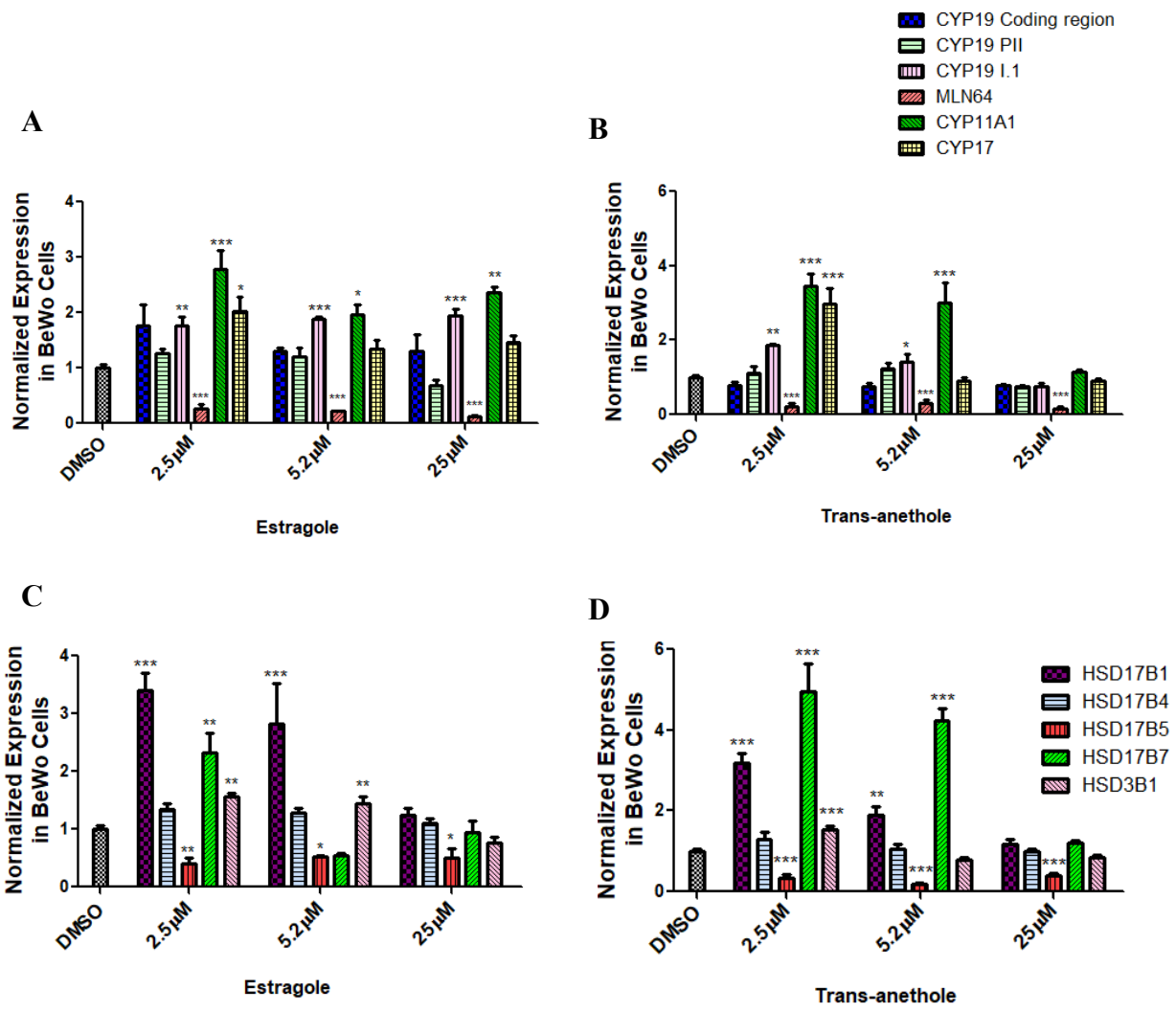

Figure 3-4: *The relative expression (mean ± SEM) of genes involved in feto-placental steroidogenesis in BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole. (A, B) CYP450 enzymes, MLN64, and (C, D) HSD enzymes.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*
Note: *Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

3.4.5. Catalytic activity of aromatase

In order to confirm whether the effects of estragole and trans-anethole on the promoter-specific expression of *CYP19* was associated with comparable changes in catalytic activity of the

enzyme, each compound was tested for its ability to induce CYP19 activity in H295R and BeWo cells in co-culture. Estragole, at concentrations of 2.5 and 5.2 µM, increased CYP19 activity significantly in H295R (1.4- and 1.2-fold, respectively) and BeWo cells (1.3- and 1.2-fold, respectively) (Fig. 3-5A). Trans-anethole significantly increased CYP19 activity in H295R cells at 2.5 µM (1.4-fold) and in BeWo cells at 2.5 and 5.2 µM (1.3-fold) (Fig 3-5B).

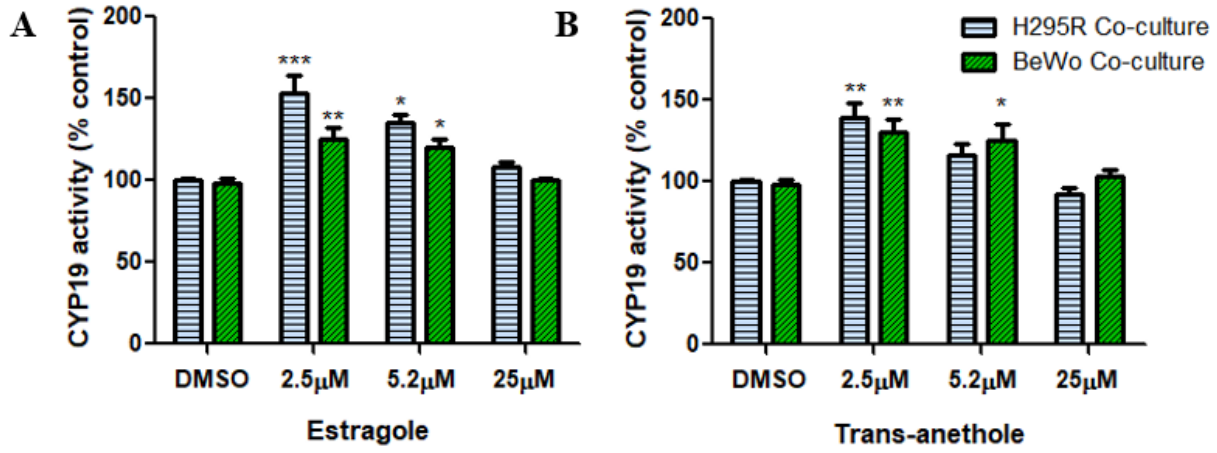

Figure 3-5: *Relative CYP19 (aromatase) activity (mean ± SEM) in H295R and BeWo cells in co-culture after a 24h exposure to (A) estragole or (B) trans-anethole (2.5, 5.2 and 25 µM), expressed as a percentage of DMSO.*

*Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *p < 0.05; **p < 0.01; ***p < 0.001.*

3.4.6. Kinase Activities and cAMP

To investigate whether estragole and trans-anethole increased the promoter-specific expression of *CYP19* in H295R and BeWo cells (PII- and I.1-derived) via the PKA and/or PKC kinase pathways, the co-culture was exposed to selective kinase inhibitors for 4 h prior to a 24h exposure to estragole and trans-anethole. The 24 h exposure to our treatments was considered the ideal timepoint by Thibeault et al. (2014) to observed maximum *CYP19* expression, while a 4 h pre-treatment of kinase inhibitors was suggested by the manufacturer of the kinase activity kits.

In the feto-placental co-culture, 2.5 µM estragole or trans-anethole increased PKC kinase activity by about 50% relative to DMSO control in both H295R and BeWo cells (Fig. 3-6A,B).

PKC activity was not increased by estragole or trans-anethole in either cell type when the co-culture was pre-treated with PKC inhibitor, chelerythrine chloride (CC) (Fig. 3-6C,D).

Estragole and trans-anethole at 2.5 and 5.2 μM increased PKA activity by about 50% relative to DMSO control in H295R cells in co-culture (Fig. 3-7A). Estragole and trans-anethole did not affect relative PKA activity in BeWo cells in co-culture (Fig. 3-7B). PKA activity was not increased by estragole or trans-anethole in H295R cells after the co-culture was pre-treated with PKA inhibitor, H89 (Fig. 3-7C). Pre-treatment with H89 did not affect PKA activity in BeWo cells which remain unchanged relative to DMSO control (Fig. 3-7D).

Intracellular cAMP levels were increased by a 100% in both H295R and BeWo cells after treatment with estragole and trans-anethole at concentrations of 2.5, 5.2, and 25 μM (Fig. 3-8A,B).

To determine whether the rise in PKA and PKC activity was linked to the increases in promoter-specific expression of *CYP19* we detected in response to a 2.5μM concentration of estragole or trans-anethole, we looked at changes to gene expression in the presence of CC and H89. After pre-treatment with CC, there was no significant change to pII-derived *CYP19* expression in H295R cells (Fig. 3-9A). However, there was a significant reduction in pII-derived *CYP19* transcripts after pre-treatment with H89 (Fig. 9A). In BeWo cells, there was a significant reduction in I.1-derived *CYP19* expression in response to estragole and trans-anethole after pre-treatment with CC, while no change in *CYP19 I.1* expression was detected after an H89 pre-treatment (Fig. 3-9B).

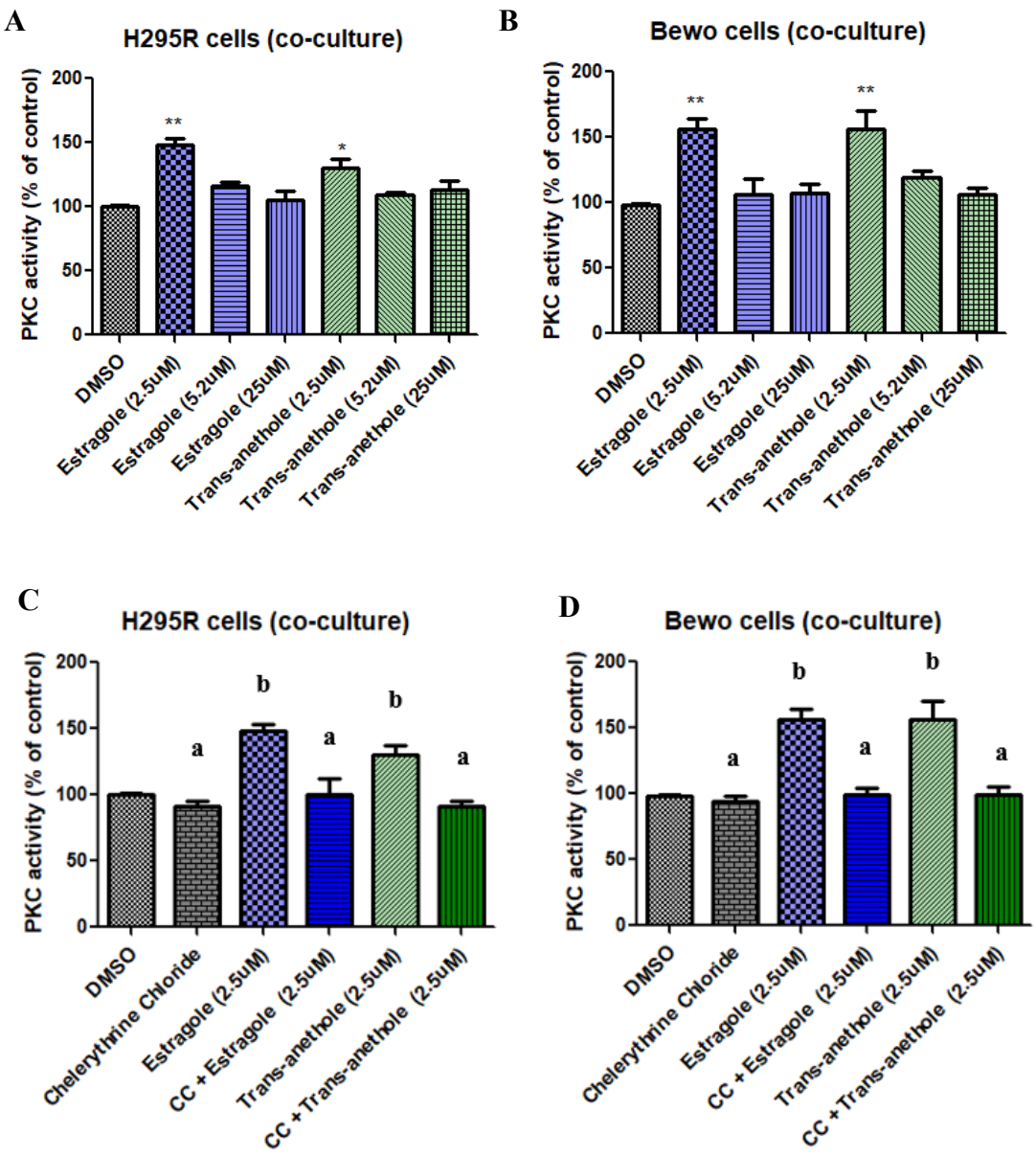

Figure 3-6: *Relative PKC activity (mean ± SEM; n=3) in (A) H295R and (B) BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole (2.5, 5.2 and 25 µM) expressed as a percentage of DMSO. Relative PKC activity in (C) H295R and (D) BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole (2.5 µM), in the presence or absence of 10 µM of the selective PKC inhibitor chelerythrine chloride (CC), expressed as a percentage of DMSO.*

*Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (a) No statistically significant difference between treatment and DMSO control; (b) Statistically significant difference between treatment and DMSO control (Student t-test; $p<0.05$).*

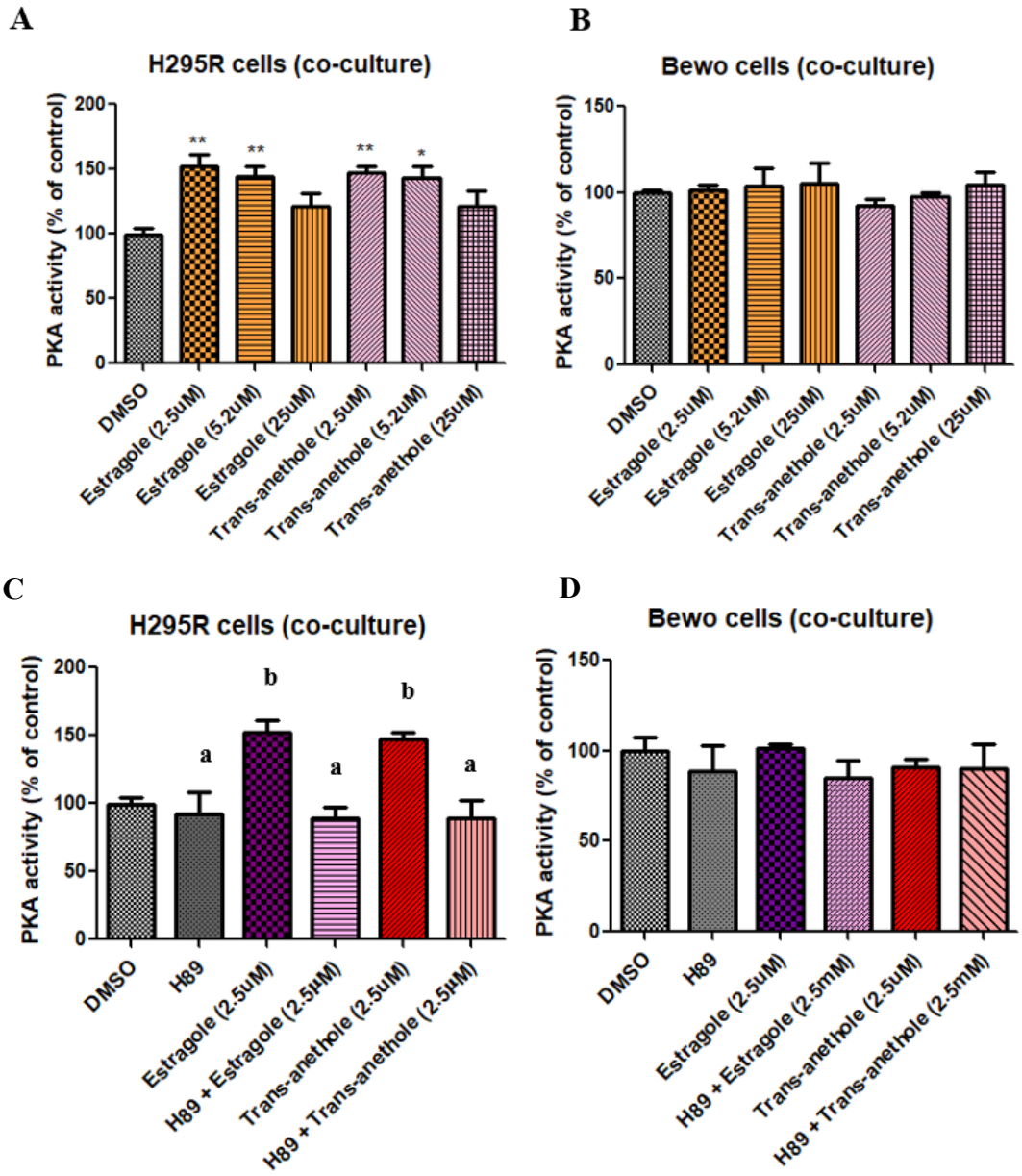

Figure 3-7: *Relative PKA activity (mean ± SEM; n=3) in (A) H295R and (B) BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole (2.5, 5.2 and 25 µM), expressed as a percentage of DMSO. Relative PKA activity in (C) H295R and (D) BeWo cells in co-culture in response to estragole or trans-anethole (2.5 µM), in the presence or absence of 10 µM of the selective PKA inhibitor H89, expressed as a percentage of DMSO.*

*Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (a) No statistically significant difference between treatment and DMSO control; (b) Statistically significant difference between treatment and control (Student t-test; $p<0.05$).*

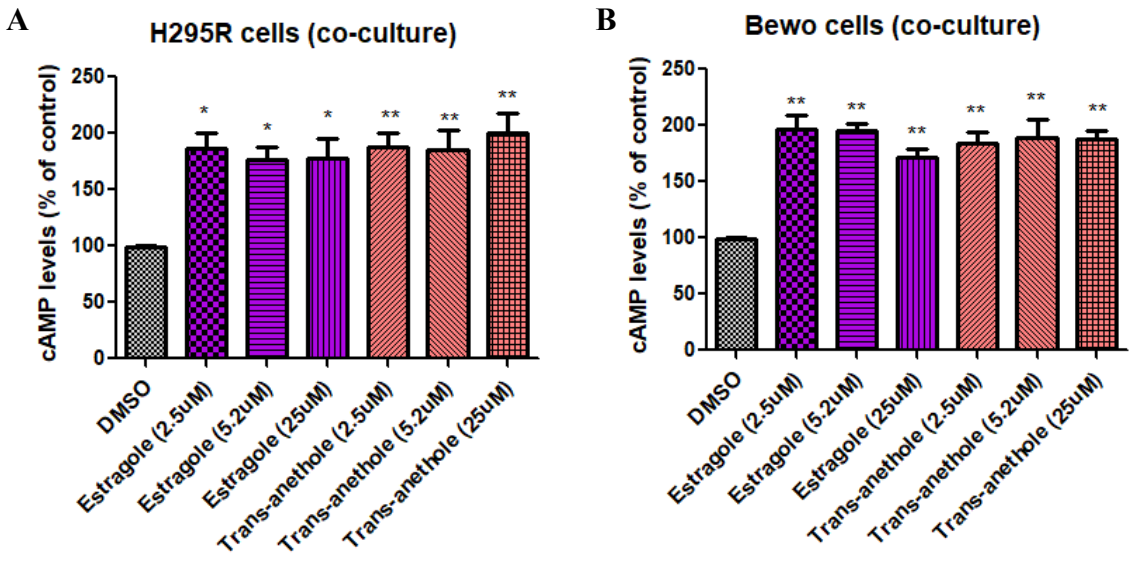

Figure 3-8: *Relative levels of intracellular cAMP (mean ± SEM; n=3) in (A) H295R and (B) BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole (2.5, 5.2 and 25 µM), expressed as a percentage of DMSO.*

Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

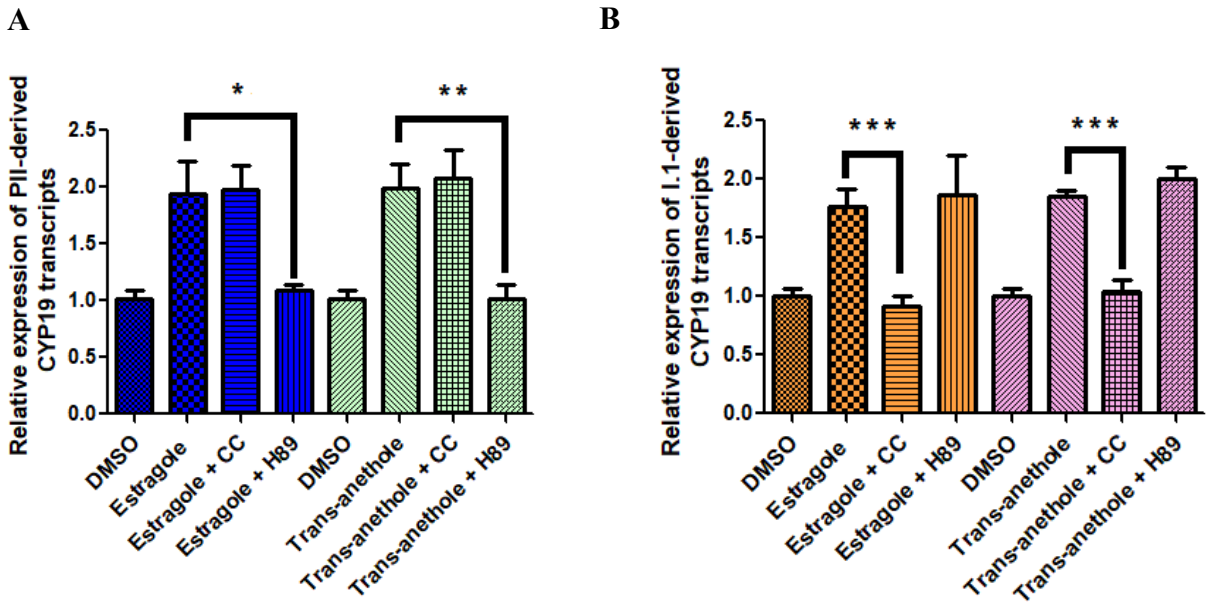

Figure 3-9: *Relative expression of PII-derived CYP19 transcripts in H295R cells (A) and I.1-derived CYP19 transcripts in BeWo cells (B) after treatment with 2.5 µM estragole or trans-anethole with or without pre-treatment with 10 µM H89, a selective PKA inhibitor or 10 µM chelerythrine chloride (CC), and expressed as a percentage of DMSO control (0.1%).*

Statistically significant differences between treatment were determine using the Student t-test
**p < 0.05; **p < 0.01; *** p < 0.005*

3.4.7. The Effect of Mifepristone on StAR Gene Expression and Progesterone Levels

StAR protein is responsible for the rate-limiting step in steroidogenesis and by controlling the movement of free-cholesterol from the outer to the inner mitochondrial membrane. Along with the action of CYP11A1 and 3β-HSD, progesterone is produced. Here, we set out to determine whether progesterone is involved in a positive feedback mechanism regulating StAR gene expression. To do so, we co-treated the feto-placental co-culture with either estragole or trans-anethole and 1 μM of mifepristone, a progesterone receptor inhibitor. Mifepristone or a co-treatment of mifepristone with estragole or trans-anethole did not affect progesterone levels in co-culture compared to vehicle control DMSO (Fig. 3-10A).

In H295R cells in co-culture, the greatest induction of *StAR* mRNA levels occurred at 2.5 μM estragole or trans-anethole (Fig. 3-3A-B, 3-10B). Co-treatment with mifepristone resulted in a significant reduction of *StAR* mRNA levels, relative to treatment with estragole or trans-anethole alone (Fig. 3-10B).

In BeWo cells in co-culture, *StAR* mRNA levels was significantly reduced at all concentrations of estragole and trans-anethole studied (2.5, 5.2 and 25 μM). Given that placental cells lack StAR protein, our interest was in *MLN64* which possesses a closely related gene sequence to *StAR* and may mediate the effects of StAR protein in placental cells. *MLN64* mRNA levels were also significantly reduced by estragole and trans-anethole treatments (Fig. 3-10C). Co-treatment of estragole or trans-anethole with mifepristone resulted in no statistically significant difference in *MLN64* mRNA levels to DMSO control (Fig. 3-10C).

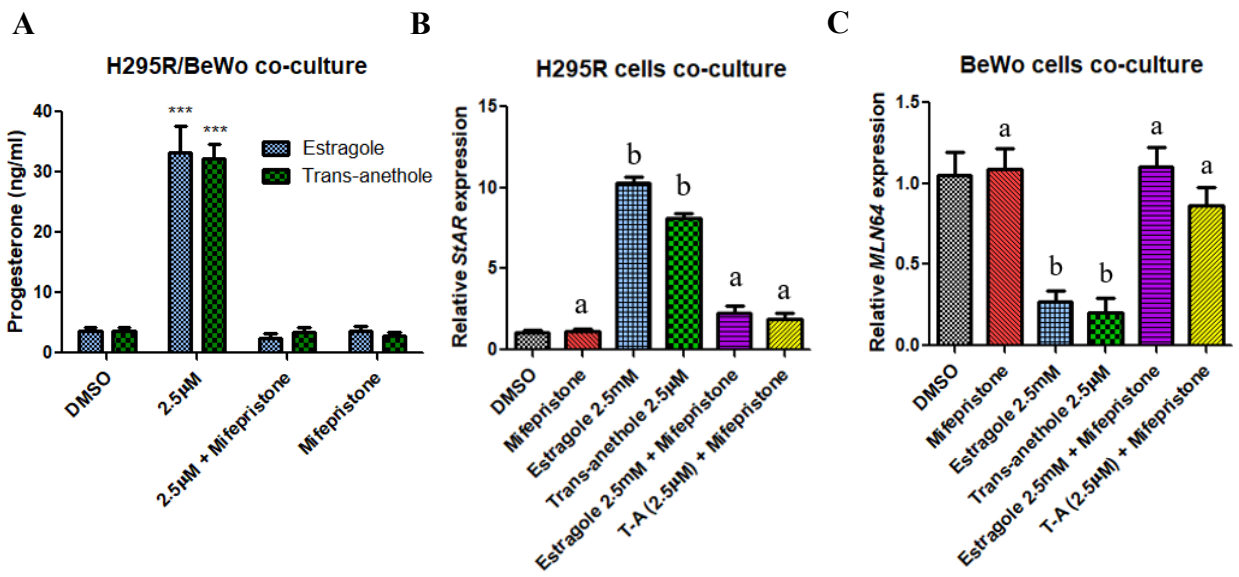

Figure 3-10: *(A) Progesterone production (mean ± SEM; n=3) by H295R and BeWo cells in co-culture after a 24h exposure to estragole or trans-anethole, in the presence or absence of 1 µM of the progesterone receptor antagonist mifepristone. (B) Relative expression levels (mean ± SEM;n=3) of StAR in H295R and (C) MLN64 in BeWo cells exposed for 24 h in co-culture to 2.5 µM estragole or trans-anethole, in the presence or absence of mifepristone.*

****p < 0.001, determined by Student t-test.*
(a) No statistically significant difference between treatment and DMSO vehicle control; (b) A statistically significant difference between treatment and control (Student t-test; p<0.05).

3.5 Discussion

3.5.1. Estragole and trans-anethole are responsible for the disruption of feto-placental steroidogenesis by basil and fennel seed essential oils, respectively

One of the most popular forms of complementary therapies used by pregnant women is aromatherapy which employs essential oils for medicinal purposes (Hall et al., 2011; Joulaeerad et al., 2018). In light of this and recent evidence that has shown that certain essential oils can cross the fetal-placental barrier (Pelkonen et al., 2017), we thought it was important to shed some light on the effects of essential oils and potential risks they could pose to mother and fetus during pregnancy. A major concern regarding the safety of essential oils is that they are composed of hundreds of different compounds among which some may be working independently and others synergistically to mediate various *in vitro* or *in vivo* biological effects. In this study, we used GC/MS reports supplied by our essential oil supplier to identify the biological compounds in

basil and fennel seed essential oil that were present in the largest amounts, to test if these individual chemicals were responsible for any of the effects of the two essential oils on feto-placental steroidogenesis. In addition, we wished to gain mechanistic insight into the role of each compound.

3.5.2. Steroid hormone levels, aromatase and kinase activities

Estragole and trans-anethole significantly increased levels of progesterone, DHEA, androstenedione, estradiol, estrone, and estriol. The observed effects were not always concentration-dependent, such as for the effects on progesterone, androstenedione, and estradiol where the lower concentrations of estragole and trans-anethole resulted in the greatest increases in these steroid hormones. This non-monotonic response was also seen for mRNA levels of steroidogenic enzymes in our feto-placental co-culture system. This was particularly true for the significant stimulation of promoter-specific expression of PII and I.1-derived *CYP19* transcripts in H295R and BeWo cells, respectively, by both estragole (Fig. 3-3A, 3-4A) and trans-anethole (Fig 3-3B, 3-4B). These non-monotonic effects were also seen in H295R cells in response to the neonicotinoids thiacloprid and thiamethoxam (Caron-Beaudoin et al., 2016), albeit in monoculture. It is possible that estragole and trans-anethole are activating intracellular signaling pathways that stimulate specific promoters involved in *CYP19* expression at the lower concentrations we studied, but may start acting upon additional pathways that impair or counteract this initial stimulation at higher concentrations. These non-monotonic effects on the promoter-specific stimulation of *CYP19* transcript were confirmed by our observation that the catalytic activity of *CYP19* increased significantly only at the lowest concentrations of estragole and trans-anethole; at higher concentrations this initial increase in aromatase activity was no longer evident (Fig. 3-5A-B). Reports in Vandenberg et al. (2012) have shown that endocrine-disrupting compounds in low doses can influence the response of an organ/system to endogenous hormones or even work synergistically with other chemicals and natural hormones to create an additive response. Moreover, it is quite difficult to ascertain the physiological relevance of aromatase induction since we cannot measure the effect in rodents; which lack the *CYP19* promoters unique to humans. Overall, a 1.2-1.4 fold induction of aromatase may not seem highly significant, we do not know *in situ* how this effects estrogen production. Moreover, pesticides

have been recently shown to possess similar effects on aromatase (Caron-Beaudoin et al., 2016; Thibeault et al., 2014).

To investigate this non-monotonic response and to better understand the mechanism behind the ability of estragole and trans-anethole to stimulate aromatase catalytic activity and promoter-specific expression of the *CYP19* gene, we looked at changes in protein kinase activities induced by our compounds. PKC activity was significantly increased in both H295R and BeWo cells in co-culture by 2.5 μM estragole or trans-anethole. The PKC signaling pathway is known to stimulate promoter I.1-derived *CYP19* mRNA production in the placenta and associated BeWo cells (Hudon Thibeault et al., 2017; Klempan et al., 2011; Tan et al., 2013). Inhibition of PKC activity with chelerythrine chloride resulted in no significant change in PKC activity relative to DMSO in both H295R and BeWo cells. It is important to note that chelerythrine chloride did not significantly alter PKC levels in DMSO-only treated cells, possibly because basal PKC levels are already low. To further understand the role played by estragole and trans-anethole at increasing promoter-specific *CYP19* expression, we also assessed PKA activity and cAMP levels. A 2.5 and 5.2 μM concentration of estragole or trans-anethole increased PKA activity in H295R cells by about 50%; and this effect was inhibited by pre-treatment with H89. The increased PKA activity in H295R cells by estragole and trans-anethole was associated with an increase in intracellular levels of cAMP, a second messenger known to activate PKA signaling. A previous study has shown that estragole and trans-anethole both significantly increase intracellular cAMP levels by 2- to 3-fold in rat smooth muscle cells (Henrique Bezerra Cabral et al., 2014). Both the PKA and PKC pathways can be activated by cAMP which leads to the phosphorylation of the cAMP responsive element binding protein 1 (CREB1). CREB1 then translocates to the nucleus and binds to a cAMP responsive-element which leads to the expression of *CYP19* through PII/I.3 (Huang et al., 2011; Mao et al., 2007; Parakh et al., 2006).

Estragole and trans-anethole increased cAMP levels in BeWo cells in our co-culture model. It has been suggested that cAMP levels can rise as a result of the activity of certain PKC isoforms in placental trophoblasts (Karl & Divald, 1996). These PKC isoforms are considered PMA-responsive (phorbol 12-myristate 13-acetate) (Karl & Divald, 1996). Other studies have also shown that PKC activation can alter cAMP levels (Heyworth et al., 1985; Houslay, 1991).

However, it is currently unclear which mechanisms allow the PMA-dependent increase in cAMP production, although it has been suggested to be caused by a direct effect on adenylate cyclase activity (Karl & Divald, 1996; Yoshimasa et al., 1987). More recent studies have shown that phorbol esters (Silinsky & Searl, 2003) and epidermal growth factor (EGF) (Connor et al., 1997) activate PKC in placental trophoblasts, but no information is available yet as to which PKC isoforms are involved and whether they are different from PMA-responsive isoforms. In all, more research is needed to better understand how PMA may increase cAMP levels in trophoblasts and which PKC isoforms are involved. In our study, our results suggests that trans-anethole and estragole are able to mimic PMA's ability to stimulate adenylate cyclase and increase cAMP levels to activate PKC. It is also important to note that while all doses of estragole and trans-anethole that we evaluated increased cAMP levels, we only detected significant rises in PKA activity in H295R cells at estragole and trans-anethole concentrations of 2.5μM and 5.2μM and increases in PKC activity in BeWo cells at 2.5μM of estragole and trans-anethole. We believe that at the lower concentrations of our treatments, cAMP levels are activating the kinase pathways we studied, while at higher concentrations of estragole and trans-anethole, the rise in cAMP could be acting on additional pathways that we have not yet been investigated by either impairing or counteracting the initial stimulation of promoter-specific expression of CYP19. More work is needed to further explore the role that increased cAMP levels may be playing in the feto-placental co-culture outside of aromatase activation.

3.5.3. Progesterone regulates StAR gene expression

In H295R cells, we observed significant upregulation of the cholesterol transport gene *StAR*, whereas in BeWo cells *MLN64* gene expression was significantly reduced. In the placenta, cholesterol entry and placental steroidogenesis is thought to be regulated by MLN64 which shares 60% sequence homology with *StAR* (Esparza-Perusquía et al., 2015; Uribe et al., 2003). Recent studies that have also identified progesterone as a possible regulator of *StAR* in steroidogenic mouse Leydig cells (Schwarzenbach et al., 2003) and it is known that that progesterone receptors are expressed in H295R cells (de Cremoux et al., 2008). In our study, co-treatment of estragole and trans-anethole with mifepristone prevented estragole- and trans-anethole-mediated stimulation of progesterone production by the co-culture as well as the increase in *StAR* mRNA levels in the H295R cells of the co-culture. Mifepristone also prevented

the estragole- and trans-anethole-mediated decrease in expression of the *StAR* homolog *MLN64* in BeWo cells. Taken together, our results provide new evidence that progesterone appears to play an important role in regulating *StAR* gene expression in H295R and *MLN64* gene expression in BeWo cells.

3.6 Conclusion

In this study, isomers estragole and trans-anethole, the most abundant chemical constituents of basil and fennel seed oil, respectively, disrupted feto-placental steroidogenesis in a co-culture model composed of fetal-like adrenocortical (H295R) and trophoblast-like (BeWo) cells. After a 24 h exposure of the co-culture to estragole or trans-anethole (2.5, 5.2, and 25 μM), levels of estradiol, estrone, DHEA, androstenedione, progesterone and estriol were significantly increased; an effect that was not always concentration-dependent. Moreover, estragole and trans-anethole altered the gene expression of several key steroidogenic enzymes, including *CYP11A1*, *HSD3B1, HSD3B2, HSD17B1, HSD17B5, HSD17B7*, as well as the steroidogenic factor *StAR* which is involved in cholesterol transport. In the co-culture, estragole and trans-anethole increased PII- and I.1-mediated *CYP19* expression in H295R and BeWo cells, respectively, as well as catalytic activity of the enzyme. Using selective protein kinase inhibitors, we were able to confirm that the increases in promoter-specific expression of *CYP19* in H295R and BeWo cells was mediated by stimulation of PKA and PKC kinase activity, respectively. This study presented new evidence that progesterone appears to play an important role in regulating *StAR* gene expression in H295R and *MLN64* expression in BeWo cells. Our results indicate that further study is necessary to determine the potential risks of using essential oils during pregnancy considering their potential to disrupt steroidogenic enzyme activity and expression *in vitro.*

3.7 Acknowledgements

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3.8 Conflicts of interest

The authors declare to have no conflicts of interest.

3.9 References

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils--a review. Food Chem Toxicol, 46(2), 446-475. doi:10.1016/j.fct.2007.09.106

Bayala, B., Bassole, I. H. N., Gnoula, C., Nebie, R., Yonli, A., Morel, L., . . . Simpore, J. (2014). Chemical Composition, Antioxidant, Anti-Inflammatory and Anti-Proliferative Activities of Essential Oils of Plants from Burkina Faso. PLoS ONE, 9(3), e92122. doi:10.1371/journal.pone.0092122

Borthwick, F., Allen, A.-M., Taylor, J. M., & Graham, A. (2010). Overexpression of STARD3 in human monocyte/macrophages induces an anti-atherogenic lipid phenotype. Clin Sci (Lond), 119(7), 265-272. doi:10.1042/CS20100266

Caron-Beaudoin, E., Denison, M. S., & Sanderson, J. T. (2016). Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. Toxicol Sci, 149(1), 134-144. doi:10.1093/toxsci/kfv220

Casey, M. L., MacDonald, P. C., & Andersson, S. (1994). 17 beta-Hydroxysteroid dehydrogenase type 2: chromosomal assignment and progestin regulation of gene expression in human endometrium. J Clin Invest, 94(5), 2135-2141. doi:10.1172/jci117569

Connor, P., Talavera, F., Kang, J. S., Burke, J., Roberts, J., & Menon, K. M. (1997). Epidermal growth factor activates protein kinase C in the human endometrial cancer cell line HEC-1-A. Gynecol Oncol, 67(1), 46-50. doi:10.1006/gyno.1997.4828

de Cremoux, P., Rosenberg, D., Goussard, J., Bremont-Weil, C., Tissier, F., Tran-Perennou, C., . . . Raffin-Sanson, M. L. (2008). Expression of progesterone and estradiol receptors in normal adrenal cortex, adrenocortical tumors, and primary pigmented nodular adrenocortical disease. Endocr Relat Cancer, 15(2), 465-474. doi:10.1677/erc-07-0081

Dolstra, H., Fredrix, H., Maas, F., Coulie, P. G., Brasseur, F., Mensink, E., . . . van de Wiel-van Kemenade, E. (1999). A Human Minor Histocompatibility Antigen Specific for B Cell Acute Lymphoblastic Leukemia. The Journal of Experimental Medicine, 189(2), 301-308. doi:10.1084/jem.189.2.301

El Asbahani, A., Miladi, K., Badri, W., Sala, M., Ait Addi, E. H., Casabianca, H., . . . Elaissari, A. (2015). Essential oils: from extraction to encapsulation. Int J Pharm, 483(1-2), 220-243. doi:10.1016/j.ijpharm.2014.12.069

Good, M., Sodhi, C. P., Yamaguchi, Y., Jia, H., Lu, P., Fulton, W. B., . . . Hackam, D. J. (2016). The human milk oligosaccharide 2'-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. Br J Nutr, 116(7), 1175-1187. doi:10.1017/s0007114516002944

Heneweer, M., van den Berg, M., & Sanderson, J. T. (2004). A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett, 146(2), 183-194.

Henrique Bezerra Cabral, P., Campos, R., Fonteles, M., Ferreira Santos, C., Leal-Cardoso, J., & Nascimento, N. (2014). Effects of the essential oil of Croton zehntneri and its major components, anethole and estragole, on the rat corpora cavernosa (Vol. 112).

Heyworth, C. M., Wilson, S. P., Gawler, D. J., & Houslay, M. D. (1985). The phorbol ester TPA prevents the expression of both glucagon desensitisation and the glucagon-mediated block of insulin stimulation of the peripheral plasma membrane cyclic AMP phosphodiesterase in rat hepatocytes. FEBS Letters, 187(2), 196-200. doi:10.1016/0014-5793(85)81241-2

Hogg, K., Robinson, W. P., & Beristain, A. G. (2014). Activation of endocrine-related gene expression in placental choriocarcinoma cell lines following DNA methylation knock-down. Mol Hum Reprod, 20(7), 677-689. doi:10.1093/molehr/gau020

Houslay, M. D. (1991). 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur J Biochem, 195(1), 9-27.

Huang, C., Wu, S., Zhang, X., Chang, H., Zhao, Y., Giesy, J. P., & Hu, J. (2011). Modulation of estrogen synthesis through activation of protein kinase A in H295R cells by extracts of estuary sediments. Environ Toxicol Chem, 30(12), 2793-2801. doi:10.1002/etc.688

Hudon Thibeault, A.-A., Laurent, L., Vo Duy, S., Sauvé, S., Caron, P., Guillemette, C., . . . Vaillancourt, C. (2017). Fluoxetine and its active metabolite norfluoxetine disrupt estrogen synthesis in a co-culture model of the feto-placental unit. Mol Cell Endocrinol, 442, 32-39. doi:https://doi.org/10.1016/j.mce.2016.11.021

Karl, P. I., & Divald, A. (1996). Protein kinase C in cultured human placental trophoblasts: identification of isoforms and role in cAMP signalling. The Biochemical journal, 320 (Pt 3)(Pt 3), 831-836.

Kim, M., Park, Y. J., Ahn, H., Moon, B., Chung, K. H., & Oh, S. M. (2016). The effects of the standardized extracts of Ginkgo biloba on steroidogenesis pathways and aromatase activity in H295R human adrenocortical carcinoma cells. Environ Health Toxicol, 31, e2016010. doi:10.5620/eht.e2016010

Klempan, T., Hudon-Thibeault, A. A., Oufkir, T., Vaillancourt, C., & Sanderson, J. T. (2011). Stimulation of serotonergic 5-HT2A receptor signaling increases placental aromatase (CYP19) activity and expression in BeWo and JEG-3 human choriocarcinoma cells. Placenta, 32(9), 651-656. doi:10.1016/j.placenta.2011.06.003

Kondoh, N., Wakatsuki, T., Ryo, A., Hada, A., Aihara, T., Horiuchi, S., . . . Yamamoto, M. (1999). Identification and characterization of genes associated with human hepatocellular carcinogenesis. Cancer Res, 59(19), 4990-4996.

Mao, L.-M., Tang, Q., & Wang, J. Q. (2007). Protein kinase C-regulated cAMP response element-binding protein phosphorylation in cultured rat striatal neurons. Brain research bulletin, 72(4-6), 302-308. doi:10.1016/j.brainresbull.2007.01.009

Oskarsson, A., Ulleras, E., Plant, K. E., Hinson, J. P., & Goldfarb, P. S. (2006). Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. J Appl Toxicol, 26(6), 484-492. doi:10.1002/jat.1166

Parakh, T. N., Hernandez, J. A., Grammer, J. C., Weck, J., Hunzicker-Dunn, M., Zeleznik, A. J., & Nilson, J. H. (2006). Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. Proceedings of the National Academy of Sciences of the United States of America, 103(33), 12435-12440. doi:10.1073/pnas.0603006103

Qin, K. N., & Rosenfield, R. L. (2000). Expression of 17 beta-hydroxysteroid dehydrogenase type 5 in human ovary: a pilot study. J Soc Gynecol Investig, 7(1), 61-64.

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun, 313(4), 856-862.

Sanderson, J. T., Seinen, W., Giesy, J. P., & van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci, 54(1), 121-127.

Schwarzenbach, H., Manna, P. R., Stocco, D. M., Chakrabarti, G., & Mukhopadhyay, A. K. (2003). Stimulatory effect of progesterone on the expression of steroidogenic acute regulatory protein in MA-10 Leydig cells. Biol Reprod, 68(3), 1054-1063.

Shehu, A., Albarracin, C., Devi, Y. S., Luther, K., Halperin, J., Le, J., . . . Gibori, G. (2011). The Stimulation of HSD17B7 Expression by Estradiol Provides a Powerful Feed-Forward Mechanism for Estradiol Biosynthesis in Breast Cancer Cells. Molecular Endocrinology, 25(5), 754-766. doi:10.1210/me.2010-0261

Shiraki, N., Yamazoe, T., Qin, Z., Ohgomori, K., Mochitate, K., Kume, K., & Kume, S. (2011). Efficient Differentiation of Embryonic Stem Cells into Hepatic Cells In Vitro Using a Feeder-Free Basement Membrane Substratum. PLoS One, 6(8), e24228. doi:10.1371/journal.pone.0024228

Silinsky, E. M., & Searl, T. J. (2003). Phorbol esters and neurotransmitter release: more than just protein kinase C? British journal of pharmacology, 138(7), 1191-1201. doi:10.1038/sj.bjp.0705213

Svoboda, K. P., & Greenaway, R. I. (2003). Lemon scented plants. International Journal of Aromatherapy, 13(1), 23-32. doi:http://dx.doi.org/10.1016/S0962-4562(03)00048-1

Takagi, S., Naito, M., Kawai, S., Okada, R., Nagata, C., Hosono, S., . . . Wakai, K. (2017). Macronutrient intakes and serum oestrogen, and interaction with polymorphisms in CYP19A1 and HSD17B1 genes: a cross-sectional study in postmenopausal Japanese women. British Journal of Nutrition, 118(6), 463-472. doi:10.1017/S0007114517002239

Tan, W., Huang, H., Wang, Y., Wong, T. Y., Wang, C. C., & Leung, L. K. (2013). Bisphenol A differentially activates protein kinase C isoforms in murine placental tissue. Toxicology and Applied Pharmacology, 269(2), 163-168. doi:https://doi.org/10.1016/j.taap.2013.03.016

Thibeault, A. A., Deroy, K., Vaillancourt, C., & Sanderson, J. T. (2014). A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals. Environ Health Perspect, 122(4), 371-377. doi:10.1289/ehp.1307518

Tratwal, J., Follin, B., Ekblond, A., Kastrup, J., & Haack-Sørensen, M. (2014). Identification of a common reference gene pair for qPCR in human mesenchymal stromal cells from different tissue sources treated with VEGF. BMC Molecular Biology, 15(1), 11. doi:10.1186/1471-2199-15-11

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, 3(7), Research0034.

von Krogh, K., Harjen, H., Almas, C., Zimmer, K. E., Dahl, E., Olsaker, I., . . . Verhaegen, S. (2010). The effect of valproate and levetiracetam on steroidogenesis in forskolin-stimulated H295R cells. Epilepsia, 51(11), 2280-2288. doi:10.1111/j.1528-1167.2010.02702.x

Wang, Y., Gho, W. M., Chan, F. L., Chen, S., & Leung, L. K. (2008). The red clover (trifolium

pretense) isoflavone biochenin A inhibits aromatase activity and expression. Brit J Nutr, 99.
Xu, D., Aka, J. A., Wang, R., & Lin, S. X. (2017). 17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation. J Steroid Biochem Mol Biol, 171, 270-280. doi:10.1016/j.jsbmb.2017.04.009
Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkowitz, R. J., & Caron, M. G. (1987). Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature, 327(6117), 67-70. doi:10.1038/327067a0

Chapter 4 : BASIL AND FENNEL SEED ESSENTIAL OILS TRIGGER A CYP19 PROMOTER-SWITCH IN HS578T CELLS BY INCREASING CAMP LEVELS AND PKA ACTIVITY

Debbie Yancu* and Thomas Sanderson*

INRS - Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada

* Corresponding author at:
INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada
Laval, QC H7V 1B7, Canada.
E-mail addresses: debbie.yancu@iaf.inrs.ca (D. Yancu) and thomas.sanderson@iaf.inrs.ca (J.T. Sanderson)

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Author contributions:
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Prof. Thomas Sanderson obtained the research funding, supervised the project and participated in writing and revising the manuscript

4.1 Abstract

It is estimated that 40-60% of women suffering from breast cancer choose to use complementary and alternative medicines (CAMs) with the goal of avoiding toxic side-effects associated with their cancer treatment. Natural products and dietary supplements are the most frequently used CAMs among breast cancer patients and recent evidence suggests some of these products exhibit estrogenic effects. Aromatase (CYP19) is a key enzyme in the synthesis of estrogens and its expression is regulated in a tissue-specific manner with the help of different promoters. In the normal breast, *CYP19* is regulated by its promoter I.4. However, a promoter-switch takes place in hormone-dependent breast cancer whereby I.4 activity is decreased in favor of increases in promoter I.3, PII, and I.7 activity. In this study, we evaluated the effects of basil and fennel seed essential oil in Hs578t cells; a representative model of human estrogen-dependent breast cancer. We determined that both essential oils triggered a promoter-switch in Hs578t cells and increased *CYP19* expression and aromatase catalytic activity which was associated with a decrease in I.4 activity and a rise in PII activity. Furthermore, we showed that the promoter-switch was mediated by an increase in cAMP levels and PKA activity.

Keywords

Hs578t
Breast cancer
Aromatase
Essential oils
Basil
Fennel seed

4.2 Introduction

Humans are exposed to a wide variety of structurally diverse chemicals from many sources, including environmental contaminants, food additives, therapeutic agents, and chemicals from industrial processes in consumer products, such as personal care products, etc. A significant number of these chemicals have been identified as endocrine disruptors and can produce a variety of adverse effects, which includes enhancing the risk of development and progression of breast cancer (Macon & Fenton, 2013; Neuhouser et al., 2016). Breast cancer is the most prevalent cancer worldwide in women and the second leading cause of cancer-related deaths in

females (World Cancer Research Fund, 2018). Although the exact etiology is unknown, approximately 70% of breast tumors are estrogen-dependent (Lumachi et al., 2015). In estrogen-dependent breast cancer, growth and proliferation of the mammary tumor may be stimulated by endocrine disrupting chemicals that directly activate the estrogen receptor (ER) and its downstream signaling pathway. Another highly relevant mechanism of endocrine disruption is the potential of chemicals to increase the biosynthesis of potent endogenous steroid hormones that stimulate breast cancer growth, such as the production of estrogens by inducing the enzyme aromatase (CYP19).

The enzyme aromatase is regulated in a highly complex, tissue-specific manner through the alternate use of at least 10 distinct promoters located upstream of the coding exon of the *CYP19* gene (Bulun et al., 2003; Chen et al., 2009). Four of these promoters have been shown to be modulated in breast cancer (Chen et al., 2009; Odawara et al., 2009). In healthy breast stromal cells (adipocytes and preadipocytes), *CYP19* expression (and resultant aromatase activity) is regulated via the adipose stromal-specific I.4 promoter. The estrogens produced locally by stromal aromatase ensure the differentiation of breast epithelial cells and maintain ER-mediated functions of the breast epithelium. However, stromal cells, including fibroblasts, inflammatory, and endovascular cells, have been shown to support ER-positive breast cancer progression to metastasis. Essentially, stromal cells have inducible aromatase activity (Meng et al., 2001; Zhou et al., 2001). In hormone-dependent breast cancer, a promoter-switch occurs in which the tumor cells (almost always of epithelial origin) secrete a variety of factors (tumor necrosis factor-α, prostaglandins E2, interleukin-6, and interleukin-11) that repress the activity of the I.4 promoter of *CYP19* and activate the normally silent promoters PII, I,3 and I.7 (Chen et al., 2009; Sebastian et al., 2002).

It is estimated that between 40-60% of women who are diagnosed annually with breast cancer supplement their traditional cancer therapy with the use of complementary and alternative medicines (CAMs) (Neuhouser et al., 2016; Roumeliotis et al., 2017). Breast cancer patients report that they use of CAMs to improve their overall health and well-being, symptom management, and probability of long-term survival (Adams et al., 2015; Demark-Wahnefried et al., 2015; Hedderson et al., 2004). However, very little data is available to understand the positive and potentially negative impact CAM use may have on breast cancer prognosis. Natural

products and dietary supplements are of particular concern, as they are the most frequently reported CAMs used by breast cancer patients (Eisenberg et al., 1998; Neuhouser et al., 2016; Patterson et al., 2002). Chief among concerns is the potential for the CAM treatment to affect tumor biology by exhibiting estrogenic effects, which certain herbal supplements possess (Amato et al., 2002; Kiyama, 2017; Ma et al., 2011).

In this study, we used Hs578t cells, which our laboratory has validated as a representative model of the aromatase-expressing and estrogen-producing stromal cells found in the human hormone-dependent breast cancer environment (Caron-Beaudoin et al., 2018), to determine the effects of basil (*Ocimum basilicum*) and fennel seed (*Foeniculum vulgare*) essential oils on steroidogenesis. Our goal was to determine whether basil and fennel seed oils had similar effects on steroidogenesis in a hormone-dependent breast cancer environment to those we previously observed in a model of fetal-placental steroidogenesis (Chapter 2), with a focus on the promoter-specific expression and catalytic activity of CYP19.

4.3 Materials and methods

4.3.1. Treatments

Basil and fennel seed essential oils were obtained from Rocky Mountain Oils (Orem, UT, USA) and were provided with detailed GC/MS data reports of their chemical composition (see supplementary material). Essential oils were dissolved in DMSO and added to the cells at concentration in culture medium ranging from 0.0005% to 0.05%, always maintaining a final DMSO concentration of 0.1%.

4.3.2. Cell culture

Hs578t (American Type Culture Collection, ATCC; cat. no. HTB-126) are non-ER expressing, aromatase-containing cancer (or cancer-associated) cells derived from a carcinosarcoma; a rare stromal-like tumor. They are considered a “mesenchymal-like” or “stromal-like” breast cancer cell line because they have low to negligible expression of epithelial luminal markers (such as E-cadherin) and express high levels of mesenchymal markers (such as vimentin and N-cadherin) (Lacroix & Leclercq, 2004), and display a fibroblast-like phenotype in

cell culture (Lacroix & Leclercq, 2004; Prat et al., 2010). Most critically for our study, Hs578t cells exhibit activity of all breast cancer-relevant *CYP19* promoters (I.4, PII, I.3, I.7), which makes them particularly suitable as a research model to understand the interactions that take place in the ER-dependent breast cancer microenvironment (Caron-Beaudoin et al., 2018).

Hs578t cells were grown in ATCC-recommended culture medium, consisting of Dulbecco's modified Eagle medium (DMEM #30-2002) modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate. This base medium was supplemented with 0.01 mg/ml bovine insulin (Gibco, Luzern, Switzerland), 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ) and 100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco). Cells were incubated in 75 cm^2 filter-cap flasks (Thermo Fisher Scientific, Waltham, MA) at 37°C in a humidified atmosphere containing 5% CO_2. For steroid hormone measurements and RNA extraction, 7.5 x 10^5 Hs578t cells were added to the wells of 6-well plates (Corning Life Sciences, Corning, NY) in 2 ml of phenol red-free DMEM supplemented with 4 mM glutamine, 0.01 mg/ml insulin (Gibco), 100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco), and 1% dextran-coated charcoal-stripped (steroid-free) FBS (Hyclone). After 24 h, the culture medium was removed and replaced with medium containing various concentration of essential oils and incubated for an additional 24 h before hormone measurements and RNA extraction. The DMSO vehicle (0.1%) was used as a negative control. Experiments were performed using cells under passage 10.

4.3.3. Cell viability

The toxicity of basil and fennel seed essential oils to Hs578t cells was determined using a WST-1 cell viability kit (Roche, Basel, Switzerland), which is based on the cleavage of a tetrazolium salt by the mitochondria in metabolically active cells. Hs578t cells were added to 96-well plates (Corning) at a density of 10^4 cells/well in 200 µl of culture medium (supplement phenol red-free DMEM medium with stripped FBS) for 24 h. After 24 h, cells were exposed to fresh medium containing increasing concentrations of essential oils for another 24 h. Cells were then incubated for 1.5 h with 20 ul of WST-1 reagent and the linear formation of formazan was measured using the SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California) at a wavelength of 450 nm.

4.3.4. Steroid hormone quantification

Steroid hormone concentrations were determined using ELISA kits obtained from DRG Diagnostics (Marburg, Germany) according to manufacturer's recommendations; kits included estradiol (EIA-2693), estrone (EIA-4174), progesterone (EIA-1561), androstenedione (EIA-3265) and dehydroepiandrosterone (DHEA; EIA-3415).

4.3.5. RNA extraction and amplification by RT-qPCR

Real-time quantitative PCR (RT-qPCR) was used to assess gene expression of steroidogenic enzymes. After a 24 h exposure to essential oils, culture medium was removed from the wells and Hs578t cells were washed twice with PBS and detached using TrypLE Express (1X) (Thermofisher), RNA was isolated using RNeasy mini kits (Qiagen, Mississauga, ON) and stored at -80°C until further analysis. After -80°C storage RNA purity was assessed by determining the 260/280 nm absorbance ratio (~2.0) using a Nanodrop (Thermofisher). Reverse transcription of 0.5 µg RNA was performed using an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON) and T3000 Thermocycler (Biometra, Göttingen, Germany); resultant cDNA was stored at -20°C. Pre-amplification of the obtained cDNA was performed using a PreAmp Kit (BioRad, Hercules, CA). Pre-amplification is an additional step that amplifies cDNA for 10 cycles using classic PCR, along with the necessary primer pairs (for reference genes and genes of interest). This method is useful to determine the level of expression of genes that are expressed at endogenously low levels, which is the case for several promoter-specific *CYP19* transcripts in Hs578t cells. The (pre-amplified) cDNA obtained was then used to perform quantitative PCR using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with a CFX96 real-time PCR Detection System (Bio-Rad). Suitable reference genes to normalize target gene expression were selected using geNorm software (Biogazelle, Zwijnaarde, Belgium) (Table 4-1). Primer pair sequences used to amplify promoter-specific *CYP19* transcripts and promoter non-distinct *CYP19* mRNA are listed in Table 4-1.

Table 4-1: Primer pair sequences used to determine the promoter-specific and promoter non-distinct expression of *CYP19*

Steroidogenic gene	**Primer Pairs (5' → 3')**	**Reference:**
CYP19 coding region (exon II-X)	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC Rv: TCACCAATAACAGTCTGGATTTCC	(Sanderson et al., 2000)
CYP19 I.4	Fw: GGATCTTCCAGACGTCGCGA Rv: CATGGCTTCAGGCACGATGC	(Klempan et al., 2011)
CYP19 PII	Fw: TCTGTCCCTTTGATTTCCACAG Rv: GCACGATGCTGGTGATGTTATA	(Heneweer et al., 2004)
CYP19 I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA Rv: AGAGGGGGCAAT TTAGAGTCTGTT	(Wang et al., 2008)
CYP19 I.7	Fw: ACACTCAGCTTTTTCCCAACA Rv: TTTCACCCCTTTCTCCGGTC	(Caron-Beaudoin et al., 2018)
Reference gene	**Primer Pairs (5' → 3')**	**Reference:**
TBP	Fw: TGCACAGGAGCCAAGAGTGAA Rv: CACATCACAGCTCCCCACCA	(Tratwal et al., 2014)
RPLO	Fw: GGCGACCTGGAAGTCCAACT Rv: CCATCAGCACCACAGCCTTC	(Good et al., 2016)
PBGD	Fw: GGCAATGCGGCTGCAA Rv: GGGTACCCACGCGAATCAC	(Dolstra et al., 1999)

4.3.6. Aromatase catalytic activity

After a 24 h period of cell acclimatization following plating in 24-well plates, Hs578t cells were exposed to different concentrations of essential oils for an additional 24 h. After, the treatment medium was removed, cells were washed twice with PBS, and exposed to 54 nM of 1β-3H- androstenedione in serum-free culture medium (250ml per well) for 1.5h at 37°C. Additional steps were as described previously (Lephart & Simpson, 1991; Sanderson et al., 2000). The amount of tritiated water released, as a measure of aromatase activity, was determined using a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Negative controls contained radioactive substrate but no cells. A positive control radioactive substrate was directly added to scintillation cocktail. Cellular aromatase activity was expressed as a percent of vehicle control activity (cells exposed to 0.1% DMSO). Formestane (4-hydroxyandrostenedione) (1 μM), a selective aromatase inhibitor, was used to verify the specificity of the assay for the enzymatic aromatization reaction

4.3.7. Kinase activity and cAMP levels

Activation of PKA was evaluated using a PKA kinase activity assay kit and cAMP levels using a direct cAMP immunoassay kit (Abcam, Cambridge, UK) following the manufacturer's

instructions. After Hs578t cells were exposed to basil or fennel seed essential oil for 24 h, cells were lysed according to manufacturer's instructions in culture plates and supernatants were collected after 15 min of centrifugation at 15,000g to remove cell debris. The supernatants were stored at -80°C prior to measurements of kinase activity and cAMP levels.

4.3.8. Statistical analysis

All data were analyzed using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA). Results are presented as means with standard error (SEM) of three different experiments; per experiment each treatment was tested in triplicate. Statistically significant differences from control (*p<0.05; **p<0.01; ***p<0.001) were determined using one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test.

4.4 Results

4.4.1. The effects of basil and fennel seed essential oils on Hs578t cell viability

A 24 h exposure to increasing concentrations of basil or fennel seed essential oils (0.0005%, 0.001%, 0.005%, 0.01%, and 0.05%) did not statistically affect the viability of Hs578t cells based on mitochondrial reductase activity (Fig. 4-1).

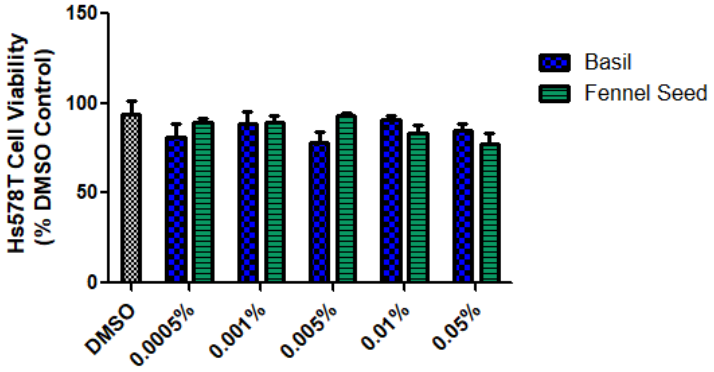

Figure 4-1*: Viability of Hs578t cells exposed for 24 h to various concentrations of basil or fennel seed essential oil as a percentage of DMSO vehicle control (0.1%).*

Results are presented as the mean ± SEM (n=3).

4.4.2. The effects of basil and fennel seed essential oils on hormone production in Hs578t cells

Basil essential oil significantly increased progesterone production by 2.1 fold at concentrations of 0.01% (0.49 ± 0.1 ng/ml) and 0.05% (0.5 ± 0.1 ng/ml) compared to DMSO control (0.23 ± 0.1 ng/ml) (Fig. 4-2A). There was no significant effect on DHEA and androstenedione production after exposure to all concentrations of basil essential oil studied (Fig. 4-2B,C). Basil essential oil significantly decreased estradiol production at a concentration of 0.001% (63.3 ± 5.8 pg/ml), 0.005% (62.2 ± 3.8 pg/ml), 0.01% (56.6 ± 2.9 pg/ml), and 0.05% (61.0 ± 6.7 pg/ml) compared to vehicle control (81.5 ± 5.8 pg/ml) (Fig. 4-2D). Estrone levels were significantly increased by basil essential oil treatment by approximately 4.1 fold for concentrations of 0.001%, 0.005%, and 0.01% (43.8 ± 4.5 pg/ml, 44.4 ± 4.1 pg/ml, and 42.4 ± 6.4 pg/ml, respectively) and by 3.2 fold for a concentration of 0.05% (34.5 ± 4.5 pg/ml) compared to DMSO control (10.8 ± 1.1 pg/ml) (Fig. 4-2E).

Fennel seed essential oil significantly increased progesterone production by 1.9 fold at concentrations of 0.005% and 0.01% (0.45 ± 0.1 ng/ml and 0.45 ±0.1 ng/ml, respectively) and by 2.3 fold at 0.05% (0.54 ± 0.1 ng/ml) compared to DMSO control (Fig. 4-2A). DHEA and androstenedione production was not effected by fennel seed essential oil treatment (Fig. 4-2B-C). Estradiol production significantly increased by fennel seed essential oil at concentration of 0.001% (59.9 ± 4.9 pg/ml), 0.005% (60.6 ± 4.6 pg/ml), 0.01% (52.4 ± 3.7 pg/ml), and 0.05% (60.7 ± 3.8 pg/ml) compared to vehicle control (Fig. 4-2D). On the other hand, fennel seed essential oil treatment significantly increased estrone production levels by 3.2 fold at 0.005% (34.3 ± 4.9 pg/ml) and by 3.6 fold at 0.01% (38.6 ± 8.4 pg/ml) (Fig. 4-2E).

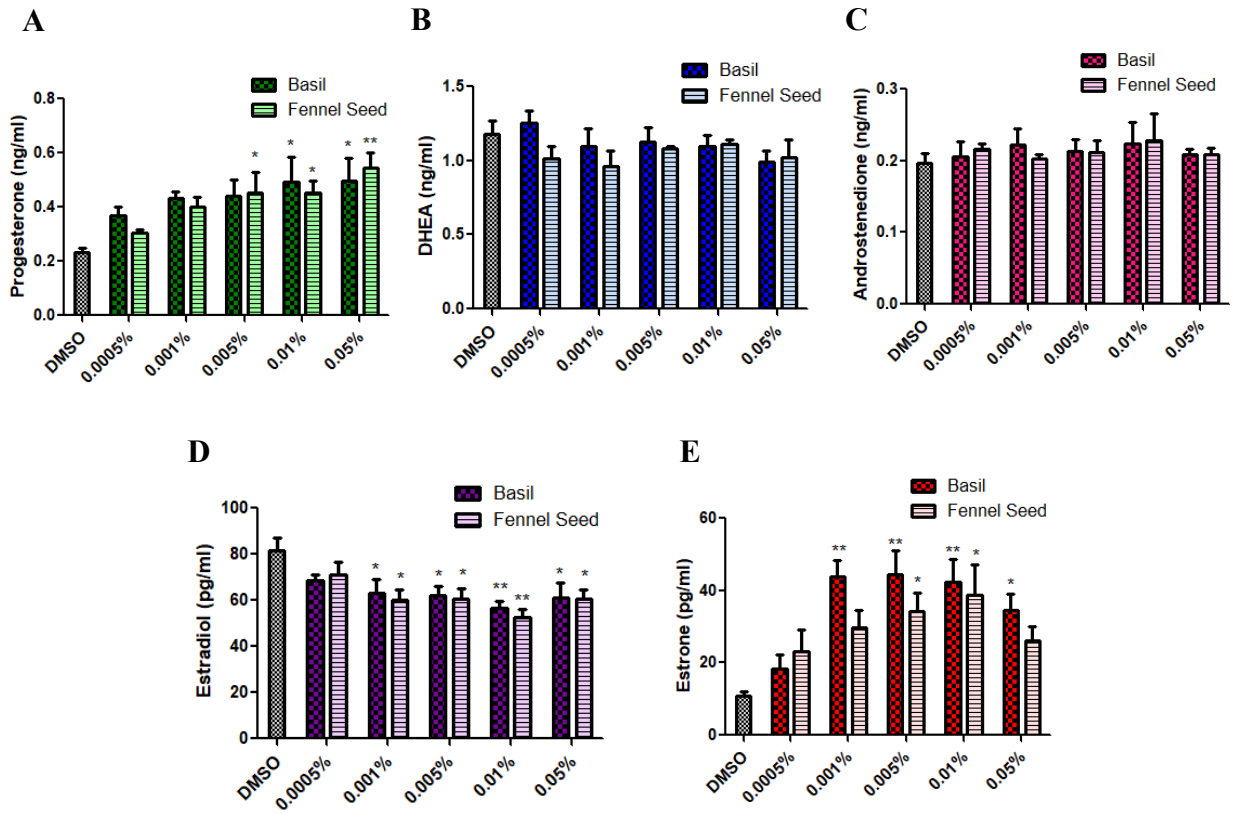

Figure 4-2: *Progesterone (A), DHEA (B), androstenedione (C), estradiol (D), and estrone (E) production (mean ± SEM; n=3) by Hs578t cells after a 24 h treatment with basil or fennel seed essential oil.*

Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

4.4.3. Promoter-specific expression of CYP19 in Hs578t cells

In Hs578t cells, basil essential oil concentrations of 0.001%, 0.005% and 0.01% significantly increased expression of promoter non-distinct *CYP19* (exon II-X coding region) by 3.6 ± 0.5 fold, 3.6 ± 0.6 fold, and 4.0 ± 0.4 fold, respectively, compared to DMSO vehicle control (Fig. 4-3A). This was associated with a concomitant increase in PII-mediated *CYP19* expression at the same concentrations by 3.0 ± 0.4 fold, 3.5 ± 0.5 fold, and 3.7 ± 0.7 fold, respectively. (Fig. 4-3A). The increases in promoter PII-mediated and promoter non-distinct *CYP19* expression also concurred with significant decreases in promoter I.4-mediated *CYP19* expression, which is a signature characteristic of the promoter-switch that occurs in breast cancer tissues. Basil essential oil concentrations of 0.001%, 0.005%, and 0.01% decreased I.4-mediated

CYP19 expression to about 0.5 ± 0.1 fold of DMSO control in for compared to DMSO control (Fig. 4-3A). At 0.05%, the statistically significant effects of basil oil on PII-, I.4- and promoter non-distinct *CYP19* expression were no longer observed (Fig. 4-3A). We did not detect any statistically significant changes in promoter I.3- and I.7-mediated *CYP19* expression (Fig. 4-3A).

Fennel seed essential oil concentrations of 0.001%, 0.005%, 0.01% and 0.05% significantly increased expression of promoter non-distinct *CYP19* (exon II-X coding region) by 3.9 ± 0.3 fold, 4.5 ± 0.5 fold, 4.9 ± 0.3 fold and 3.3 ± 0.7 fold respectively, compared to DMSO control (Fig. 4-3B). This was associated with a concomitant increase in PII-mediated *CYP19* expression at these concentrations by 3.1 ± 0.4, 3.7 ± 0.8 and 3.1 ± 0.6 fold, respectively, compared to DMSO control (Fig. 4-3B). The increase in PII-mediated *CYP19* expression also concurred with significant decreases of I.4-mediated *CYP19* expression by 20% at a fennel seed oil concentration of 0.001% and by 50% at concentrations of 0.005% and 0.01%; this observation is characteristic of a breast cancer-relevant promoter-switch in *CYP19* regulation. (Fig. 4-3B). We did not detect any statistically significant changes in I.3- and I.7-mediated *CYP19* expression (Fig. 4-3B).

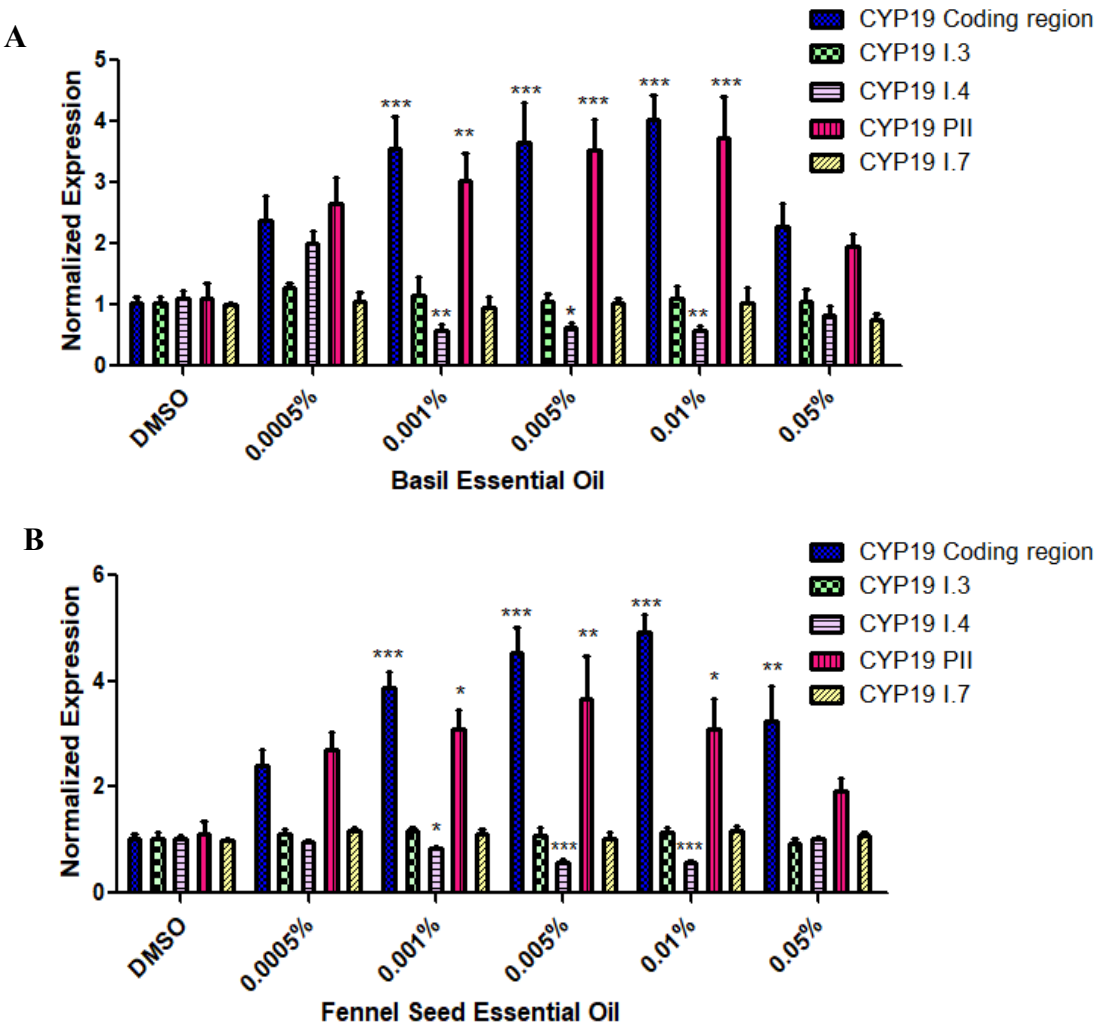

Figure 4-3*: Relative expression of promoter non-specific CYP19 coding region and I.3, I.4, PII and I.7 promoter-specific CYP19 mRNA levels in Hs578t cells after treatment with basil (A) and fennel seed (B) essential oil.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
**p < 0.05; **p < 0.01; ***p < 0.001.*
***Note**: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

4.4.4. Catalytic activity of aromatase

The catalytic activity of CYP19 was determined to confirm whether the effects of basil and fennel seed essential oils on the (promoter-specific) expression of *CYP19* in Hs578t cells translated into changes in aromatase activity. Both essential oils increased aromatase activity; basil oil by 1.6 ± 0.2 and 1.8 ± 0.1 fold, and fennel seed oil by 1.9 ± 0.1 and 1.8 ± 0.1 fold, at 0.005% and 0.01%, respectively (Fig. 4-4).

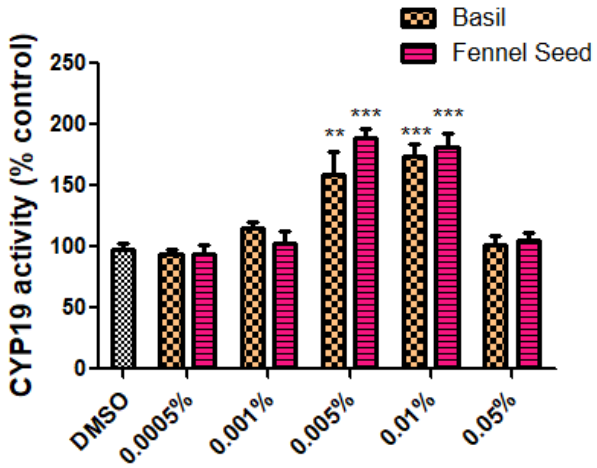

Figure 4-4: *Relative CYP19 activity in Hs578t cells after a 24 h exposure to basil or fennel seed essential oils and expressed as a percentage of DMSO.*

*Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *p < 0.05; **p < 0.01; ***p < 0.001.*

4.4.5. PKA activity and cAMP levels

To investigate whether the rise in aromatase catalytic activity and promoter PII-mediated expression of *CYP19* was caused by the action of basil and fennel seed essential oils on the PKA signaling pathway; we determined their effects on cAMP levels and PKA kinase activity in the cells.

Basil essential oil at 0.005% and 0.01% significantly increased cAMP levels by 1.7 ± 0.2 fold relative to DMSO vehicle control (Fig. 4-5A). The same concentrations increased PKA activity by 1.6 ± 0.2 and 1.7 ± 0.1 fold, respectively, relative to control (Fig. 4-5B).

Fennel seed essential oil at 0.005% and 0.001% significantly increased cAMP levels by 2.0 ± 0.1 and 1.9 ± 0.1 fold, respectively, relative to DMSO control (Fig. 4-5A). The increase in cAMP levels was lower (about 1.5 ± 0.1 fold) but significant at the higher fennel seed oil concentrations of 0.01% and 0.05% (Fig. 4-5A). PKA activity, at fennel seed concentrations of 0.005% and 0.01%, increased by an average of 1.7 ± 0.2 fold relative to DMSO (Fig. 4-5B).

At 0.05%, there was no statistically significant effect on PKA activity for both basil and fennel seed essential oil relative to DMSO control.

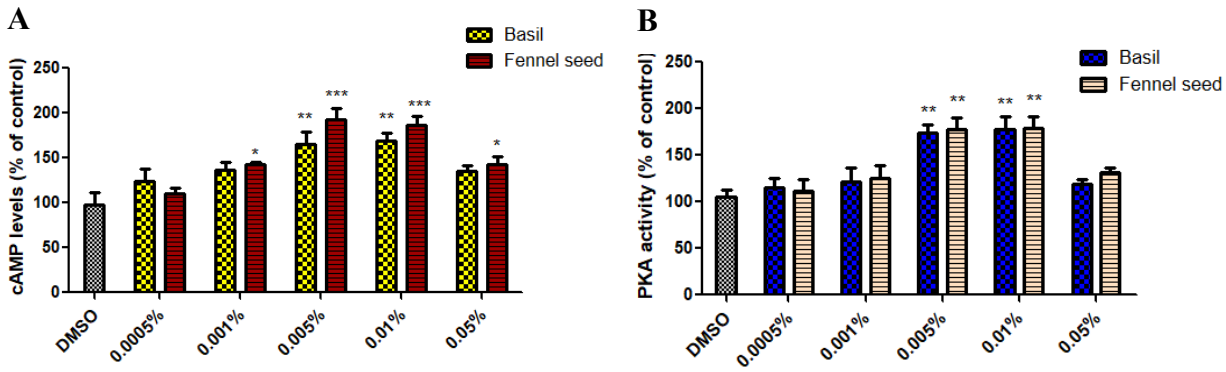

Figure 4-5: *Intracellular cAMP levels (A) and PKA activity (B) in Hs578t cells after a 24-h exposure to basil and fennel seed essential oils expressed as a percentage of DMSO.*

*Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *p < 0.05; **p < 0.01; ***p < 0.001.*

4.5 Discussion

In this study, we used Hs578t cells as representative model of the *CYP19*-expressing and estrogen-producing stromal cells found in the human estrogen-dependent breast cancer environment. Our laboratory recently demonstrated that Hs578t cells possess transcriptional activity mediated by all four breast-cancer relevant *CYP19* promoters, which include the normally active I.4 promoter and the breast cancer-associated promoters PII, I.3 and I.7 (Caron-Beaudoin et al., 2018). We used the Hs578t cells to determine whether basil and fennel seed essential oils could induce a promoter-switch in the promoter usage similar to that which occurs in the tumor tissues of hormone-dependent breast cancer patients.

Indeed this study found that basil and fennel seed essential oils triggered a promoter switch in Hs578t cells, which resulted in an increase in *CYP19* expression and overall catalytic activity of the enzyme. Both essential oils decreased I.4-mediated expression of *CYP19*, whilst simultaneously increasing expression of *CYP19* via PII. Interestingly, despite being in close proximity to PII on the *CYP19* gene and being activated via a similar mechanism of action, we did not detect a significant change in I.3-mediated *CYP19* expression, although this has been previously observed n Hs578t cells exposed to the neonicotinoids thiacloprid and imidacloprid (Caron-Beaudoin et al., 2018).

Expression of *CYP19* via I.3/PII is dependent on prostaglandin E2 (PGE2) activation of PKA and PKC through binding of the prostanoid receptors, EP1 and EP2, located on breast adipose fibroblasts (D. Chen et al., 2009). EP1 and EP2 are G-protein coupled receptors (GPCRs) that stimulate the enzyme adenylate cyclase which catalyzes the formation of cAMP which then binds and activates PKA. PKA activation is crucial for aromatase expression via promoter I.3/II, but the PKC pathway also contributes to the activity of these promoters by potentiating PKA-mediated aromatase expression (Chen et al., 2009). Once PKA and PKC pathways are activated, phosphorylation of cAMP-responsive element binding protein 1 (CREB1) occurs, which results in its translocation to the nucleus and binding to CRE-like sequences in the I.3/PII promoter regions, leading to their activation (Chen et al., 2011). We have previously showed (see Chapter 3) that the main compounds of basil and fennel seed essential oils, estragole and trans-anethole, respectively, increase cAMP levels and stimulate PKA activity in a feto-placental co-culture system of sterodiogenesis during human pregnancy, resulting in increased expression of *CYP19* through PII and placental-derived I.1. In the present study, we confirmed that basil and fennel seed essential oils significantly increased promoter PII-mediated *CYP19* expression in Hs578t cells by stimulating cAMP levels and PKA activity, with significant increases at 0.005% and 0.01%.

In this study, we have also shown that basil and fennel seed essential oil induced aromatase activity in Hs578t cells, which resulted in increased estrone levels. In addition, basil and fennel seed oil treatment resulted in a significant reduction of estradiol levels. Estrone, produced via the aromatization of androstenedione, is the major estrogen in post-menopausal women that no longer possess functional ovaries. In these women, estrogen production relies

solely on aromatase-mediated conversion of androgens precursors, mostly DHEA(-sulfate) and some androstenedione, which occurs in the adrenal cortex and adipose tissues (Grodin et al., 1973; Judd et al., 1974). Typically, estradiol levels are markedly reduced in post-menopausal women, however, decreased estradiol levels are not associated with increases in estrone levels (Kim et al., 2017). Postmenopausal women with high circulating estrone levels have increased risk of ER-positive breast cancer development and progression (Miyoshi et al., 2003). Our observation that basil and fennel seed essential oil can increase estrone production in an *in vitro* model of the stromal cell in the human breast cancer microenvironment, suggests that further studies of the impact of these oils on estrogen-dependent breast cancer risk may be warranted.

4.6 Conclusion

In conclusion, we looked at the effects of basil and fennel seed essential oils in Hs578t cells; stromal cell line found in the estrogen-dependent breast cancer. Basil and fennel seed essential oils activated a promoter-switch in Hs578t cells by increasing promoter PII- and decreasing promoter I.4-mediated *CYP19* expression, which is a hallmark of hormone-dependent breast cancer progression in humans. This promoter-switch involved increases in intracellular cAMP levels and PKA activity. Moreover, basil and fennel seed essential oils stimulated increased catalytic activity of the enzyme aromatase, resulting in increased estrone levels; major estrogen subtype in post-menopausal women. Further work needs to be conducted to better understand the effects of basil and fennel seed essential oils on estrogen biosynthesis and estrogen receptor-mediated responses in human hormone-dependent breast cancer, especially with the rising popularity of CAM use by breast cancer patients.

4.7 Acknowledgements

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4.8 Conflicts of interest

The authors declare to have no conflicts of interest.

4.9 References

Adams, R. N., Mosher, C. E., Blair, C. K., Snyder, D. C., Sloane, R., & Demark-Wahnefried, W. (2015). Cancer survivors' uptake and adherence in diet and exercise intervention trials: an integrative data analysis. Cancer, 121(1), 77-83. doi:10.1002/cncr.28978

Amato, P., Christophe, S., & Mellon, P. L. (2002). Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. Menopause, 9(2), 145-150.

Bulun, S. E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H., & Shozu, M. (2003). The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. J Steroid Biochem Mol Biol, 86(3-5), 219-224.

Caron-Beaudoin, E., Denison, M. S., & Sanderson, J. T. (2016). Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. Toxicol Sci, 149(1), 134-144. doi:10.1093/toxsci/kfv220

Caron-Beaudoin, E., Viau, R., & Sanderson, J. T. (2018). Effects of Neonicotinoid Pesticides on Promoter-Specific Aromatase (CYP19) Expression in Hs578t Breast Cancer Cells and the Role of the VEGF Pathway. Environ Health Perspect, 126(4), 047014. doi:10.1289/ehp2698

Chen, D., Reierstad, S., Fang, F., & Bulun, S. E. (2011). JunD and JunB Integrate Prostaglandin E(2) Activation of Breast Cancer-Associated Proximal Aromatase Promoters. Molecular Endocrinology, 25(5), 767-775. doi:10.1210/me.2010-0368

Chen, D., Reierstad, S., Lu, M., Lin, Z., Ishikawa, H., & Bulun, S. E. (2009). Regulation of breast cancer-associated aromatase promoters. Cancer Lett, 273(1), 15-27. doi:10.1016/j.canlet.2008.05.038

Demark-Wahnefried, W., Rogers, L. Q., Alfano, C. M., Thomson, C. A., Courneya, K. S., Meyerhardt, J. A., . . . Ligibel, J. A. (2015). Practical clinical interventions for diet, physical activity, and weight control in cancer survivors. CA Cancer J Clin, 65(3), 167-189. doi:10.3322/caac.21265

Dolstra, H., Fredrix, H., Maas, F., Coulie, P. G., Brasseur, F., Mensink, E., . . . van de Wiel-van Kemenade, E. (1999). A Human Minor Histocompatibility Antigen Specific for B Cell Acute Lymphoblastic Leukemia. The Journal of Experimental Medicine, 189(2), 301-308. doi:10.1084/jem.189.2.301

Eisenberg, D. M., Davis, R. B., Ettner, S. L., Appel, S., Wilkey, S., Van Rompay, M., & Kessler, R. C. (1998). Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. Jama, 280(18), 1569-1575.

Good, M., Sodhi, C. P., Yamaguchi, Y., Jia, H., Lu, P., Fulton, W. B., . . . Hackam, D. J. (2016). The human milk oligosaccharide 2'-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. Br J Nutr, 116(7), 1175-1187. doi:10.1017/s0007114516002944

Grodin, J. M., Siiteri, P. K., & MacDonald, P. C. (1973). Source of estrogen production in postmenopausal women. J Clin Endocrinol Metab, 36(2), 207-214. doi:10.1210/jcem-36-2-207

Hedderson, M. M., Patterson, R. E., Neuhouser, M. L., Schwartz, S. M., Bowen, D. J., Standish, L. J., & Marshall, L. M. (2004). Sex differences in motives for use of complementary and alternative medicine among cancer patients. Altern Ther Health Med, 10(5), 58-64.

Heneweer, M., Muusse, M., Dingemans, M., de Jong, P. C., van den Berg, M., & Sanderson, J.

T. (2005). Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci, 83(2), 257-263. doi:10.1093/toxsci/kfi025
Heneweer, M., van den Berg, M., & Sanderson, J. T. (2004). A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett, 146(2), 183-194.
Judd, H. L., Judd, G. E., Lucas, W. E., & Yen, S. S. (1974). Endocrine function of the postmenopausal ovary: concentration of androgens and estrogens in ovarian and peripheral vein blood. J Clin Endocrinol Metab, 39(6), 1020-1024. doi:10.1210/jcem-39-6-1020
Kim, C., Harlow, S. D., Zheng, H., McConnell, D. S., & Randolph, J. F., Jr. (2017). Changes in androstenedione, dehydroepiandrosterone, testosterone, estradiol, and estrone over the menopausal transition. Womens Midlife Health, 3. doi:10.1186/s40695-017-0028-4
Kiyama, R. (2017). Estrogenic Potentials of Traditional Chinese Medicine. The American Journal of Chinese Medicine, 45(07), 1365-1399. doi:10.1142/s0192415x17500756
Klempan, T., Hudon-Thibeault, A. A., Oufkir, T., Vaillancourt, C., & Sanderson, J. T. (2011). Stimulation of serotonergic 5-HT2A receptor signaling increases placental aromatase (CYP19) activity and expression in BeWo and JEG-3 human choriocarcinoma cells. Placenta, 32(9), 651-656. doi:10.1016/j.placenta.2011.06.003
Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., & Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology, 138(3), 863-870. doi:10.1210/endo.138.3.4979
Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat, 83(3), 249-289. doi:10.1023/B:BREA.0000014042.54925.cc
Lumachi, F., Santeufemia, D. A., & Basso, S. M. (2015). Current medical treatment of estrogen receptor-positive breast cancer. World J Biol Chem, 6(3), 231-239. doi:10.4331/wjbc.v6.i3.231
Ma, H., Sullivan-Halley, J., Smith, A. W., Neuhouser, M. L., Alfano, C. M., Meeske, K., . . . Bernstein, L. (2011). Estrogenic botanical supplements, health-related quality of life, fatigue, and hormone-related symptoms in breast cancer survivors: a HEAL study report. BMC Complement Altern Med, 11, 109. doi:10.1186/1472-6882-11-109
Macon, M. B., & Fenton, S. E. (2013). Endocrine disruptors and the breast: early life effects and later life disease. Journal of mammary gland biology and neoplasia, 18(1), 43-61. doi:10.1007/s10911-013-9275-7
Meng, L., Zhou, J., Sasano, H., Suzuki, T., Zeitoun, K. M., & Bulun, S. E. (2001). Tumor necrosis factor alpha and interleukin 11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively down-regulating CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma: mechanism of desmoplastic reaction. Cancer Res, 61(5), 2250-2255.
Miyoshi, Y., Tanji, Y., Taguchi, T., Tamaki, Y., & Noguchi, S. (2003). Association of serum estrone levels with estrogen receptor-positive breast cancer risk in postmenopausal Japanese women. Clin Cancer Res, 9(6), 2229-2233.
Neuhouser, M. L., Smith, A. W., George, S. M., Gibson, J. T., Baumgartner, K. B., Baumgartner,

R., . . . Ballard, R. (2016). Use of complementary and alternative medicine and breast cancer survival in the Health, Eating, Activity, and Lifestyle Study. Breast Cancer Res Treat, 160(3), 539-546. doi:10.1007/s10549-016-4010-x

Odawara, H., Iwasaki, T., Horiguchi, J., Rokutanda, N., Hirooka, K., Miyazaki, W., . . . Koibuchi, N. (2009). Activation of aromatase expression by retinoic acid receptor-related orphan receptor (ROR) alpha in breast cancer cells: identification of a novel ROR response element. J Biol Chem, 284(26), 17711-17719. doi:10.1074/jbc.M109.009241

Patterson, R. E., Neuhouser, M. L., Hedderson, M. M., Schwartz, S. M., Standish, L. J., Bowen, D. J., & Marshall, L. M. (2002). Types of alternative medicine used by patients with breast, colon, or prostate cancer: predictors, motives, and costs. J Altern Complement Med, 8(4), 477-485. doi:10.1089/107555302760253676

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., . . . Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res, 12(5), R68. doi:10.1186/bcr2635

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun, 313(4), 856-862.

Roumeliotis, G. A., Dostaler, G., & Boyd, K. U. (2017). Complementary and Alternative Medicines and Patients With Breast Cancer: A Case of Mortality and Systematic Review of Patterns of Use in Patients With Breast Cancer. Plast Surg (Oakv), 25(4), 275-283. doi:10.1177/2292550317716126

Sanderson, J. T., Seinen, W., Giesy, J. P., & van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci, 54(1), 121-127.

Sebastian, S., Takayama, K., Shozu, M., & Bulun, S. E. (2002). Cloning and characterization of a novel endothelial promoter of the human CYP19 (aromatase P450) gene that is up-regulated in breast cancer tissue. Mol Endocrinol, 16(10), 2243-2254. doi:10.1210/me.2002-0123

Tratwal, J., Follin, B., Ekblond, A., Kastrup, J., & Haack-Sørensen, M. (2014). Identification of a common reference gene pair for qPCR in human mesenchymal stromal cells from different tissue sources treated with VEGF. BMC Molecular Biology, 15(1), 11. doi:10.1186/1471-2199-15-11

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, 3(7), Research0034.

Wang, Y., Gho, W. M., Chan, F. L., Chen, S., & Leung, L. K. (2008). The red clover (trifolium pretense) isoflavone biochenin A inhibits aromatase activity and expression. Brit J Nutr, 99.

Waumans, D., Bruneel, N., & Tytgat, J. (2003). Anise oil as para-methoxyamphetamine (PMA) precursor. Forensic Science International, 133(1), 159-170. doi:https://doi.org/10.1016/S0379-0738(03)00063-X

Zhou, J., Gurates, B., Yang, S., Sebastian, S., & Bulun, S. E. (2001). Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. Cancer Res, 61(5), 2328-2334.

Chapter 5 : DEVELOPMENT OF AN ESTROGEN-DEPENDENT BREAST CANCER CO-CULTURE MODEL AS A TOOL FOR STUDYING ENDOCRINE DISRUPTORS

Debbie Yancu*, Rachel Viau and Thomas Sanderson*

INRS - Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada

* Corresponding author at:
INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC H7V 1B7, Canada
Laval, QC H7V 1B7, Canada.
E-mail addresses: debbie.yancu@iaf.inrs.ca (D. Yancu) and thomas.sanderson@iaf.inrs.ca (J.T. Sanderson)

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Author contributions:
Debbie Yancu developed the hypothesis and objectives, designed and performed the experiments, performed the statistical analyses and wrote the manuscript.
Rachel Viau assisted in designing and performing experiments.
Prof. Thomas Sanderson obtained the research funding, supervised the project and participated in writing and revising the manuscript.

5.1 Abstract

We developed an innovative co-culture system composed of Hs578t human mammary stromal-like cells and T47D hormone-dependent breast epithelial tumor cells as a representative *in vitro* model of the human hormone-dependent mammary tumor microenvironment. Hs578t cells expressed aromatase (CYP19) mainly via the healthy stromal *CYP19* promoter I.4, but also to a lesser extent via the breast cancer-relevant promoters PII, I.3 and I.7, and produced estrogens from androgen precursors. These estrogens stimulated T47D cell proliferation and estrogen receptor-dependent expression of trefoil factor-1 (*TFF1*), which is known to stimulate mammary tumor cell proliferation and migration, but the cells can undergo a "promoter-switch" where the normally silent *CYP19* promoters PII, I.3 and I.7 become activated, which mimics the *in vivo* situation in human breast cancer patients. This positive feedback loop is the hallmark of the hormone-dependent breast tumor microenvironment. Using the co-culture model we designed, we evaluated the promoter-specific expression of *CYP19*, expression of estrogen-dependent gene *TFF1*, and determined the effects exhibited by basil and fennel seed essential oils on steroidogenesis in the tumor microenvironment.

Keywords:

Estrogen-dependent breast cancer
Aromatase
Hs578t
T47D
Co-culture
Essential oils

5.2 Introduction

The notion of cross-talk between a tumor and its direct environment is not new, with reports dating back to the 19th century describing the complexity of the cancer microenvironment, in which numerous cell types play an important role in the progression of the disease (Paget, 1889). Despite cancerous cell lines being available since the middle of the 1950s, *in vitro* experiments using only a single cell type cannot effectively mimic a cancer microenvironment. To better replicate an *in vivo* human tumor microenvironment, co-culture systems composed of at least two cell types relevant to the tumor have been either been proposed

or established. Breast cancer is the most common cancer worldwide in women and is, in 60% of cases, initially hormone-dependent, the majority of which are dependent on estrogen and progesterone (World Cancer Research Fund, 2018; Lumachi et al., 2015) Hormone-dependent breast cancer in women involves multiple interactions between epithelial carcinoma cells and non-tumorigenic stromal cells (Yamaguchi & Hayashi, 2009); thus, the use of co-culture systems using these different cell types is warranted to appropriately study potential therapeutic interventions, particularly since it has been established that the cancer microenvironment is a major factor prompting treatment resistance (Siemann, 2011). The few breast cancer co-culture systems that have been developed have focused on the communication between epithelial breast tumor cells and healthy primary human mammary associated fibroblasts (Heneweer et al., 2005; Wang et al., 2015). However, the cross-talk that exists between breast cancer cells with epithelial phenotype and those that have acquired mesenchymal morphology within the multifaceted breast cancer microenvironment is only beginning to be explored and will have significant impact in our understanding of any beneficial or adverse effects new treatments or environmental exposures could have on breast cancer growth and progression.

The stromal-tumoral interchange of steroid hormones and growth/inflammatory factors plays and important role in human mammary tumor growth and progression (Imagawa et al., 2002). The stroma contains aromatase, the key enzyme catalyzing the final step in the biosynthesis of estrogens from androgen precursors (Bulun et al., 2003). Mammary stromal cells, including fibroblasts (pre-adipocytes) and adipocytes have been shown to support estrogen-receptor(ER)α-positive breast cancer progression to metastasis. Fibroblasts have inducible aromatase activity before they differentiate into mature adipocytes which have far less aromatase activity (Meng et al., 2001; Zhou et al., 2001a). In breast cancer, a desmoplastic reaction is initiated when malignant epithelial tumor cells secrete a variety of inflammatory factors (tumor necrosis factor-α, prostaglandin E2, interleukin-6 and interleukin-11) into the tumor microenvironment, which are able to stimulate *CYP19* gene transcription and aromatase activity in fibroblasts as well as preventing their differentiation into mature adipocytes, resulting in an overproduction of estrogens (Meng et al., 2001; Zhou et al., 2001a). A positive feedback mechanism is created when the estrogens synthesized by the undifferentiated fibroblasts bind to ERs on the epithelial tumor cells, in turn stimulating further production of factors to activate fibroblast aromatase expression leading to ER-positive breast cancer progression (Fig. 5-1)

(Bulun et al., 2005; Saha Roy & Vadlamudi, 2012). Importantly, these stromal-tumoral interactions and the positive feedback loop they form cannot be replicated using cell-based reporter assays with single cell types in monoculture and currently require animal testing. Experimental rodents are poor models for the stromal-tumoral microenvironment of human breast cancer since they do not express mammary aromatase, but they are used nonetheless or studies resort to non-human primates, such as the Macaque monkey (Cline & Wood, 2005; Dewi et al., 2016; Wood et al., 2013).

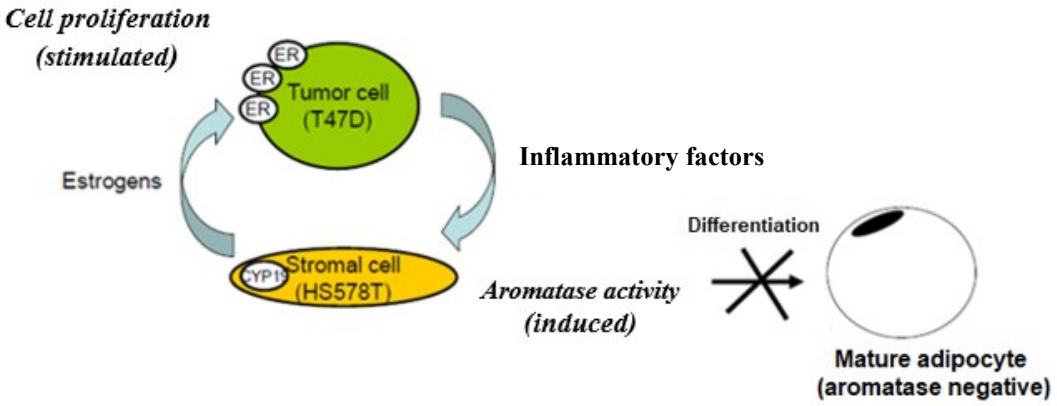

Figure 5-1: *Stromal-tumor microenvironment; a positive feedback mechanism for tumor growth. Hs578t: human stromal-like mammary cells; T47D: human hormone-dependent mammary tumor cells.*

The aromatase enzyme is regulated in a highly complex, tissue-specific manner through the alternate use of at least 10 distinct promoters located in the first exon of the *CYP19* gene (Bulun et al., 2003). Four of these promoters have been shown to be active in breast cancer (Chen et al., 2009; Odawara et al., 2009; Sebastian et al., 2002). In healthy breast stromal cells, *CYP19* expression (and thus aromatase activity) is regulated via the adipose-specific I.4 promoter. The estrogens produced locally by stromal aromatase ensure the differentiation of breast epithelial cells and maintain ER-mediated functions of the breast epithelium. In hormone-dependent breast cancer, however, a promoter switch occurs in which the tumor cells (usually of epithelial origin) secrete factors that repress the activity of the I.4 promoter of *CYP19* and activate the normally silent promoters PII, I,3 and I.7 (Sebastian et al., 2002; Zhou et al., 2001b).

To study the interactions that take place in the stromal-tumoral microenvironment of estrogen-dependent breast tumors (Khamis et al., 2012), we developed a co-culture model consisting of Hs578t human stromal-like and T47D human estrogen-dependent epithelial cancer cells that allowed us to recreate the stromal-tumoral positive feedback mechanism *in vitro* . Hs578t cells have several unique properties: (1) they are 'triple negative', not expressing estrogen (ER), progesterone (PR) or HER2/neu receptors; (2) they are derived from a carcinosarcoma, a rare stromal-like tumor and are considered "mesenchymal- or stromal-like", because the cells have low to negligible expression of epithelial luminal markers (such as E-cadherin, P-cadherinand claudins) and express high levels of mesenchymal markers (such as vimentin and N-cadherin); they also display a fibroblast-like phenotype in cell culture (Prat et al., 2010); and (3) our laboratory demonstrated that Hs578t cells express aromatase regulated by the mammary stromal I.4 promoter of *CYP19* (Caron-Beaudoin et al., 2018). Furthermore, we discovered that a breast cancer-associated promoter-switch, such as occurs *in vivo*, can be induced in these cells (Caron-Beaudoin et al., 2018). The cells proliferate relatively slowly, but do not undergo senescence after several passages as do primary cells or its related cell line Hs578Bst. Taken together, these characteristics render this cell line an excellent model of the human mammary stroma, or more precisely, the cancer-associated fibroblasts that surround an epithelial breast tumor (Alkasalias et al., 2018).

The other component of the co-culture model, T47D cells, express estrogen and androgen receptors, but do not produce their own estrogens and are dependent on estrogens provided by the surrounding stroma (the Hs578t component) in order to proliferate and secrete the various inflammatory factors required to 'condition' the surrounding stroma into increasing its aromatase activity and producing more estrogens. We chose T47D cells over MCF-7 cells as they express higher levels of ER and are more responsive to stimulation by estrogens, making them a more sensitive biosensor of estrogenic activity (Lacroix & Leclercq, 2004). To demonstrate an application of the developed co-culture model, we evaluated the effects of basil and fennel seed essential oils, which we recently showed to disrupt aromatase expression and estrogen production in Hs578t cells in monoculture (see Chapter 4).

5.3 Materials and methods

5.3.1. Treatments

A number of pharmacological compounds with established mechanisms of action were included as controls to delineate the effects of activation or inhibition of known specific cell signaling pathways on promoter-specific *CYP19* expression, aromatase activity and estrogen biosynthesis (Table 5-1). Dexamethasone, a glucocorticoid known to induce I.4-mediated *CYP19* expression (Sigma-Aldrich, St-Louis, MO) and forskolin (Sigma-Aldrich), a known inducer of PII/I.3-mediated *CYP19* expression, were used as positive control. Testosterone (Sigma), a substrate for aromatase, and estradiol (Sigma) were used to determine effects on *CYP19* expression in Hs578t cells and estrogenic responses in T47D cells. Basil and fennel seed essential oil were obtained from Rocky Mountain Oils (Orem, UT, USA) and supplied with lot-specfic GC/MS reports of their chemical composition (see supplementary material). Essential oil concentrations ranged from 0.0005% to 0.05%. Basil and fennel seed essential oils were dissolved in DMSO and the final DMSO concentration in culture medium was 0.1%.

Table 5-1: Pharmacological compounds selected to determine the promoter-specific expression of *CYP19* and expression of ER-responsive gene, *TFF1*, in a co-culture of Hs578t and T47D cells

Treatment	Concentration	Known endocrine activity
Forskolin	20 µM	PKA stimulant that activates *CYP19* PII and I.3
Dexamethasone	1 µM	Glucocorticoid that stimulates *CYP19* I.4
Testosterone	1-100 µM	Androgen precursor for estrogen biosynthesis
Estradiol	0.1-100 pM	Estrogen receptor (ER) agonist

5.3.2. Cell culture and co-culture model assembly

T47D cells (American Type Culture Collection (ATCC), no. HTB-133) are tumorigenic epithelial cells isolated from a ductal carcinoma and used to represent ER-positive hormone-dependent breast cancer. Hs578t cells (ATCC, no. HTB-126) are triple-negative breast cancer cells with mesenchymal properties derived from a carcinosarcoma; a rare stromal-like tumor. For propagation, T47D and Hs578t cells were cultured in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12 (DMEM/F12) with phenol red, containing 1.2 g/L sodium bicarbonate and 2 mg/L pyridoxine HCl (Gibco, Luzern, Switzerland). Medium was completed with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ), 0.01 mg/mL of bovine insulin, (Sigma-Aldrich) and

100 IU/ml of penicillin and 100ug/ml of streptomycin (Gibco). Cells were cultured in 75cm^2 filter-cap culture flasks placed in 37°C humidified atmosphere containing 5% CO_2.

One of the major challenges in the development of a co-culture model is the ability to maintain the characteristics of the individual cell types under co-culture conditions. For this, an optimal co-culture medium was created based on the ATCC-recommended culture medium for Hs578t cells with some modifications. The co-culture media was DMEM/F12 without phenol red (Gibco) supplemented with 1% stripped FBS (Hyclone). For monoculture experiments, T47D cells were seeded in 6-well plates at a density of 1.0 x 10^5 cells/well and Hs578t cells were seeded in transwell inserts (polycarbonate membrane with 0.4 µM pores, Corning Life Sciences, Corning, NY) at a density of 2.0 x 10^5 cells/well with 2 ml of the co-culture media each. For co-culture experiments, T47D cells were seeded in 6-well plates at a density of 1.0 x 10^5 cells/well in 1.5 ml of co-culture media and Hs578t cells were seeded at a density of 2.0 x 10^5 cells/well in transwell inserts of 6-well culture plates in 1 ml of co-culture media with 1.5 ml of culture media put into the wells. For both monoculture and co-culture experiments, cells were allowed to acclimatize for 24 h prior to treatments and/or assembly of the transwell system (Fig. 5-2). For certain co-culture experiments, the co-culture medium was supplemented with testosterone. Exposures to pharmacological compounds was for 72 h. T47D and Hs578t cells were used between passages 5 and 10.

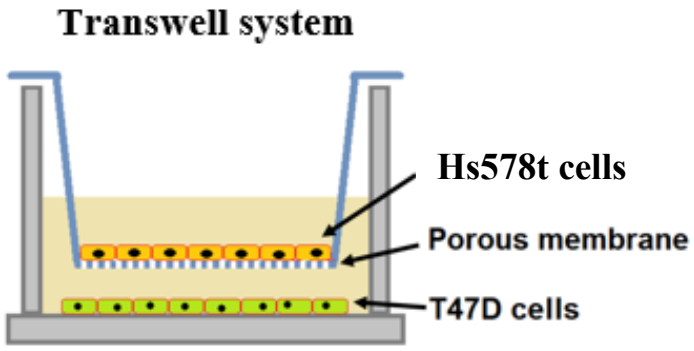

Figure 5-2: *Breast cancer co-culture model set-up. Hs578t cells were added to transwell inserts placed into the wells of 6-well culture plates containing T47D cells. Intercellular communication was achieved through the porous membrane of the transwell inserts.*

5.3.3. Cell viability

The effect of the co-culture medium on Hs578t and T47D cell proliferation/viability was compared to that of cells in their recommended monoculture media using a WST-1 assay kit (Roche, Basil, Switzerland). Hs578t and T47D cells were seeded separately in 96-well plates at a density of $1x10^4$ cells/well in 200 µl of co-culture media for 48 h, after which they were exposed to WST-1 reagent Absorbance (450nm) was measured at 30 min intervals to determine changes in cell viability over time using the SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA).

5.3.4. RNA isolation and RT-qPCR analysis

Real-time quantitative PCR (RT-qPCR) was used to assess levels of gene expression of steroidogenic enzymes. Cells were added to 6-well plates and acclimatized for 24 h, after which they were exposed for 72 h to various treatments. DMSO (0.1%) was used as a vehicle control. For monoculture experiments, T47D cells were removed from the wells and Hs578t cells from the inserts of separate plates (with wells containing co-culture medium only), washed with PBS and then detached with trypsin. In co-culture experiments, Hs578t cells in the inserts were isolated separately from the T47D cells in the wells below. RNeasy mini kits (Qiagen, Mississauga, ON) were used to isolate RNA, which was then stored at -80°C. RNA purity was assessed by determining the 260/280 nm absorbance ratio (~2.0) using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using 0.5 µg RNA with an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) and T3000 Thermocycler (Biometra, Göttingen, Germany); resultant cDNA was stored at -20°C. Preamplification of the obtained cDNA was performed using the PreAmp Kit (BioRad). Pre-amplification is an additional step that amplifies cDNA for 10 cycles using classic PCR, along with the necessary primer pairs (for reference genes and genes of interest). This method is useful to determine the level of expression of genes that are expressed at endogenously low levels, which is the case for several promoter-specific *CYP19* transcripts in Hs578t cells. The (pre-amplified) cDNA obtained was then used to perform quantitative PCR using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with a CFX96 real-time PCR Detection System (Bio-Rad). Suitable reference genes to normalize target gene expression were selected using geNorm software

(Biogazelle, Zwijnaarde, Belgium) (Table 4-1). Primer pair sequences used to amplify promoter-specific CYP19 transcripts and promoter non-distinct *CYP19* mRNA are listed in Table 5-2.

In this study, the primer pairs used were designed to amplify mRNA species containing an untranslated 5' region uniquely derived from the promoters I.4, PII, I.3 or I.7 of *CYP19*. A primer pair designed to recognize only the coding region (exons II-X) was used to amplify overall (promoter non-distinct) *CYP19* transcript. The primer pair used for the amplification of the ER-dependent gene *TFF1* in estrogen-dependent breast cancer has been reported previously (Gillesby & Zacharewski, 1999; Heneweer et al., 2005; Li et al., 2009). A minimum of two suitable reference genes were included for reliable quantification using the geNorm algorithm method (Biogazelle qbase Plus software, Zwijnaarde, Belgium). All validated primer pair sequences are listed in Table 5-2.

Table 5-2: Primer pair sequences used to determine promoter-specific expression of *CYP19 and ER-responsive gene, TFF1*.

Steroidogenic gene	**Primer Pairs (5' → 3')**	**Reference:**
CYP19 coding region (exon II-X)	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC Rv: TCACCAATAACAGTCTGGATTTCC	(Sanderson et al., 2000)
CYP19 I.4	Fw: GGATCTTCCAGACGTCGCGA Rv: CATGGCTTCAGGCACGATGC	(Klempan et al., 2011)
CYP19 PII	Fw: TCTGTCCCTTTGATTTCCACAG Rv: GCACGATGCTGGTGATGTTATA	(Heneweer et al., 2004)
CYP19 I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA Rv: AGAGGGGGCAAT TTAGAGTCTGTT	(Wang et al., 2008)
CYP19 I.7	Fw: ACACTCAGCTTTTTCCCAACA Rv: TTTCACCCCTTTCTCCGGTC	(Caron-Beaudoin et al., 2018)
TFF1	S : TTTGGAGCAGAGAGGAGGCAATGG A : TGGTATTAGGATAGAAGCACCAGGG	(Gillesby and Zacharewski, 1999)
Reference gene	**Primer Pairs (5' → 3')**	**Reference:**
TBP	Fw: TGCACAGGAGCCAAGAGTGAA Rv: CACATCACAGCTCCCCACCA	(Tratwal et al., 2014)
RPLO	Fw: GGCGACCTGGAAGTCCAACT Rv: CCATCAGCACCACAGCCTTC	(Good et al., 2016)
PBGD	Fw: GGCAATGCGGCTGCAA Rv: GGGTACCCACGCGAATCAC	(Dolstra et al., 1999)

5.3.5. Aromatase catalytic activity

Hs578t cells were seeded at a density of 1 x 10^4 cells into transwell inserts of a 24-well plate in 0.2ml/insert and 0.8ml of co-culture media was added to the wells underneath the inserts. In another 24-well plate, T47D cells were seeded at a density of 2.5 x 10^4 cells/well in 1 ml of co-culture media. After a 24 h hour period of cell acclimatization following plating, the co-culture was assembled and exposed to essential oils for an additional 72 h time period. After, the treated medium was removed, inserts were then washed twice with PBS, placed into 12-well plates, and exposed to 54nM of 1β-3H- androstenedione in serum-free culture medium (50 µl) for 1.5h at 37°C. Additional steps were as described previously (Lephart & Simpson, 1991; Sanderson et al., 2000). The amount of tritiated water released, as a measure of aromatase activity, was determined using a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Negative controls contained radioactive substrate but no cells. A positive control radioactive substrate was directly added to scintillation cocktail. Cellular aromatase activity was expressed as a percent of vehicle control activity (cells exposed to 0.1% DMSO). Formestane (4-hydroxyandrostenedione) (1 µM), a selective aromatase inhibitor, was used to verify the specificity of the assay for the enzymatic aromatization reaction.

5.3.6. Statistical analysis

Results will be presented as means with standard errors of three independent experiments using different cell passages; per experiment, each treatment will be tested in triplicate. Statistically significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) from control will be determined by one-way ANOVA or two-way ANOVA followed by a Dunnett or Bonferroni post-hoc test to correct for multiple comparisons to control using GraphPad Prism v5.04 (GraphPad Software, San Diego, CA).

5.4 Results

5.4.1. Cell viability in co-culture medium

In order to verify optimal Hs578t and T47D cell growth in the co-culture media, prior to co-culture assembly, we performed a WST-1 assay. There was no significant difference between

the viability of Hs578t and T47D cells plated in their respective native media compared to co-culture medium (Fig. 5-3).

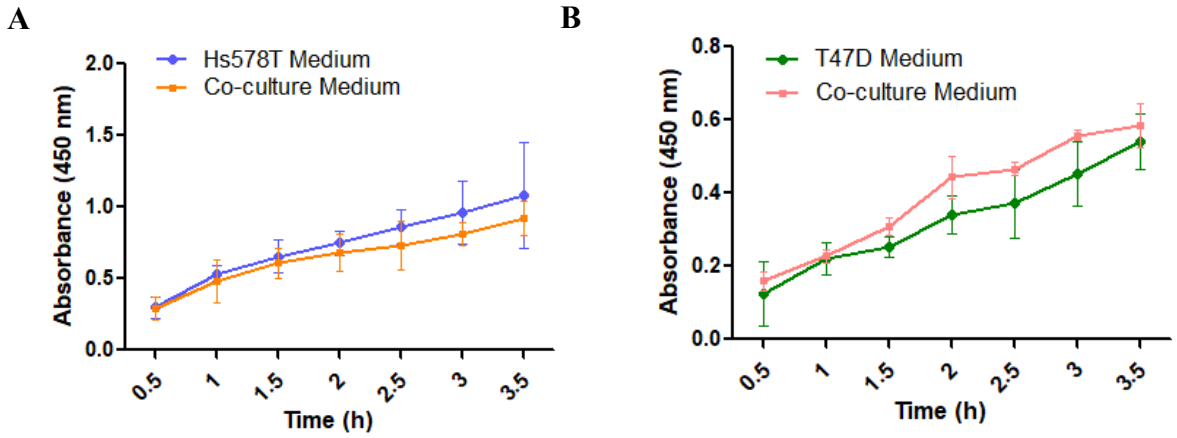

Figure 5-3: *Viability of Hs578t (A) and T47D (B) cells after exposure to their respective native media and to our co-culture media for 72 h.*

After 72 h, cells were treated with the WST-1 reagent. At 30 min intervals for 4 consecutive hours thereafter, absorbance (450nm) was measured. Data was analyzed using two-way ANOVA.

5.4.2. Estrogenic response of T47D cells in the co-culture model

It is essential for the T47D tumor cells to respond to estrogens produced by the Hs578t cells to properly mimic the communication between the cell compartments of the co-culture and reproduce the positive feedback mechanism that supports breast cancer growth. The effect of estradiol on *TFF1* expression in T47D cells in co-culture with Hs578t cells was measured to confirm the estrogen-responsiveness of the tumor cells in the presence of the stromal compartment and was compared to their response in monoculture without stromal influence (Fig 5-4A,B). *TFF1* expression was greater and increased at the lower estradiol concentrations in co-culture than in monoculture (Fig. 5-4A,B). This confirms that the T47D cell compartment is highly sensitive to estrogenic stimulation in our co-culture model. Although estradiol also

increased T47D cell proliferation in mono- and co-culture, this response was less sensitive (had a lower fold increase over 72 h) than the induction of *TFF1* expression.

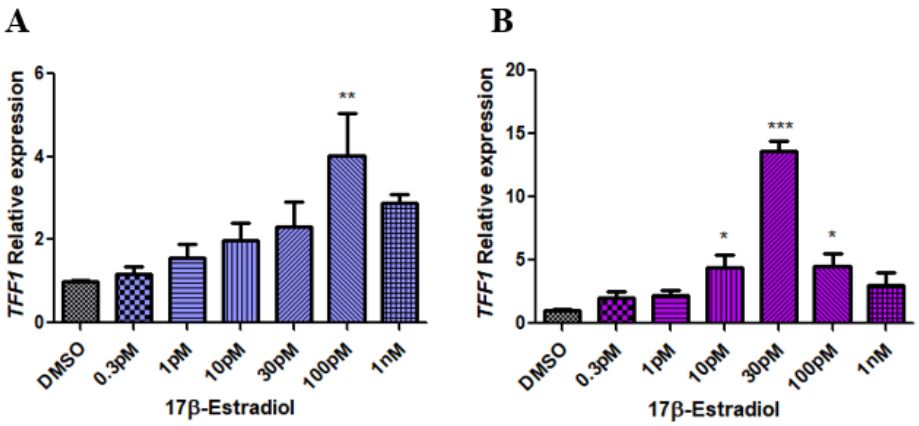

Figure 5-4*: Relative expression of TFF1 in T47D cells in response to a 72 h treatment of 17β-estradiol in (A) monoculture and (B) co-culture..*

*Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001.*

5.4.3. Promoter-specific CYP19 expression in monoculture and in the co-culture model

To determine the specific *CYP19* promoters normally activated in Hs578t cells in response to an estrogenic compound; estradiol was administered to the cells in monoculture and co-culture. Under monoculture conditions, estradiol treatment of Hs578t cells resulted in a significant induction of promoter non-distinct *CYP19* mRNA levels at concentrations of 0.3 pM, 1 pM, and 10 pM (Fig. 5-5A). A significant decrease in I.4-derived *CYP19* expression was detected at concentrations of 1 pM and 10 pM (Fig. 5-5A). There was also significant increases of promoter specific *CYP19* expression via PII, I.3, and I.7 at 0.3 pM, 1 pM, and 10 pM (Fig. 5-5A). In co-culture, estradiol treatment resulted in the significant increase in the expression of promoter non-distinct *CYP19* mRNA levels (1 pM-100 pM) and activation of promoter-specific *CYP19* expression via PII, I.3, and I.7 at concentrations of 1 pM to 30 pM (Fig. 5-5B). Furthermore, I.4-derived *CYP19* transcripts were decreased to almost negligible levels in co-culture at concentrations ranging from 1 pM to 100 pM (Fig. 5-5B).

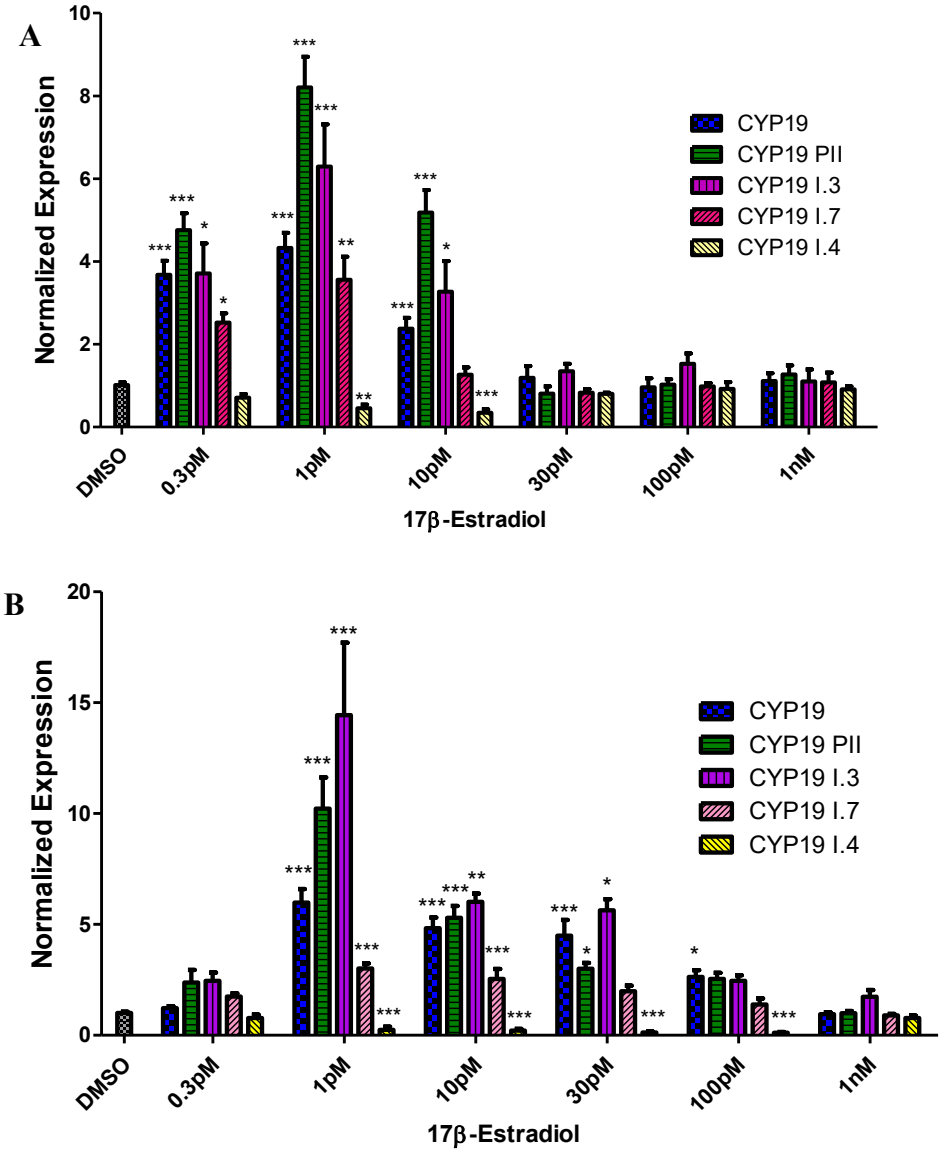

Figure 5-5*: Relative expression of promoter non-distinct and PII, I.3, I.7 and I.4 promoter-specific CYP19 expression in Hs578t cells after a 72 h exposure to 17β-estradiol in (A) monoculture and (B) co-culture.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test
**p < 0.05; **p < 0.01; ***p < 0.001.*
***Note**: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.*

5.4.4. Effect of testosterone on promoter-specific CYP19 expression in Hs578t cells and TFF1 gene expression in T47D cells

Testosterone is an androgen that is converted to estradiol in relevant tissues via the activation of CYP19. To further validate that intercellular communication is occurring between T47D and Hs578t cells in co-culture, we added testosterone to monoculture and co-culture medium to study the effects. Testosterone addition to Hs578t cells in monoculture resulted in a significant rise in *CYP19* mRNA levels, both through increases of the promoter non-distinct coding region and stimulation by promoters PII and I.3 at concentrations of 1 nM to 100 nM (Fig. 5-6A). Testosterone also significantly decreased expression of *CYP19* via promoter I.4 at these concentrations (Fig. 5-6A). A similar trend in *CYP19* expression was observed in Hs578t cells isolated from monoculture and co-culture, but the stimulatory effects of testosterone were more pronounced in the latter (Fig. 5-6B).

We also determined the effect of testosterone on the expression of *TFF1* in T47D cells in monoculture and co-culture to determine the influence of the presence of Hs578t cells on the ER-mediated gene response in T47D cells. Testosterone did not significantly affect the expression of *TFF1* in T47D monocultures relative to DMSO vehicle control (Fig. 5-7A). However, in co-culture, testosterone significantly increased *TFF1* expression levels in T47D cells with the highest increase noted at 20 nM (Fig. 5-7B). These results suggests that testosterone is successfully converted to estradiol in the Hs578t compartment and subsequently activates ER-mediated *TFF1* gene expression in T47D cells. Furthermore, testosterone also increased aromatase catalytic activity in Hs578t cells isolated from co-culture (Fig. 5-8).

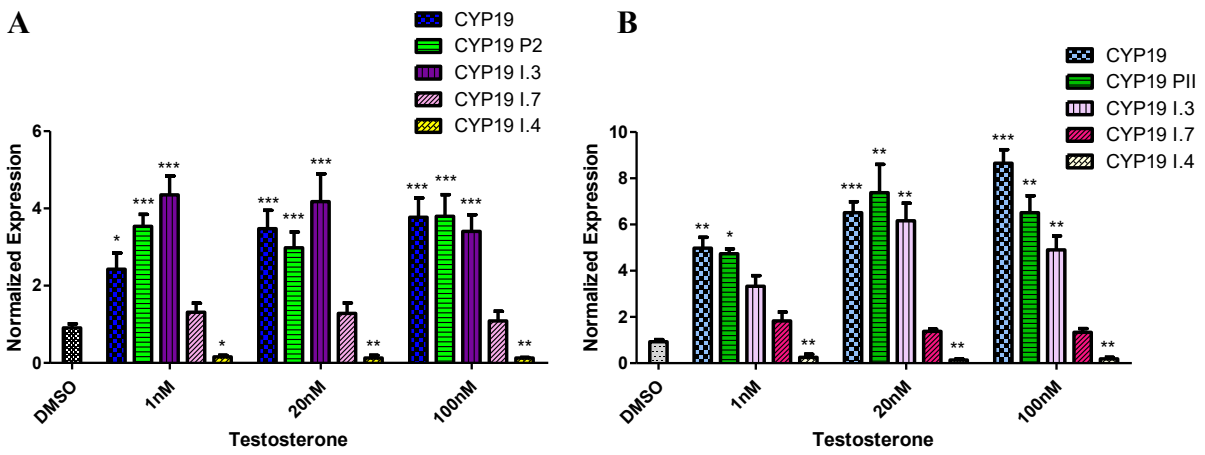

Figure 5-6: *Relative expression of promoter non-specific and PII, I.3, I., and I.4 promoter-specific CYP19 in Hs578t cells from (A) monoculture and (B) co-culture after a 72 h treatment with testosterone.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test
**p < 0.05; **p < 0.01; ***p < 0.001.*
Note: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.

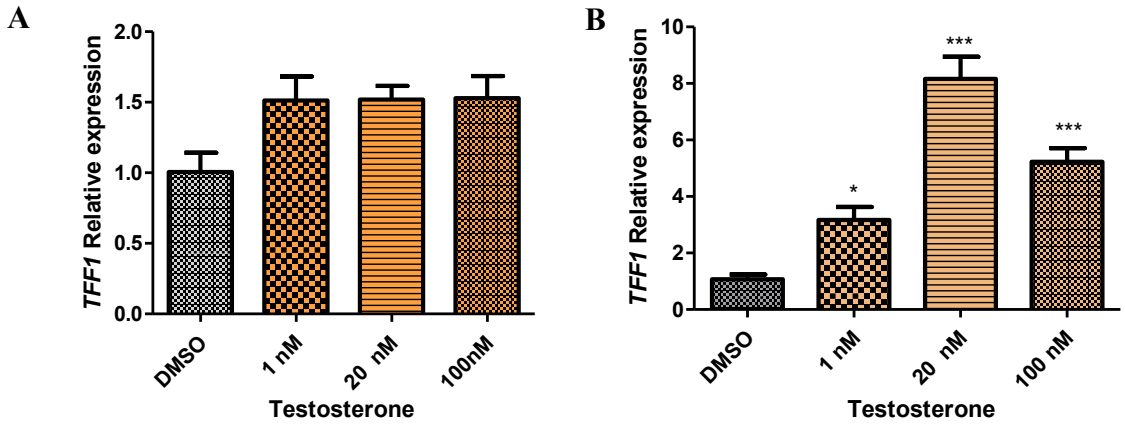

Figure 5-7*: Relative expression of ER-responsive gene, TFF1, in T47D cells after a 72 h treatment with testosterone in (A) monoculture and (B) co-culture.*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test.
**p < 0.05; ***p < 0.001.*

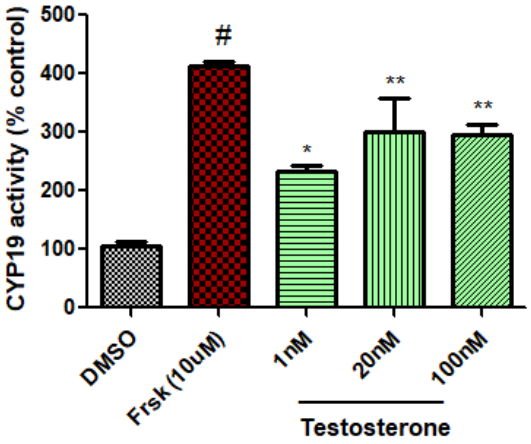

Figure 5-8*: Relative CYP19 activity in Hs578t cells in co-culture after a 72 h exposure to testosterone and expressed as a percentage of DMSO.*

*Forskolin (Frsk) was used as a positive control of aromatase induction. Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test, *p < 0.05, **p < 0.01, or Student t-test, # p< 0.05.*

5.4.5. Effects of basil and fennel seed essential oils on promoter-specific expression of CYP19 in Hs578t cells and TFF1 expression in T47D cells in co-culture

We have previously shown that both basil and fennel seed essential oils disrupted steroidogenesis in a feto-placental co-culture model (see Chapter 2). Here, we evaluated whether basil and fennel seed essential oil treatment alters promoter-specific expression of *CYP19* in Hs578t cells isolated from co-culture after addition of testosterone to the co-culture medium. After a 72 h treatment of basil essential oil concentrations ranging from 0.0005%-0.05%, Hs578t cells showed a significant increase in the expression of the *CYP19* coding region at concentrations of 0.0005%, 0.001%, and 0.005% (2.7 ± 0.4 fold, 4.7 ± 0.7 fold, and 4.5 ± 0.5 fold, respectively) relative to DMSO control (Fig. 5-9A). This was associated with a rise in PII-derived *CYP19* mRNA at the same concentrations by 2.7 ± 0.4 fold, 4.8 ± 0.3 fold, and 4.3 ± 0.6 fold, respectively, relative to DMSO control (Fig. 5-9A). We also detected increased activity of I.3 at a concentration of 0.001% by 2.3 ± 0.3 fold (Fig. 5-9A). Basil essential oil was also able to significantly decrease I.4-derived *CYP19* mRNA levels at concentrations of 0.001% and 0.005% by approximately 70% to 0.3 ± 0.1 fold (Fig. 5-9A). Under the same conditions, fennel seed

essential oil treatment resulted in a similar pattern of effects, with increases to the *CYP19* coding region and PII-derived *CYP19* transcripts and decreases to I.4-derived *CYP19* levels, while increasing expression of I.3-derived *CYP19* mRNA levels at a concentration of 0.005% as well (Fig. 5-9B). Our results produced a an inverted U-shaped curve as there was no significant change to the promoter-specific expression of *CYP19* relative to DMSO control at concentration over 0.01% (Fig. 5-9A,B).

Moreover, we wanted to determine whether basil and fennel seed essential oil treatment altered expression of the ER-responsive gene *TFF1* in T47D cells in monoculture and co-culture after testosterone addition. In monoculture, both basil and fennel seed oils significantly increased *TFF1* expression in T47D cells at a concentration of 0.01% and 0.05% by 1.9 ± 0.1 fold and 2.8 ± 0.3 fold, respectively (Fig. 5-10A). In co-culture, *TFF1* expression increased as well, but with the highest increases shifting to lower concentrations of basil and fennel seed oils at 0.001% and 0.005% (2.6 ± 0.3 fold and 2.8 ± 0.1 fold, respectively) (Fig. 5-10B). In effect, our co-culture increased the sensitivity of T47D cells to induce *TFF1* with greater efficacy at lower concentrations of essentials oils.

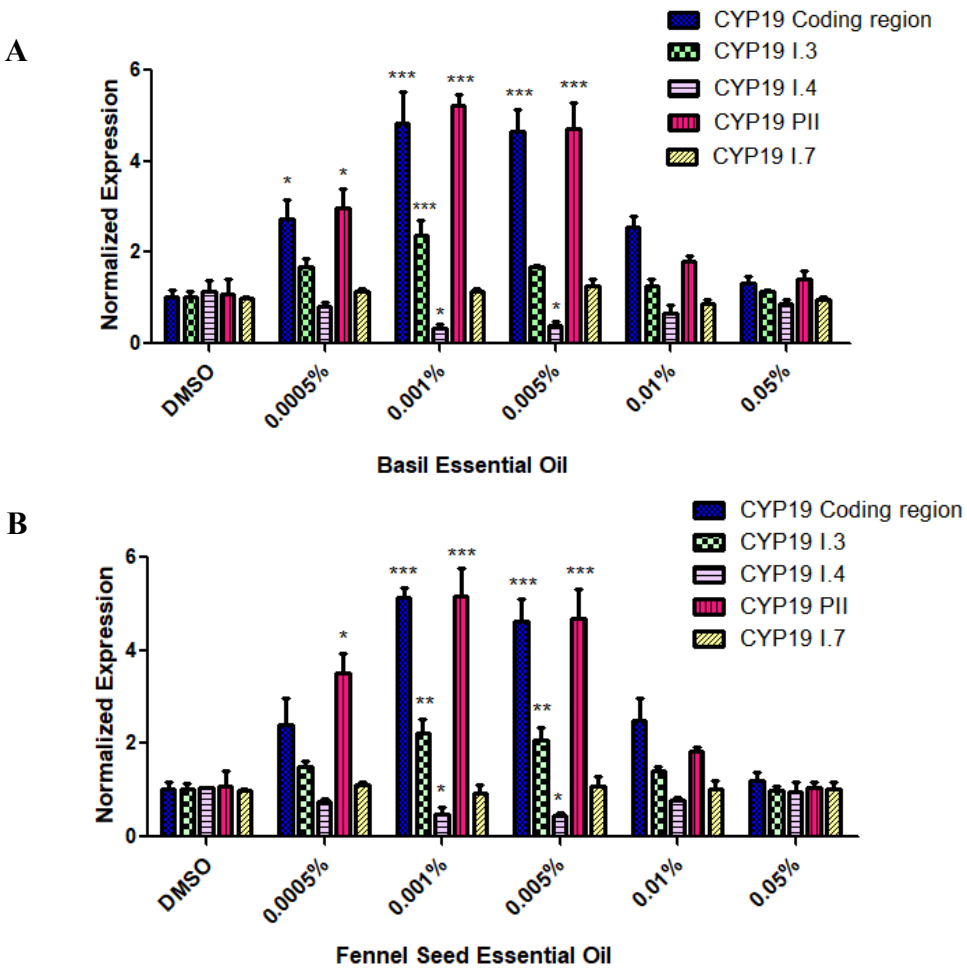

Figure 5-9: *Relative expression of promoter non-specific CYP19 coding region and I.3, I.4, PII and I.7 promoter-specific CYP19 mRNA levels in Hs578t cells from co-culture after 72 h treatment with basil (A) and fennel seed (B) essential oil (after addition of 20 μM of testosterone).*

*Experiments were performed 3 times using cells of different passage. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test *p < 0.05; **p < 0.01; ***p < 0.001*
Note: Individual gene expression levels are relative to their own basal (DMSO) level of expression. Expression levels cannot be compared among genes.

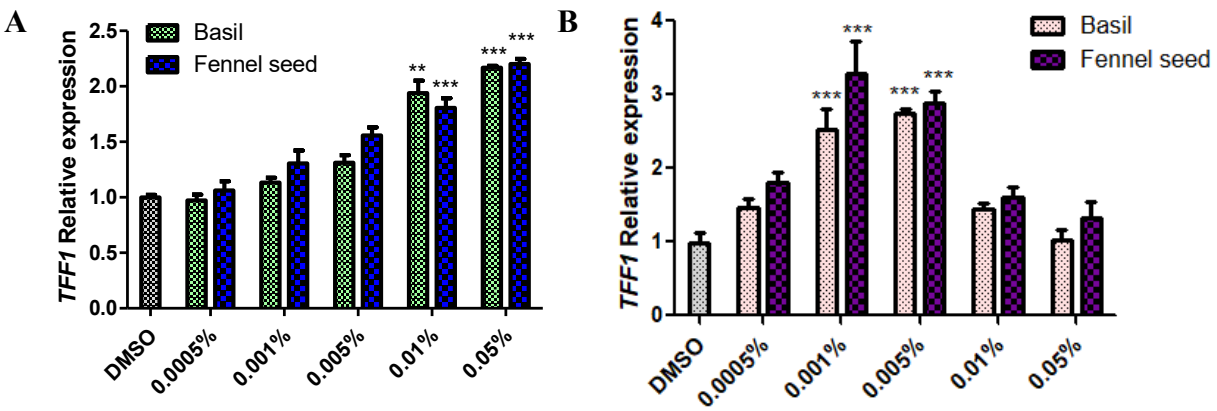

Figure 5-10: *Relative expression of TFF1 in T47D cells (A) monoculture and (B) co-culture after a 72 h exposure to basil and fennel seed essential oils (after addition of 20µM of testosterone).*

Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test
***p < 0.01; ***p < 0.001*

5.4.6. Effect of basil and fennel seed essential oils on CYP19 catalytic in the Hs578t cells from co-culture

The catalytic activity of CYP19 was determined to confirm whether the effects of basil and fennel seed essential oils on the promoter-specific expression of *CYP19* in Hs578t cells translated into changes in aromatase activity. Both basil and fennel seed oils significantly increased aromatase activity in Hs578t cells from co-culture at concentrations of 0.001%, 0.005%, and 0.01% by 1.8 ± 0.4 fold, 1.8 ± 0.2 fold, and by 1.4 ± 0.2 fold, respectively (Fig. 5-11).

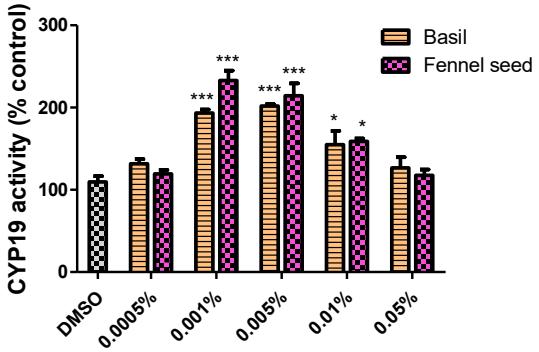

Figure 5-11: *Relative CYP19 activity in Hs578t cells after a 72-h exposure of basil or fennel seed essential oils.*

*Experiments were performed 3 times using cells of different passage; per experiment each treatment was tested in triplicate. Statistically significant differences from DMSO (0.1%) vehicle control were determined using one-way ANOVA followed by a Dunnett post-hoc test. *$p < 0.05$. ***$p < 0.001$.*

5.5 Discussion

5.5.1. A co-culture of Hs578t and T47D cells mimics the in vivo estrogen-dependent breast cancer microenvironment

We have developed a co-culture system of Hs578t and T47D cells, which is more sensitive and more responsive to endocrine disrupting effects of chemicals (related to estrogen biosynthesis and estrogen-signaling) because it offers far more mechanistic opportunities for endocrine disruption than any single cell type alone. In our co-culture model, Hs578t cells, under the influence of T47D cells, expressed greater levels of *CYP19* and produced larger quantities of estrogens (in the presence of an androgen precursor) than Hs578t cells in monoculture. In turn, T47D cells exhibited greater ER-mediated gene expression (*TFF1*), than in monoculture. Essentially, the co-culture system mimics the positive feedback system that takes place *in vivo,* whereby potential estrogenic effects of chemicals on ER signaling in T47D cells are amplified resulting in increased secretion of factors that stimulate aromatase expression and estrogen biosynthesis in Hs578t cells. On the other side, the co-cultures also responds to chemicals that are not innately estrogenic at the receptor level, but that can induce *CYP19* expression, aromatase activity and biosynthesis of estrogens, which in turn activate ER-responsive genes in T47D cells resulting in enhanced tumor cell proliferation and growth. Our estrogen-dependent breast cancer co-culture model will be a useful tool of human relevance to screen large numbers of chemicals for their potential endocrine disruption effects via mechanisms other than direct activation of the ER, thus refining and reducing the need for animal experimentation

5.5.2. Evaluating the effects of basil and fennel seed essential oils on promoter-specific CYP19 and TFF1 expression in the breast cancer co-culture model

The first application of our estrogen-dependent breast cancer model was to detect potential endocrine disrupting effects of basil and fennel seed essential oils on the biosynthesis of estrogens in the Hs578t/T47D co-culture. We have previously shown that basil and fennel seed

oils, whose main components are estragole and trans-anethole, respectively, interfered with steroidogenesis in a feto-placental model of pregnancy consisting of a co-culture of adrenocortical H295R cells and placental BeWo cells (see Chapters 2 and 3). In the current study, both basil and fennel seed oils increased promoter-specific expression of *CYP19* and stimulated a promoter-switch by increasing PII and I.3-derived *CYP19* transcripts and decreasing levels of I.4-derived transcripts in Hs578t cells in co-culture. Basil and fennel seed oils stimulated a concentration-dependent increase in *TFF1* expression in T47D cells from monoculture. However, in co-culture, a non-monotonic dose response curve was produced whereby increased expression of *TFF1* in T47D cells occurred at lower concentrations; matching concentrations of basil and fennel seed that increased *CYP19* expression levels in Hs578t cells. Taken together, these results indicate that Hs578t cells enhance the effects of essential oils on the activation of ER-responsive genes in T47D cells.

5.5.3. Essentials oils and breast cancer

Studying the effects of essential oils on breast cancer is a relatively new field of study with most attention being devoted to potential anti-cancer activities that certain oils and their main constituents may possess. Previous research has described potential antioxidant, anti-inflammatory and anti-proliferative effects of certain essential oils in cancer (Blowman et al., 2018). In the context of our present study, others have identified estragole and trans-anethole as promising new anti-cancer agents that can regulate growth and proliferation of MCF-7 breast cancer cells (Chen & deGraffenried, 2012). However, in that particular study, concentrations of estragole and trans-anethole were more than 1000-fold greater than that used in our study. With regards to steroidogenesis, we were the first to show that estragole and trans-anethole can disrupt feto-placental steroidogenesis and in the present study, we provide further evidence to this effect in the context of estrogen-dependent breast cancer. Moreover, our results are supported by previous work done by Nakagawa & Suzuki (2003) that showed the potential for hydroxylated intermediates of trans-anethole to competitively displace estradiol from the estrogen receptor and enhance cell proliferation of MCF-7 breast cancer cells, with lower concentrations proving more effective. At present, very little work is available outlying the potential metabolism of essential oils and their major constituents in humans, although various metabolites, for example, estragole and trans-anathole may have a wide-range of unpredictable effects. Future work will be devoted

to uncovering the various biotransformations that essentials oils may undergo *in vivo* and the effects that metabolic intermediates may have on steroidogenesis and steroid hormone signaling, using physiologically relevant co-culture systems, such as the one in the present study

5.5.4. Perspectives

In this study, we have developed an *in vitro* model of estrogen-dependent breast cancer whereby interactions between stromal and tumor cells can be monitored at the level of estrogen biosynthesis and aromatase. Our breast cancer co-culture model will significantly reduce the requirement for animal testing as it is, mechanistically, a far more relevant experimental system of human estrogen-dependent breast cancer. The interactions between aromatase and estrogen biosynthesis in the stromal compartment and ER signaling in the tumoral compartment are more representative of the human disease than any experimental breast cancer model using rodents. To further validate our model, more work is currently being done to determine the response of the co-culture to various aromatase inducers and inhibitors, as well as, measurements to the changes in secretion of inflammatory and steroidogenic factors that are characteristically released during a desmoplastic reaction. Moreover, we have also begun experimentation to potentially replace T47D cells in our co-culture with T47D-KBluc transfected cells (ATCC, no: CRL-2865), which respond to increased estradiol levels with increased expression of luciferase, and therefore provide an additional means of validating estrogenic response in conjunction with *TFF1* expression. In conclusion, our breast cancer co-culture model could be a fundamental new tool for the screening of potential endocrine disrupting compounds and will be useful for evaluating drugs effective against estrogen-dependent breast cancer by interfering with the uniquely human mammary tumor microenvironment.

5.6 Acknowledgements

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5.7 Conflicts of interest

The authors declare to have no conflicts of interest.

5.8 References

Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M., & Lehti, K. (2018). Fibroblasts in the Tumor Microenvironment: Shield or Spear?. *International journal of molecular sciences*, *19*(5), 1532. doi:10.3390/ijms19051532

Blowman, K., Magalhães, M., Lemos, M. F. L., Cabral, C., & Pires, I. M. (2018). Anticancer Properties of Essential Oils and Other Natural Products. Evid Based Complement Alternat Med, 2018, 3149362-3149362. doi:10.1155/2018/3149362

Bulun, S. E., Lin, Z., Imir, G., Amin, S., Demura, M., Yilmaz, B., . . . Deb, S. (2005). Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. Pharmacol Rev, 57.

Bulun, S. E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H., & Shozu, M. (2003). The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. J Steroid Biochem Mol Biol, 86(3-5), 219-224.

Caron-Beaudoin, É., Viau, R., & Sanderson, J. T. (2018). Effects of Neonicotinoid Pesticides on Promoter-Specific Aromatase (CYP19) Expression in Hs578t Breast Cancer Cells and the Role of the VEGF Pathway. Environ Health Perspect, 126(4), 047014-047014. doi:10.1289/EHP2698

Chen, C. H., & deGraffenried, L. A. (2012). Anethole suppressed cell survival and induced apoptosis in human breast cancer cells independent of estrogen receptor status. Phytomedicine, 19(8), 763-767. doi:https://doi.org/10.1016/j.phymed.2012.02.017

Chen, D., Reierstad, S., Lu, M., Lin, Z., Ishikawa, H., & Bulun, S. E. (2009). Regulation of breast cancer-associated aromatase promoters. Cancer Lett, 273(1), 15-27. doi:10.1016/j.canlet.2008.05.038

Cline, J. M., & Wood, C. E. (2005). Hormonal effects on the mammary gland of postmenopausal nonhuman primates. Breast Dis, 24, 59-70.

Dewi, F. N., Wood, C. E., Willson, C. J., Register, T. C., Lees, C. J., Howard, T. D., . . . Cline, J. M. (2016). Effects of Pubertal Exposure to Dietary Soy on Estrogen Receptor Activity in the Breast of Cynomolgus Macaques. Cancer Prev Res (Phila), 9(5), 385-395. doi:10.1158/1940-6207.capr-15-0165

Dolstra, H., Fredrix, H., Maas, F., Coulie, P. G., Brasseur, F., Mensink, E., . . . van de Wiel-van Kemenade, E. (1999). A Human Minor Histocompatibility Antigen Specific for B Cell Acute Lymphoblastic Leukemia. The Journal of Experimental Medicine, 189(2), 301-308. doi:10.1084/jem.189.2.301

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., . . . Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, 136(5), E359-386. doi:10.1002/ijc.29210

Gillesby, B. E., & Zacharewski, T. R. (1999). pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. Breast Cancer Res Treat, 56(3), 253-265.

Good, M., Sodhi, C. P., Yamaguchi, Y., Jia, H., Lu, P., Fulton, W. B., . . . Hackam, D. J. (2016). The human milk oligosaccharide 2'-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. Br J Nutr, 116(7), 1175-1187. doi:10.1017/s0007114516002944

Heneweer, M., Muusse, M., Dingemans, M., de Jong, P. C., van den Berg, M., & Sanderson, J. T. (2005). Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci, 83(2), 257-263. doi:10.1093/toxsci/kfi025

Heneweer, M., van den Berg, M., & Sanderson, J. T. (2004). A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett, 146(2), 183-194.

Imagawa, W., Pedchenko, V. K., Helber, J., & Zhang, H. (2002). Hormone/growth factor interactions mediating epithelial/stromal communication in mammary gland development and carcinogenesis. J Steroid Biochem Mol Biol, 80(2), 213-230.

Khamis, Z. I., Sahab, Z. J., & Sang, Q.-X. A. (2012). Active roles of tumor stroma in breast cancer metastasis. Int J Breast Cancer, 2012, 574025-574025. doi:10.1155/2012/574025

Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat, 83(3), 249-289. doi:10.1023/B:BREA.0000014042.54925.cc

Li, J., Chen, L., Xu, S., Yin, W., Chen, Y., Shao, Z., . . . Zeng, X. (2009). c-myb activates CXCL12 transcription in T47D and MCF7 breast cancer cells. Acta Biochimica et Biophysica Sinica, 42(1), 1-7. doi:10.1093/abbs/gmp108

Marinov, V., & Valcheva-Kuzmanova, S. (2015). Review on the pharmacological activities of anethole. 2015, 2(2). doi:10.14748/ssp.v2i2.1141

Meng, L., Zhou, J., Sasano, H., Suzuki, T., Zeitoun, K. M., & Bulun, S. E. (2001). Tumor Necrosis Factor α and Interleukin 11 Secreted by Malignant Breast Epithelial Cells Inhibit Adipocyte Differentiation by Selectively Down-Regulating CCAAT/Enhancer Binding Protein α and Peroxisome Proliferator-activated Receptor γ. Mechanism of Desmoplastic Reaction, 61(5), 2250-2255.

Odawara, H., Iwasaki, T., Horiguchi, J., Rokutanda, N., Hirooka, K., Miyazaki, W., . . . Koibuchi, N. (2009). Activation of aromatase expression by retinoic acid receptor-related orphan receptor (ROR) alpha in breast cancer cells: identification of a novel ROR response element. J Biol Chem, 284(26), 17711-17719. doi:10.1074/jbc.M109.009241

Paget, S. (1889). The distribution of secondary growths in cancer of the breast. Lancet, 1, 571-573.

Pipe, A., & Ayotte, C. (2002). Nutritional supplements and doping. Clin J Sport Med, 12(4), 245-249.

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., . . . Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res, 12(5), R68. doi:10.1186/bcr2635

Saha Roy, S., & Vadlamudi, R. K. (2012). Role of estrogen receptor signaling in breast cancer metastasis. Int J Breast Cancer, 2012, 654698. doi:10.1155/2012/654698

Sanderson, J. T., Seinen, W., Giesy, J. P., & van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci, 54(1), 121-127.

Sebastian, S., Takayama, K., Shozu, M., & Bulun, S. E. (2002). Cloning and characterization of a novel endothelial promoter of the human CYP19 (aromatase P450) gene that is up-regulated in breast cancer tissue. Mol Endocrinol, 16(10), 2243-2254. doi:10.1210/me.2002-0123

Subramanian, A., Salhab, M., & Mokbel, K. (2008). Oestrogen producing enzymes and mammary carcinogenesis: a review. Breast Cancer Res Treat, 111.

Tratwal, J., Follin, B., Ekblond, A., Kastrup, J., & Haack-Sørensen, M. (2014). Identification of a common reference gene pair for qPCR in human mesenchymal stromal cells from different tissue sources treated with VEGF. BMC Molecular Biology, 15(1), 11. doi:10.1186/1471-2199-15-11

Wang, X., Sang, X., Diorio, C., Lin, S. X., & Doillon, C. J. (2015). In vitro interactions between mammary fibroblasts (Hs 578Bst) and cancer epithelial cells (MCF-7) modulate aromatase, steroid sulfatase and 17beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol, 412, 339-348. doi:10.1016/j.mce.2015.05.032

Wang, Y., Gho, W. M., Chan, F. L., Chen, S., & Leung, L. K. (2008). The red clover (trifolium pretense) isoflavone biochenin A inhibits aromatase activity and expression. Brit J Nutr, 99.

Wood, C. E., Branstetter, D., Jacob, A. P., Cline, J. M., Register, T. C., Rohrbach, K., . . . Dougall, W. C. (2013). Progestin effects on cell proliferation pathways in the postmenopausal mammary gland. Breast Cancer Res, 15(4), R62. doi:10.1186/bcr3456

Yamaguchi, Y., & Hayashi, S.-i. (2009). Estrogen-Related Cancer Microenvironment of Breast Carcinoma. Endocrine Journal, 56(1), 1-7. doi:10.1507/endocrj.K08E-099

Zhou, J., Gurates, B., Yang, S., Sebastian, S., & Bulun, S. E. (2001a). Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. Cancer Res, 61.

Zhou, J., Gurates, B., Yang, S., Sebastian, S., & Bulun, S. E. (2001b). Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. Cancer Res, 61(5), 2328-2334.

Chapter 6 : GENERAL CONCLUSION

With the increasing popularity of alternative modes of treatments, especially naturally-derived products, such as essential oils, it was of utmost importance to have insights into their biological activity beyond folk medicine claims. Altogether, this research project encompasses new information regarding steroidogenic activity exhibited by a few commonly used essentials oils in the context of their most frequently reported users: women. The application of an established feto-placental co-culture system and the development of an innovative breast cancer co-culture model, have provided us with *in vitro* mechanistic research tools that not only respond to the need to find alternative models to animal testing, but are more effective in replicating certain steroidogenic interactions that are uniquely relevant to humans. These tools will be useful in determining alternative and hitherto poorly studied, mechanisms of action of endocrine disrupting compounds.

6.1. Implications of the effects of essential oils on steroidogenesis in pregnancy

The first objective of this thesis was to characterize the effects of common essential oils and their respective active ingredients on steroidogenesis in an *in vitro* model representative of fetal-placental steroidogenesis developed in our laboratory (Thibeault et al., 2014) and described in section *1.6.1*. The use of CAMs during pregnancy has increased substantially in recent years with some cohort studies estimating usage of 60 to 78% (Hall et al., 2011). Pregnant women have been shown to prefer alternative medicine due to concerns that pharmacological drugs may cause birth defects (Hall et al., 2011). One of the most popular forms of complementary therapy used by pregnant women is aromatherapy, which employs essential oils for medicinal purposes (Hall et al., 2011; Joulaeerad et al., 2018). Studies have shown that certain essential oils can alleviate pregnancy symptoms, such as nausea and vomiting (Joulaeerad et al., 2018), stress (Igarashi, 2013) and lower back pain (Shirazi et al., 2017), among other conditions. Of concern, however is that most women do not inform their medical practitioners of their CAM use (Hall et al., 2011). In light of this and recent evidence that has shown that certain essential oils can cross the fetal-placental barrier (Pelkonen et al., 2017), our objective was to shed some light on the effects of essential oils and potential risks they could pose to mother and fetus during pregnancy.

Of the five common essential oils (basil, fennel seed, sweet orange, black pepper and common sage) we initially chose, only basil and fennel seed oil altered steroid hormone production in the feto-placental co-culture system by increasing levels of progesterone, DHEA, androstenedione, estradiol, estrone, and estriol. We also determined that basil and fennel seed essential oils disrupt steroidogenesis by altering the expression of enzymes involved in cholesterol transport (*StAR and CYP11A1*) and those involved in hormone production (*HSD3B1/2*, *SULT2A1*, and *HSD17B1*, *-4*, and *-5)*. Moreover, basil and fennel seed essential oils altered the promoter-specific expression of aromatase and increased its catalytic activity at the lowest concentrations we evaluated. Provided with the GC/MS data of our essential oils, we determined that trans-anethole and estragole, respectively, were mediating the effects of fennel seed and basil essential oils. Essentially, the aromatase gene is activated via different promoters that are regulated by a unique set of hormones, cytokines, and second messengers. We determined that trans-anethole and estragole were able to increase expression of I.1 and pII-derived *CYP19* mRNA levels and this was caused by a rise in PKC and PKA levels in the feto-placental model BeWo and H295R cells, respectively; an effect which was inhibited when essential oil treatments were administered along with PKC and PKA pathway inhibitors. Furthermore, we were also able to uncover a new relationship between the *StAR* gene, and its homolog MLN64, and progesterone in H295R and BeWo cells. In effect, a rise in progesterone levels, triggered by trans-anethole and estragole, feedbacks into the feto-placental system by upregulating and inhibiting expression of *StAR* in H295R and BeWo cells, respectively; an effect that is eliminated in the presence of mifepristone (progesterone receptor inhibitor). More work is necessary to properly delineate the important role progesterone may have in regulating *StAR* gene expression in steroidogenic cell lines. Overall our results supported our hypothesis and provided more evidence that certain essential oils may pose a risk to mother and fetus during pregnancy by not only altering expression of key steroidogenic enzymes, but by also indirectly effecting estrogen biosynthesis.

6.2. Implication of essential oil use in estrogen-dependent breast cancer patients

The second and third objective of this thesis were to characterize the effects of essential oils on steroidogenesis in two different cell-culture models of estrogen-dependent breast cancer.

It has been reported that 40-60% of breast cancer patients, more than any other type of cancer patient, use complementary and alternative medicine at different stages of their traditional cancer therapy (Garg et al., 2005; Konkimalla & Efferth, 2008; Neuhouser et al., 2016; Roumeliotis et al., 2017). Among breast cancer patients, use of alternative therapies is believed to improve long-term survival, prevent recurrence, help in recovery, and manage stress (Hann et al., 2005; Liao et al., 2013). In a cohort study of 1000 breast cancer patients, 47% reported using natural and herbal products as a form of CAM modality after being diagnosed (Greenlee et al., 2009). A more recent cohort study of 707 breast cancer patients reported that 51% of CAMs used were plant-based products (Neuhouser et al., 2016). Favored among natural and herbal products are essentials oils which are available in various forms: applied topically in creams, ointments, or mixed with a carrier oil, through inhalation using a diffuser or humidifier, and ingested in beverages and certain foods. At present, two essential oils, lavender and tea tree oils, have been shown to cause reversible pre-pubertal gynecomastia formation in young boys through topical administration (Henley et al., 2007) via estrogen and androgen receptor activity. To our knowledge, we are the first to provide mechanistic insight into the effects exhibited by basil and fennel seed essential oils on steroidogenesis in two relevant models of estrogen-dependent breast cancer. To that effect, the first model we utilized for our evaluations was the Hs578t cell line. This cell line described in section *1.6.2.1.* was previously characterized in our lab (Caron-Beaudoin et al., 2018) and shown to possess all breast cancer relevant aromatase promoters and the capability of inducing the promoter switch described in section *1.5.4* Studying the effects of essential oils on promoter-specific expression of aromatase as well as resulting estrogen production changes is an important step to establishing new guidelines for exposure to these natural compounds during critical steroidogenic processes such as hormone-dependent breast cancer. Our results showed that basil and fennel seed essential oils trigger a promoter switch in Hs578t cells whereby aromatase expression and estrogen production is increased. More specifically, basil and fennel seed essential oils triggered a rise in pII-derived *CYP19* mRNA levels and catalytic activity while decreasing I.4-derived transcripts; and effect mediated by a rise in cAMP levels and PKA activity.

To better represent the cellular interactions that take place in a tumor microenvironment, we developed a co-culture system described in section *1.6.2.* consisting of fibroblast-like Hs578t

cells and T47D cancerous epithelial cells. In chapter 5 of this thesis, we showed that aromatase-containing Hs578t cells can stimulate estrogen production, after addition of androgen precursors to cell culture medium, which bind to ERs located on T47D cells and subsequently trigger expression of estrogen-responsive genes. The breast cancer co-culture model we have developed could be an excellent tool for high-throughput screening of potential endocrine disrupting compounds and for evaluating drugs effective against estrogen-dependent breast cancer. The stromal-tumoral interactions of our co-culture model cannot be replicated using cell-based reporter assays with single cell types in monoculture and experimental rodents are poor models for this interaction since they do not express human mammary aromatase. We believe that our co-culture system could reduce requirement for animal testing as it is a more relevant experimental system of a human breast cancer microenvironment. Given the importance of maintaining hormonal stimulation at a minimum when dealing with conditions of estrogen-dependent growth and proliferation of breast cancer cells, we believe more work is needed to determine the risks associated with the use basil and fennel seed essential oils, and their respective active compounds, by breast cancer patients. Despite our newly-developed breast cancer co-culture model being an excellent tool for assessing endocrine disrupting compounds, it is difficult to determine whether the concentrations that resulted in steroidogenic effects in our cell lines are equivalent to doses that humans are exposed to.

6.3. Perspectives

6.3.1. Risks of combining essential oils with conventional medical treatments

Herbal products, including essential oils, have a widespread use worldwide and are often associated with minimal risk because of their natural origins. A growing concern given the popularity and self-initiated usage of these herbal products is possible interactions with concomitantly taken drugs with reports estimating between 20-75% of CAM users take prescribed medications (Awortwe et al., 2019; Eisenberg et al., 2001; Jermini et al., 2019; Samojlik et al., 2016). Given that essential oils are mixtures of compounds, certain constituents may interact with drugs and possibly influence pharmacodynamic or pharmacokinetic effects. In terms of cancer, herbal products could decrease exposure to a cancer drug concentration or interfere with the drug's effector site (Tascilar et al., 2006). Recently, the increasing number of

reports on herb-drug interactions in clinical investigations has resulted in a new-wave of pharmacological studies on herbal products (Asher et al., 2017; Bossi et al., 2017; Cai et al., 2017). However, very few studies have explored the potential drug-interactions of essential oils. At present using rodent models, there is evidence to support peppermint essential oil and caraway oil interfere with gastro-intestinal drugs by affecting motility (Goerg & Spilker, 2003), caraway oil affects the bioavailability of the acetaminophen paracetamol (Samojlik et al., 2012a), and that anise seed oil interacts with central nervous system drugs and acetaminophen by impacting their analgesic potential and effects on motor activity (Samojlik et al., 2012b; Samojlik et al., 2016). To our knowledge, there is no current research available studying the effect of essential oils on drugs regulating steroidogenic pathways such as oral contraceptives, drugs for hormone replacement, and for treatments of hormone-dependent breast cancer.

There are two major forms of oral contraceptives: a combination birth control pill which contains estrogen and progestin (synthetic progesterone that is more hydrophilic and can enter the bloodstream) and the mini-pill which contains only progestin. The CYP450 enzyme system, especially the major subtype CYP3A4 found in the liver and digestive system, has a very important role in drug metabolism; inhibition and induction of this system plays a role in drug interactions (Galetin et al., 2006). Initial drug screenings are often done *in vitro* to determine potential pharmacokinetic interactions with oral contraceptives and whether the compound of interest induces or inhibits the CYP450 system (Rhoda Lee, 2009). In the feto-placental model used in chapter 2 and 3, we identified that basil and fennel seed essential oil, and their respective active compounds, significantly increased the expression of CYP3A7: a major P450 enzyme found in the fetal liver and detected in H295R cells. As a perspective study, investigations should be made using liver hepatocytes and essential oil treatments concurrently administered with contraceptive steroids to determine whether the activity of CYP3A4 subtype is affected. A compound that can influence the metabolism of an oral contraceptive is very concerning because of the possibility of reduced contraceptive efficacy. Moreover, it would be interesting to investigate whether the constituents found within essential oils can interfere with the action of the contraceptive steroids at the biological receptor site. To do this, there are numerous molecular modeling softwares available (Pagadala et al., 2017), such as Dock, which would be

able to determine if the geometric shapes of certain compounds within essential oils could bind to the hormone receptor targets of oral contraceptives.

6.3.2. Biotransformation of essential oils

Recommended human quantities for the use of essential oils vary between 1-5% depending on the absorption method according to pharmacopeias (Tisserand & Balacs, 2014) . However, it is currently unknown what concentration actually reaches circulation and the targeted tissue. This is concerning in light of evidence that essentials oils are able to cross the blood-brain and fetal-placental barriers (Pelkonen et al., 2017; Tillett & Ames, 2010). Unfortunately, due to the various forms available for essential oil application or consumption, the possible routes for the biotransformation of constituents within essentials *in vivo* has still not been fully elucidated. Studies have investigated the possible routes of estragole and its isomer trans-anethole biotransformation *in vitro* and using rodent models and have uncovered four major pathways: O-demethylation, epoxidation of the double bond, and 1'-hydroxilation, and oxidative degradation of the side chain to carboxylic acids (Gori et al., 2012a; Nakagawa & Suzuki, 2003). As described in section *1.2.5.1.* and *1.2.6.1.*, more research into estragole and trans-anethole began after initial reports suggested that these compounds can cause DNA damage and promote cancer formation. However, it is often suggested that studies assessing estragole and trans-anethole, among other constituents in essential oils, individually is misleading and not always a true representation of the activities exhibited by the oils (Gori et al., 2012b). In chapter 2 and 3, we have shown that estragole and trans-anethole exhibit the same steroidogenic behavior as their respective essential oils basil and fennel seed. We are currently unaware if compounds undergo any biotransformation after being administered to the fetal-placental co-culture model, especially in light of evidence that H295R cells possess fetal liver P450 enzymes that could be involved in drug metabolism.

As a perspective study, we believe it is important to investigate whether the essential oil solutions we have utilized and their respective constituents remain unaltered within the fetal-placental and breast cancer co-culture models. To do this, we propose collecting co-culture media after exposure to essential oils and analyzing the concentrations of each constituent of the media via GC/MS. We would then be able to compare the results to our initial GC/MS reports to

determine if any biotransformation of the essential oil compounds occurs in the co-culture systems and delineate the metabolic pathways involved. At present, both co-culture systems have not been evaluated for the ability to mimic *in vivo* drug metabolism; we believe this is an important future direction to take to further validate the models as useful tools mimicking human activity.

6.4. Project outcome

The main objective of this PhD thesis was to evaluate the effects of a few commonly used essential oils on steroidogenesis in physiologically relevant human cell-culture systems, representing either the feto-placental environment during pregnancy or the hormone-dependent breast cancer microenvironment. Using a well-validated feto-placental co-culture model, we successfully accomplished our first objective and determined that basil and fennel seed essential oil and their major compounds estragole and trans-anethole, respectively, increased hormone production levels, promoter-specific expression of *CYP19*, and aromatase catalytic activity. Furthermore, we were able to delineate certain pathways involved in the latter using pathway-specific inhibitors. To accomplish our second objective, we developed an estrogen-dependent breast cancer model that mimics *in vivo* interactions between stromal breast cancer cells and tumor epithelial cells. After successfully characterizing steroidogenic properties of this co-culture system, we showed that basil and fennel seed essential oils exhibited endocrine-disrupting effects in this hormone-dependent breast cancer microenvironment. Our results raise concern regarding the safety of using basil or fennel seed essential oil formulations in the context of women who are pregnant or suffering from an estrogen-dependent breast cancer. Overall, we set out to provide more information regarding potential effects exhibited by essential oils on steroidogenesis in women, who more often use alternative forms of therapy then men. We believe that we have accomplished this goal by not only providing a new tool for the study of endocrine-disrupting compounds, but also by highlighting a new application of the feto-placental co-culture system for evaluating the safety of natural products.

Chapter 7 : GENERAL REFERENCES

Abbas, M. A., Taha, M. O., Zihlif, M. A., & Disi, A. M. (2013). beta-Caryophyllene causes regression of endometrial implants in a rat model of endometriosis without affecting fertility. *Eur J Pharmacol, 702*(1-3), 12-19. doi:10.1016/j.ejphar.2013.01.011

About Natural Health Product Regulation in Canada. (2017). Retrieved from https://www.canada.ca/en/health-canada/services/drugs-health-products/natural-non-prescription/regulation.html.

Abu-Darwish, M. S., Cabral, C., Ferreira, I. V., Goncalves, M. J., Cavaleiro, C., Cruz, M. T., . . . Salgueiro, L. (2013). Essential oil of common sage (Salvia officinalis L.) from Jordan: assessment of safety in mammalian cells and its antifungal and anti-inflammatory potential. *Biomed Res Int, 2013*, 538940. doi:10.1155/2013/538940

Aburjai, T., & Natsheh, F. (2003). *Plants Used in Cosmetics* (Vol. 17).

Aka, J. A., & Lin, S. X. (2012). Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. *PLoS ONE, 7*(2), e31532. doi:10.1371/journal.pone.0031532

Aka, J. A., Zerradi, M., Houle, F., Huot, J., & Lin, S.-X. (2012). 17beta-hydroxysteroid dehydrogenase type 1 modulates breast cancer protein profile and impacts cell migration. *Breast Cancer Res, 14*(3), R92-R92. doi:10.1186/bcr3207

Akgül, A. (1989). Volatile oil composition of sweet basil (Ocimum basilicum L.) cultivating in Turkey (Short communication). *Food / Nahrung, 33*(1), 87-88. doi:doi:10.1002/food.19890330129

Al-Windi, A. (2004). Predictors of herbal medicine use in a Swedish health practice. *Pharmacoepidemiol Drug Saf, 13*(7), 489-496. doi:10.1002/pds.901

Albrecht, E. D., & Pepe, G. J. (2010). Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy. *Int J Dev Biol, 54*(2-3), 397-408. doi:10.1387/ijdb.082758ea

Alexandrovich, I., Rakovitskaya, O., Kolmo, E., Sidorova, T., & Shushunov, S. (2003). The effect of fennel (Foeniculum Vulgare) seed oil emulsion in infantile colic: a randomized, placebo-controlled study. *Altern Ther Health Med, 9*(4), 58-61.

Ali, B., Al-Wabel, N. A., Shams, S., Ahamad, A., Khan, S. A., & Anwar, F. (2015). Essential oils used in aromatherapy: A systemic review. *Asian Pacific Journal of Tropical Biomedicine, 5*(8), 601-611. doi:10.1016/j.apjtb.2015.05.007

Anthony, A., Caldwell, J., Hutt, A. J., & Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1′-hydroxyestragole. *Food and Chemical Toxicology, 25*(11), 799-806. doi:https://doi.org/10.1016/0278-6915(87)90257-2

Arias, B. A., & Ramon-Laca, L. (2005). Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. *J Ethnopharmacol, 97*(1), 89-95. doi:10.1016/j.jep.2004.10.019

Asher, G. N., Corbett, A. H., & Hawke, R. L. (2017). Common Herbal Dietary Supplement-Drug Interactions. *Am Fam Physician, 96*(2), 101-107.

Atashgaran, V., Wrin, J., Barry, S. C., Dasari, P., & Ingman, W. V. (2016). Dissecting the Biology of Menstrual Cycle-Associated Breast Cancer Risk. *Frontiers in oncology, 6*, 267-267. doi:10.3389/fonc.2016.00267

Awortwe, C., Bruckmueller, H., & Cascorbi, I. (2019). Interaction of herbal products with prescribed medications: A systematic review and meta-analysis. *Pharmacological Research, 141*, 397-408. doi:https://doi.org/10.1016/j.phrs.2019.01.028

Ayan, D., Maltais, R., Roy, J., & Poirier, D. (2012). A new nonestrogenic steroidal inhibitor of 17beta-hydroxysteroid dehydrogenase type I blocks the estrogen-dependent breast cancer tumor growth induced by estrone. *Mol Cancer Ther, 11*(10), 2096-2104. doi:10.1158/1535-7163.mct-12-0299

Ayaz, M., Sadiq, A., Junaid, M., Ullah, F., Subhan, F., & Ahmed, J. (2017). Neuroprotective and Anti-Aging Potentials of Essential Oils from Aromatic and Medicinal Plants. *Frontiers in aging neuroscience, 9*, 168-168. doi:10.3389/fnagi.2017.00168

Ayers, J. F. (2000). The use of alternative therapies in the support of breastfeeding. *J Hum Lact, 16*(1), 52-56. doi:10.1177/089033440001600111

Bag, A., & Chattopadhyay, R. R. (2018). Evaluation of antioxidant potential of essential oils of some commonly used Indian spices in in vitro models and in food supplements enriched with omega-6 and omega-3 fatty acids. *Environ Sci Pollut Res Int, 25*(1), 388-398. doi:10.1007/s11356-017-0420-5

Bagheri, H., Abdul Manap, M. Y., & Solati, Z. (2014). Antioxidant activity of Piper nigrum L. essential oil extracted by supercritical CO(2) extraction and hydro-distillation. *Talanta, 121*, 220-228. doi:10.1016/j.talanta.2014.01.007

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils--a review. *Food Chem Toxicol, 46*(2), 446-475. doi:10.1016/j.fct.2007.09.106

Bayala, B., Bassole, I. H. N., Gnoula, C., Nebie, R., Yonli, A., Morel, L., . . . Simpore, J. (2014). Chemical Composition, Antioxidant, Anti-Inflammatory and Anti-Proliferative Activities of Essential Oils of Plants from Burkina Faso. *PLoS ONE, 9*(3), e92122. doi:10.1371/journal.pone.0092122

Bengtsson, B. (1978). Estrogen induced inhibition of 3H-noradrenaline release in the uterus and portal vein of the rat. *Acta Physiol Scand, 104*(3), 287-298. doi:10.1111/j.1748-1716.1978.tb06281.x

Bhalla, Y., Gupta, V. K., & Jaitak, V. (2013). Anticancer activity of essential oils: a review. *J Sci Food Agric, 93*(15), 3643-3653. doi:10.1002/jsfa.6267

Boon, W. C., Chow, J. D., & Simpson, E. R. (2010). The multiple roles of estrogens and the enzyme aromatase. *Prog Brain Res, 181*, 209-232. doi:10.1016/s0079-6123(08)81012-6

Bossi, P., Cortinovis, D., Fatigoni, S., Cossu Rocca, M., Fabi, A., Seminara, P., . . . Roila, F. (2017). A randomized, double-blind, placebo-controlled, multicenter study of a ginger extract in the management of chemotherapy-induced nausea and vomiting (CINV) in patients receiving high-dose cisplatin. *Ann Oncol, 28*(10), 2547-2551. doi:10.1093/annonc/mdx315

Brodribb, W. (2018). ABM Clinical Protocol #9: Use of Galactogogues in Initiating or Augmenting Maternal Milk Production, Second Revision 2018. *Breastfeed Med, 13*(5), 307-314. doi:10.1089/bfm.2018.29092.wjb

Bulun, S. E., Lin, Z., Imir, G., Amin, S., Demura, M., Yilmaz, B., . . . Deb, S. (2005). Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. *Pharmacol Rev, 57*.

Bulun, S. E., Lin, Z., Zhao, H., Lu, M., Amin, S., Reierstad, S., & Chen, D. (2009). Regulation of aromatase expression in breast cancer tissue. *Ann NY Acad Sci, 1155*.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *Int J Food Microbiol, 94*(3), 223-253. doi:10.1016/j.ijfoodmicro.2004.03.022

Cai, Y., Xing, G., Shen, T., Zhang, S., Rao, J., & Shi, R. (2017). Effects of 12-week supplementation of Citrus bergamia extracts-based formulation CitriCholess on cholesterol and body weight in older adults with dyslipidemia: a randomized, double-blind, placebo-controlled trial. *Lipids Health Dis, 16*(1), 251. doi:10.1186/s12944-017-0640-1

Campbell. (2009). Campbell Reproductive Biology Site. Retrieved from http://kcampbell.bio.umb.edu/oklogin.html

Canadian Cancer Statistics 2017. *Canadian Cancer Statistics Advisory Committee.* Retrieved from https://www.cancer.ca/en/cancer-information/cancer-type/breast/statistics/?region=on

Caron-Beaudoin, E., Denison, M. S., & Sanderson, J. T. (2016). Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. *Toxicol Sci, 149*(1), 134-144. doi:10.1093/toxsci/kfv220

Caron-Beaudoin, E., Viau, R., & Sanderson, J. T. (2018). Effects of Neonicotinoid Pesticides on Promoter-Specific Aromatase (CYP19) Expression in Hs578t Breast Cancer Cells and the Role of the VEGF Pathway. *Environ Health Perspect, 126*(4), 047014. doi:10.1289/ehp2698

Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., . . . Rogan, E. G. (1997). Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proceedings of the National Academy of Sciences, 94*(20), 10937-10942. doi:10.1073/pnas.94.20.10937

Celik, L., Davey, J., Lund, D., & Schiott, B. (2008). Exploring interactions of endocrine-disrupting compounds with different conformations of the human estrogen receptor alpha ligand binding domain: a molecular docking study. *Chem Res Toxicol, 21*(11), 2195-2206. doi:10.1021/tx800278d

Chen, D., Reierstad, S., Fang, F., & Bulun, S. E. (2011). JunD and JunB Integrate Prostaglandin E(2) Activation of Breast Cancer-Associated Proximal Aromatase Promoters. *Molecular Endocrinology, 25*(5), 767-775. doi:10.1210/me.2010-0368

Chen, D., Reierstad, S., Lu, M., Lin, Z., Ishikawa, H., & Bulun, S. E. (2009). Regulation of breast cancer-associated aromatase promoters. *Cancer Lett, 273*(1), 15-27. doi:10.1016/j.canlet.2008.05.038

Choi, E. M., & Hwang, J. K. (2004). Antiinflammatory, analgesic and antioxidant activities of the fruit of Foeniculum vulgare. *Fitoterapia, 75*(6), 557-565. doi:10.1016/j.fitote.2004.05.005

Choi, S. Y., Kang, P., Lee, H. S., & Seol, G. H. (2014). Effects of Inhalation of Essential Oil of Citrus aurantium L. var. amara on Menopausal Symptoms, Stress, and Estrogen in Postmenopausal Women: A Randomized Controlled Trial. *Evid Based Complement Alternat Med, 2014*, 796518. doi:10.1155/2014/796518

Chuang, C. H., Doyle, P., Wang, J. D., Chang, P. J., Lai, J. N., & Chen, P. C. (2006). Herbal medicines used during the first trimester and major congenital malformations: an analysis of data from a pregnancy cohort study. *Drug Saf, 29*(6), 537-548.

<Citation_World Cancer Research Fund International 2018.pdf>.

Clarke, R. B., Howell, A., Potten, C. S., & Anderson, E. (1997). Dissociation between Steroid Receptor Expression and Cell Proliferation in the Human Breast. *Cancer Res, 57*(22), 4987-4991.

Clarke, T. C., Black, L. I., Stussman, B. J., Barnes, P. M., & Nahin, R. L. (2015). Trends in the use of complementary health approaches among adults: United States, 2002-2012. *Natl Health Stat Report*(79), 1-16.

Cuckle, H. S. (1992). Measuring unconjugated estriol in maternal serum to screen for fetal down syndrome. *Clin Chem, 38*(9), 1687-1689.

de Cremoux, P., Rosenberg, D., Goussard, J., Bremont-Weil, C., Tissier, F., Tran-Perennou, C., . . . Raffin-Sanson, M. L. (2008). Expression of progesterone and estradiol receptors in normal adrenal cortex, adrenocortical tumors, and primary pigmented nodular adrenocortical disease. *Endocr Relat Cancer, 15*(2), 465-474. doi:10.1677/erc-07-0081

Delaram, & Heydarnejad, M. S. (2011). *Masoumeh Herbal Remedy for Premenstrual Syndrome with Fennel (Foeniculum vulgare) – Randomized, Placebo-Controlled Study* (Vol. 20).

Demura, M., Reierstad, S., Innes, J. E., & Bulun, S. E. (2008). Novel promoter I.8 and promoter usage in the CYP19 (aromatase) gene. *Reprod Sci, 15*(10), 1044-1053. doi:10.1177/1933719108322441

Dennehy, C., Tsourounis, C., Bui, L., & King, T. L. (2010). The use of herbs by california midwives. *J Obstet Gynecol Neonatal Nurs, 39*(6), 684-693. doi:10.1111/j.1552-6909.2010.01193.x

Devi, K., Vanithakumari, G., Anusya, S., Mekala, N., Malini, T., & Elango, V. (1985). *Effect of Foeniculum vulgare seed extract on mammary glands and oviducts of ovariectomised rats* (Vol. 5).

Dhar, S. K. (1995). Anti-fertility activity and hormonal profile of trans-anethole in rats. *Indian J Physiol Pharmacol, 39*(1), 63-67.

Dou, Y.-D., Zhao, H., Huang, T., Zhao, S.-G., Liu, X.-M., Yu, X.-C., . . . Chen, Z.-J. (2016). STMN1 Promotes Progesterone Production Via StAR Up-regulation in Mouse Granulosa Cells. *Scientific Reports, 6*, 26691. doi:10.1038/srep26691

https://www.nature.com/articles/srep26691#supplementary-information

Eisenberg, D. M., Kessler, R. C., Van Rompay, M. I., Kaptchuk, T. J., Wilkey, S. A., Appel, S., & Davis, R. B. (2001). Perceptions about complementary therapies relative to conventional therapies among adults who use both: results from a national survey. *Ann Intern Med, 135*(5), 344-351.

Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Pharmacol, 4*, 177-177. doi:10.3389/fphar.2013.00177

El Asbahani, A., Miladi, K., Badri, W., Sala, M., Ait Addi, E. H., Casabianca, H., . . . Elaissari, A. (2015). Essential oils: from extraction to encapsulation. *Int J Pharm, 483*(1-2), 220-243. doi:10.1016/j.ijpharm.2014.12.069

Enan, E. E. (2005). Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils. *Arch Insect Biochem Physiol, 59*(3), 161-171. doi:10.1002/arch.20076

Ernst, E. (2002). Herbal medicinal products during pregnancy: are they safe? *BJOG: An International Journal of Obstetrics & Gynaecology, 109*(3), 227-235. doi:doi:10.1111/j.1471-0528.2002.t01-1-01009.x

Falah, N., Torday, J., Quinney, S., & Haas, D. (2015). *Estriol review: Clinical applications and potential biomedical importance* (Vol. 1).

Fariba, J., Ghannadi, A., & Najafzadeh, H. (2006). *Evaluation of the Prophylactic Effect of Fennel Essential Oil on Experimental Osteoporosis Model in Rats* (Vol. 2).

Findlay, J. K., Liew, S. H., Simpson, E. R., & Korach, K. S. (2010). Estrogen signaling in the regulation of female reproductive functions. *Handbook of experimental pharmacology*(198), 29-35. doi:10.1007/978-3-642-02062-9_2

Fink, G. (1988). *Gonadotropin secretion and its control*.

Förster, C., Mäkela, S., Wärri, A., Kietz, S., Becker, D., Hultenby, K., . . . Gustafsson, J.-Å. (2002). Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium. *Proceedings of the National Academy of Sciences, 99*(24), 15578-15583. doi:10.1073/pnas.192561299

Frass, M., Strassl, R. P., Friehs, H., Mullner, M., Kundi, M., & Kaye, A. D. (2012). Use and acceptance of complementary and alternative medicine among the general population and medical personnel: a systematic review. *Ochsner J, 12*(1), 45-56.

Galetin, A., Burt, H., Gibbons, L., & Houston, J. B. (2006). PREDICTION OF TIME-DEPENDENT CYP3A4 DRUG-DRUG INTERACTIONS: IMPACT OF ENZYME DEGRADATION, PARALLEL ELIMINATION PATHWAYS, AND INTESTINAL INHIBITION. *Drug Metabolism and Disposition, 34*(1), 166-175. doi:10.1124/dmd.105.006874

Garg, A. K., Buchholz, T. A., & Aggarwal, B. B. (2005). Chemosensitization and radiosensitization of tumors by plant polyphenols. *Antioxid Redox Signal, 7*(11-12), 1630-1647. doi:10.1089/ars.2005.7.1630

Gerhard, I., Fitzer, C., Klinga, K., Rahman, N., & Runnebaum, B. (1986). Estrogen screening in evaluation of fetal outcome and infant's development. *J Perinat Med, 14*(5), 279-291.

Ghosh, S., Wu, Y., Li, R., & Hu, Y. (2005). *Jun proteins modulate the ovary-specific promoter of aromatase gene in ovarian granulosa cells via a cAMP-responsive element* (Vol. 24).

Gillesby, B. E., & Zacharewski, T. R. (1999). pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. *Breast Cancer Res Treat, 56*(3), 253-265.

Girgert, R., Emons, G., & Gründker, C. (2019). Estrogen Signaling in ERα-Negative Breast Cancer: ERβ and GPER. *Front Endocrinol (Lausanne), 9*, 781-781. doi:10.3389/fendo.2018.00781

Goerg, K. J., & Spilker, T. (2003). Effect of peppermint oil and caraway oil on gastrointestinal motility in healthy volunteers: a pharmacodynamic study using simultaneous determination of gastric and gall-bladder emptying and orocaecal transit time. *Aliment Pharmacol Ther, 17*(3), 445-451.

Gori, L., Gallo, E., Mascherini, V., Mugelli, A., Vannacci, A., & Firenzuoli, F. (2012a). Can estragole in fennel seed decoctions really be considered a danger for human health? A fennel safety update. *Evid Based Complement Alternat Med, 2012*, 860542. doi:10.1155/2012/860542

Gori, L., Gallo, E., Mascherini, V., Mugelli, A., Vannacci, A., & Firenzuoli, F. (2012b). Can Estragole in Fennel Seed Decoctions Really Be Considered a Danger for Human Health? A Fennel Safety Update. *Evid Based Complement Alternat Med, 2012*, 860542. doi:10.1155/2012/860542

Gracia, T., Hilscherova, K., Jones, P. D., Newsted, J. L., Zhang, X., Hecker, M., . . . Giesy, J. P. (2006). The H295R system for evaluation of endocrine-disrupting effects. *Ecotoxicol Environ Saf, 65*(3), 293-305. doi:10.1016/j.ecoenv.2006.06.012

Greenlee, H., Kwan, M. L., Ergas, I. J., Sherman, K. J., Krathwohl, S. E., Bonnell, C., . . . Kushi, L. H. (2009). Complementary and alternative therapy use before and after breast cancer diagnosis: the Pathways Study. *Breast cancer research and treatment, 117*(3), 653-665. doi:10.1007/s10549-009-0315-3

Hajagos-Toth, J., Hodi, A., Seres, A. B., & Gaspar, R. (2015). Effects of d- and l-limonene on the pregnant rat myometrium in vitro. *Croat Med J, 56*(5), 431-438.

Haldosen, L. A., Zhao, C., & Dahlman-Wright, K. (2014). Estrogen receptor beta in breast cancer. *Mol Cell Endocrinol, 382*(1), 665-672. doi:10.1016/j.mce.2013.08.005

Hall, H. G., Griffiths, D. L., & McKenna, L. G. (2011). The use of complementary and alternative medicine by pregnant women: A literature review. *Midwifery, 27*(6), 817-824. doi:https://doi.org/10.1016/j.midw.2010.08.007

Hann, D., Baker, F., Denniston, M., & Entrekin, N. (2005). Long-term breast cancer survivors' use of complementary therapies: perceived impact on recovery and prevention of recurrence. *Integr Cancer Ther, 4*(1), 14-20. doi:10.1177/1534735404273723

Harrison, R. A., Holt, D., Pattison, D. J., & Elton, P. J. (2004). Who and how many people are taking herbal supplements? A survey of 21,923 adults. *Int J Vitam Nutr Res, 74*(3), 183-186. doi:10.1024/0300-9831.74.3.183

Hayes, E. L., & Lewis-Wambi, J. S. (2015). Mechanisms of endocrine resistance in breast cancer: an overview of the proposed roles of noncoding RNA. *Breast Cancer Res, 17*, 40. doi:10.1186/s13058-015-0542-y

Heneweer, M., Muusse, M., Dingemans, M., de Jong, P. C., van den Berg, M., & Sanderson, J. T. (2005). Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. *Toxicol Sci, 83*(2), 257-263. doi:10.1093/toxsci/kfi025

Henley, D. V., Lipson, N., Korach, K. S., & Bloch, C. A. (2007). Prepubertal Gynecomastia Linked to Lavender and Tea Tree Oils. *New England Journal of Medicine, 356*(5), 479-485. doi:10.1056/NEJMoa064725

Henrique Bezerra Cabral, P., Campos, R., Fonteles, M., Ferreira Santos, C., Leal-Cardoso, J., & Nascimento, N. (2014). *Effects of the essential oil of Croton zehntneri and its major components, anethole and estragole, on the rat corpora cavernosa* (Vol. 112).

Herman, A., & Herman, A. P. (2015). Essential oils and their constituents as skin penetration enhancer for transdermal drug delivery: a review. *J Pharm Pharmacol, 67*(4), 473-485. doi:10.1111/jphp.12334

Hilscherova, K., Jones, P. D., Gracia, T., Newsted, J. L., Zhang, X., Sanderson, J. T., . . . Giesy, J. P. (2004). Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Toxicol Sci, 81*(1), 78-89.

Hilton, H. N., Clarke, C. L., & Graham, J. D. (2018). Estrogen and progesterone signalling in the normal breast and its implications for cancer development. *Mol Cell Endocrinol, 466*, 2-14. doi:https://doi.org/10.1016/j.mce.2017.08.011

Huang, C., Wu, S., Zhang, X., Chang, H., Zhao, Y., Giesy, J. P., & Hu, J. (2011). Modulation of estrogen synthesis through activation of protein kinase A in H295R cells by extracts of estuary sediments. *Environ Toxicol Chem, 30*(12), 2793-2801. doi:10.1002/etc.688

Hutson, S. W., Cowen, P. N., & Bird, C. C. (1985). Morphometric studies of age related changes in normal human breast and their significance for evolution of mammary cancer. *J Clin Pathol, 38*(3), 281-287.

Igarashi, T. (2013). Physical and psychologic effects of aromatherapy inhalation on pregnant women: a randomized controlled trial. *J Altern Complement Med, 19*(10), 805-810. doi:10.1089/acm.2012.0103

Jermini, M., Dubois, J., Rodondi, P. Y., Zaman, K., Buclin, T., Csajka, C., . . . L, E. R. (2019). Complementary medicine use during cancer treatment and potential herb-drug interactions from a cross-sectional study in an academic centre. *Sci Rep, 9*(1), 5078. doi:10.1038/s41598-019-41532-3

Johnson, P. J., Kozhimannil, K. B., Jou, J., Ghildayal, N., & Rockwood, T. H. (2016). Complementary and Alternative Medicine Use among Women of Reproductive Age in the United States. *Women's health issues : official publication of the Jacobs Institute of Women's Health, 26*(1), 40-47. doi:10.1016/j.whi.2015.08.009

Jonsson, P., Katchy, A., & Williams, C. (2014). Support of a bi-faceted role of estrogen receptor beta (ERbeta) in ERalpha-positive breast cancer cells. *Endocr Relat Cancer, 21*(2), 143-160. doi:10.1530/ERC-13-0444

Joshi, R. K. (2014). Chemical composition and antimicrobial activity of the essential oil of Ocimum basilicum L. (sweet basil) from Western Ghats of North West Karnataka, India. *Ancient science of life, 33*(3), 151-156. doi:10.4103/0257-7941.144618

Joulaeerad, N., Ozgoli, G., Hajimehdipoor, H., Ghasemi, E., & Salehimoghaddam, F. (2018). Effect of Aromatherapy with Peppermint Oil on the Severity of Nausea and Vomiting in Pregnancy: A Single-blind, Randomized, Placebo-controlled trial. *Journal of reproduction & infertility, 19*(1), 32-38.

Kanno, J. (2014). Chapter 61 - Percellome toxicogenomics project as a source of biomarkers of chemical toxicity. In R. C. Gupta (Ed.), *Biomarkers in Toxicology* (pp. 1019-1032). Boston: Academic Press.

Khan, S. I., Zhao, J., Khan, I. A., Walker, L. A., & Dasmahapatra, A. K. (2011). Potential utility of natural products as regulators of breast cancer-associated aromatase promoters. *Reproductive Biology and Endocrinology, 9*(1), 1-10. doi:10.1186/1477-7827-9-91

Kim, M., Park, Y. J., Ahn, H., Moon, B., Chung, K. H., & Oh, S. M. (2016). The effects of the standardized extracts of Ginkgo biloba on steroidogenesis pathways and aromatase activity in H295R human adrenocortical carcinoma cells. *Environ Health Toxicol, 31*, e2016010. doi:10.5620/eht.e2016010

Konkimalla, V. B., & Efferth, T. (2008). Evidence-based Chinese medicine for cancer therapy. *J Ethnopharmacol, 116*(2), 207-210. doi:10.1016/j.jep.2007.12.009

Kumar, A., Malik, F., Bhushan, S., Sethi, V. K., Shahi, A. K., kaur, J., . . . Singh, J. (2008). An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells. *Chem Biol Interact, 171*(3), 332-347. doi:https://doi.org/10.1016/j.cbi.2007.10.003

Kumar, R., Zakharov, M. N., Khan, S. H., Miki, R., Jang, H., Toraldo, G., . . . Jasuja, R. (2011). The Dynamic Structure of the Estrogen Receptor. *Journal of Amino Acids, 2011*, 7. doi:10.4061/2011/812540

Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat, 83*(3), 249-289. doi:10.1023/B:BREA.0000014042.54925.cc

Lee, S., & Kelleher, S. L. (2016). Biological underpinnings of breastfeeding challenges: the role of genetics, diet, and environment on lactation physiology. *American journal of physiology. Endocrinology and metabolism, 311*(2), E405-E422. doi:10.1152/ajpendo.00495.2015

Lesgards, J. F., Baldovini, N., Vidal, N., & Pietri, S. (2014). Anticancer activities of essential oils constituents and synergy with conventional therapies: a review. *Phytother Res, 28*(10), 1423-1446. doi:10.1002/ptr.5165

Leung. (1980). Encyclopedia of common natural ingredients used in food, drugs, and cosmetics. In J. W. a. Sons (Ed.). NY.

Leuschner, J. (1997). Reproductive toxicity studies of D-camphor in rats and rabbits. *Arzneimittelforschung, 47*(2), 124-128.

Levitz, M., & Young, B. K. (1977). Estrogens in pregnancy. *Vitam Horm, 35*, 109-147.

Li, L.-X., Chen, L., Meng, X.-Z., Chen, B.-H., Chen, S.-Q., Zhao, Y., . . . Zhang, Y.-H. (2013). Exposure Levels of Environmental Endocrine Disruptors in Mother-Newborn Pairs in China and Their Placental Transfer Characteristics. *PLoS ONE, 8*(5), e62526. doi:10.1371/journal.pone.0062526

Liao, G.-S., Apaya, M. K., & Shyur, L.-F. (2013). Herbal medicine and acupuncture for breast cancer palliative care and adjuvant therapy. *Evid Based Complement Alternat Med, 2013*, 437948-437948. doi:10.1155/2013/437948

Lima, C. F., Andrade, P. B., Seabra, R. M., Fernandes-Ferreira, M., & Pereira-Wilson, C. (2005). The drinking of a Salvia officinalis infusion improves liver antioxidant status in mice and rats. *J Ethnopharmacol, 97*(2), 383-389. doi:10.1016/j.jep.2004.11.029

Liubomirski, Y., Lerrer, S., Meshel, T., Rubinstein-Achiasaf, L., Morein, D., Wiemann, S., . . . Ben-Baruch, A. (2019). Tumor-Stroma-Inflammation Networks Promote Pro-metastatic Chemokines and Aggressiveness Characteristics in Triple-Negative Breast Cancer. *Frontiers in Immunology, 10*(757). doi:10.3389/fimmu.2019.00757

Louik, C., Gardiner, P., Kelley, K., & Mitchell, A. A. (2010). Use of herbal treatments in pregnancy. *Am J Obstet Gynecol, 202*(5), 439.e431-439.e410. doi:10.1016/j.ajog.2010.01.055

Lumachi, F., Santeufemia, D. A., & Basso, S. M. (2015). Current medical treatment of estrogen receptor-positive breast cancer. *World J Biol Chem, 6*(3), 231-239. doi:10.4331/wjbc.v6.i3.231

Luu-The, V. (2001). *Analysis and characteristics of multiple types of human 17??-hydroxysteroid dehydrogenase* (Vol. 76).

Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr., . . . O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev, 9*(18), 2266-2278.

Macias, H., & Hinck, L. (2012). Mammary gland development. *Wiley interdisciplinary reviews. Developmental biology, 1*(4), 533-557. doi:10.1002/wdev.35

Malini, T., Vanithakumari, G., Megala, N., Anusya, S., Devi, K., & Elango, V. (1985). Effect of Foeniculum vulgare Mill. seed extract on the genital organs of male and female rats. *Indian J Physiol Pharmacol, 29*(1), 21-26.

Marino, M., Galluzzo, P., & Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. *Current genomics, 7*(8), 497-508.
Marinov, V., & Valcheva-Kuzmanova, S. (2015). Review on the pharmacological activities of anethole. *2015, 2*(2). doi:10.14748/ssp.v2i2.1141
Marshall, A. D., & Caldwell, J. (1992). Influence of modulators of epoxide metabolism on the cytotoxicity of trans-anethole in freshly isolated rat hepatocytes. *Food Chem Toxicol, 30*(6), 467-473.
Marshall, A. D., & Caldwell, J. (1996). Lack of influence of modulators of epoxide metabolism on the genotoxicity of tans-anethole in freshly isolated rat hepatocytes assessed with the unscheduled DNA synthesis assay. *Food and Chemical Toxicology, 34*(4), 337-345. doi:https://doi.org/10.1016/0278-6915(96)00109-3
Martin, I. (2007). *Aromatherapy for massage practitioners* (L. W. & & Wilkins. Eds.). Philadelphia.
Masotti, V., Juteau, F., Bessière, J. M., & Viano, J. (2003). Seasonal and Phenological Variations of the Essential Oil from the Narrow Endemic Species Artemisia molinieri and Its Biological Activities. *Journal of Agricultural and Food Chemistry, 51*(24), 7115-7121. doi:10.1021/jf034621y
Mazaheri, S., Nematbakhsh, M., Bahadorani, M., Pezeshki, Z., Talebi, A., Ghannadi, A.-R., & Ashrafi, F. (2013). Effects of Fennel Essential Oil on Cisplatin-induced Nephrotoxicity in Ovariectomized Rats. *Toxicology international, 20*(2), 138-145. doi:10.4103/0971-6580.117256
Mendonca, B. B., Arnhold, I. J., Bloise, W., Andersson, S., Russell, D. W., & Wilson, J. D. (1999). 17Beta-hydroxysteroid dehydrogenase 3 deficiency in women. *J Clin Endocrinol Metab, 84*(2), 802-804. doi:10.1210/jcem.84.2.5477
Meng, L., Zhou, J., Sasano, H., Suzuki, T., Zeitoun, K. M., & Bulun, S. E. (2001). Tumor Necrosis Factor α and Interleukin 11 Secreted by Malignant Breast Epithelial Cells Inhibit Adipocyte Differentiation by Selectively Down-Regulating CCAAT/Enhancer Binding Protein α and Peroxisome Proliferator-activated Receptor γ. *Mechanism of Desmoplastic Reaction, 61*(5), 2250-2255.
Michael, M. D., Michael, L. F., & Simpson, E. R. (1997). A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. *Mol Cell Endocrinol, 134*(2), 147-156. doi:https://doi.org/10.1016/S0303-7207(97)00178-0
Miettinen, M. M., Mustonen, M. V., Poutanen, M. H., Isomaa, V. V., & Vihko, R. K. (1996). Human 17 beta-hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression. *Biochemical Journal, 314*(Pt 3), 839-845.
Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., & Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res, 43*(3), 1124-1134.
Miller, J. A., Lang, J. E., Ley, M., Nagle, R., Hsu, C. H., Thompson, P. A., . . . Chow, H. H. (2013). Human breast tissue disposition and bioactivity of limonene in women with early-stage breast cancer. *Cancer Prev Res (Phila), 6*(6), 577-584. doi:10.1158/1940-6207.capr-12-0452

Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev, 32*(1), 81-151. doi:10.1210/er.2010-0013

Mimica-Dukic, N., Kujundzic, S., Sokovic, M., & Couladis, M. (2003). Essential oil composition and antifungal activity of Foeniculum vulgare Mill obtained by different distillation conditions. *Phytother Res, 17*(4), 368-371. doi:10.1002/ptr.1159

Mitra, P., & Audus, K. L. (2009). Expression and functional activities of selected sulfotransferase isoforms in BeWo cells and primary cytotrophoblast cells. *Biochem Pharmacol, 78*(12), 1475-1482. doi:10.1016/j.bcp.2009.07.013

Mitra, S., & Dash, R. (2018). Natural Products for the Management and Prevention of Breast Cancer. *Evidence-based Complementary and Alternative Medicine, 2018*, 23. doi:10.1155/2018/8324696

Mucci, L. A., Lagiou, P., Tamimi, R. M., Hsieh, C.-C., Adami, H.-O., & Trichopoulos, D. (2003). Pregnancy estriol, estradiol, progesterone and prolactin in relation to birth weight and other birth size variables (United States). *Cancer Causes & Control, 14*(4), 311-318. doi:10.1023/a:1023966813330

Nakagawa, Y., & Suzuki, T. (2003). Cytotoxic and xenoestrogenic effects via biotransformation of trans-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells. *Biochem Pharmacol, 66*(1), 63-73.

Nampoothiri, L. P., Neelima, P. S., & Rao, A. J. (2007). Proteomic profiling of forskolin-induced differentiated BeWo cells: an in-vitro model of cytotrophoblast differentiation. *Reprod Biomed Online, 14*(4), 477-487.

Navarra, M., Ferlazzo, N., Cirmi, S., Trapasso, E., Bramanti, P., Lombardo, G. E., . . . Gangemi, S. (2015a). Effects of bergamot essential oil and its extractive fractions on SH-SY5Y human neuroblastoma cell growth. *J Pharm Pharmacol, 67*(8), 1042-1053. doi:10.1111/jphp.12403

Navarra, M., Mannucci, C., Delbo, M., & Calapai, G. (2015b). Citrus bergamia essential oil: from basic research to clinical application. *Front Pharmacol, 6*, 36. doi:10.3389/fphar.2015.00036

Neuhouser, M. L., Smith, A. W., George, S. M., Gibson, J. T., Baumgartner, K. B., Baumgartner, R., . . . Ballard, R. (2016). Use of complementary and alternative medicine and breast cancer survival in the Health, Eating, Activity, and Lifestyle Study. *Breast Cancer Res Treat, 160*(3), 539-546. doi:10.1007/s10549-016-4010-x

Nguyen, T. (2017). Endocrine System: Basic Mechanisms and the menstrual cycle. Retrieved from http://sbi4u3.weebly.com/endocrine-hormones-basic-mechanisms-and-the-menstrual-cycle.html

Ni, H., Simile, C., & Hardy, A. M. (2002). Utilization of complementary and alternative medicine by United States adults: results from the 1999 national health interview survey. *Med Care, 40*(4), 353-358.

O'Lone, R., Frith, M. C., Karlsson, E. K., & Hansen, U. (2004). Genomic targets of nuclear estrogen receptors. *Mol Endocrinol, 18*(8), 1859-1875. doi:10.1210/me.2003-0044

Oliveira, C. B., Meurer, Y. S., Medeiros, T. L., Pohlit, A. M., Silva, M. V., Mineo, T. W., & Andrade-Neto, V. F. (2016). Anti-Toxoplasma Activity of Estragole and Thymol in Murine Models of Congenital and Noncongenital Toxoplasmosis. *J Parasitol, 102*(3), 369-376. doi:10.1645/15-848

Oskarsson, A., Ulleras, E., Plant, K. E., Hinson, J. P., & Goldfarb, P. S. (2006). Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. *J Appl Toxicol, 26*(6), 484-492. doi:10.1002/jat.1166

Ostad, S. N., Soodi, M., Shariffzadeh, M., Khorshidi, N., & Marzban, H. (2001). The effect of fennel essential oil on uterine contraction as a model for dysmenorrhea, pharmacology and toxicology study. *J Ethnopharmacol, 76*(3), 299-304.

Oussalah, M., Caillet, S., Salmieri, S., Saucier, L., & Lacroix, M. (2007). Antimicrobial effects of alginate-based films containing essential oils on Listeria monocytogenes and Salmonella typhimurium present in bologna and ham. *J Food Prot, 70*(4), 901-908.

Pagadala, N. S., Syed, K., & Tuszynski, J. (2017). Software for molecular docking: a review. *Biophysical reviews, 9*(2), 91-102. doi:10.1007/s12551-016-0247-1

Palmieri, C. (2004). *The expression of oestrogen receptor (ER)-beta and its variants, but not ERalpha, in adult human mammary fibroblasts* (Vol. 33).

Park, K. R., Nam, D., Yun, H. M., Lee, S. G., Jang, H. J., Sethi, G., . . . Ahn, K. S. (2011). beta-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. *Cancer Lett, 312*(2), 178-188. doi:10.1016/j.canlet.2011.08.001

Patel, S. (2017). Fragrance compounds: The wolves in sheep's clothings. *Med Hypotheses, 102*, 106-111. doi:10.1016/j.mehy.2017.03.025

Patwardhan, B., Warude, D., Pushpangadan, P., & Bhatt, N. (2005). Ayurveda and Traditional Chinese Medicine: A Comparative Overview. *Evidence-based Complementary and Alternative Medicine, 2*(4), 465-473. doi:10.1093/ecam/neh140

Pelkonen, O., Duez, P., Vuorela, P. M., & Vuorela, H. (2017). *Toxicology of herbal products*: Springer.

Petrie, K. A., & Peck, M. R. (2000). Alternative medicine in maternity care. *Prim Care, 27*(1), 117-136.

Pezzi, V., Mathis, J. M., Rainey, W. E., & Carr, B. R. (2003). Profiling transcript levels for steroidogenic enzymes in fetal tissues. *The Journal of Steroid Biochemistry and Molecular Biology, 87*(2), 181-189. doi:https://doi.org/10.1016/j.jsbmb.2003.07.006

Phillips, D. H. (1994). DNA adducts derived from safrole, estragole and related compounds, and from benzene and its metabolites. *IARC Sci Publ*(125), 131-140.

Picon, R., Darmoul, D., Rouiller, V., & Duranteau, L. (1988). Activity of 3 beta-hydroxysteroid dehydrogenase/isomerase in the fetal rat ovary. *J Steroid Biochem, 31*(5), 839-843.

Pinto, E., Salgueiro, L. R., Cavaleiro, C., Palmeira, A., & Gonçalves, M. J. (2007). In vitro susceptibility of some species of yeasts and filamentous fungi to essential oils of Salvia officinalis. *Industrial Crops and Products, 26*(2), 135-141. doi:http://dx.doi.org/10.1016/j.indcrop.2007.02.004

Poirier, D. (2010). 17β-Hydroxysteroid dehydrogenase inhibitors: a patent review. *Expert Opinion on Therapeutic Patents, 20*(9), 1123-1145. doi:10.1517/13543776.2010.505604

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., . . . Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res, 12*(5), R68. doi:10.1186/bcr2635

Purkait, S., Bhattacharya, A., Bag, A., & Chattopadhyay, R. R. (2018). Antibacterial and Antioxidant Potential of Essential Oils of Five Spices. *Journal of Food Quality and Hazards Control, 5*(2), 61-71. doi:10.29252/jfqhc.5.2.6

Qin, K., Ehrmann, D. A., Cox, N., Refetoff, S., & Rosenfield, R. L. (2006). Identification of a functional polymorphism of the human type 5 17beta-hydroxysteroid dehydrogenase gene associated with polycystic ovary syndrome. *J Clin Endocrinol Metab, 91*(1), 270-276. doi:10.1210/jc.2005-2012

Raal, A., Orav, A., & Arak, E. (2007). Composition of the essential oil of Salvia officinalis L. from various European countries. *Nat Prod Res, 21*(5), 406-411. doi:10.1080/14786410500528478

Rasmussen, M. K., Ekstrand, B., & Zamaratskaia, G. (2013). Regulation of 3β-Hydroxysteroid Dehydrogenase/Δ(5)-Δ(4) Isomerase: A Review. *International Journal of Molecular Sciences, 14*(9), 17926-17942. doi:10.3390/ijms140917926

Reed, B., & Carr, B. (2018). *The Normal Menstrual Cycle and the Control of Ovulation*: Endotext [Internet].

Rhoda Lee, C. (2009). *Drug interactions and hormonal contraception* (Vol. 14).

Rogan, E. G., Cavalieri, E. L., Köfeler, H., Li, K.-M., Gross, M. L., Devanesan, P., . . . Higginbotham, S. (2004). Metabolism and DNA binding studies of 4-hydroxyestradiol and estradiol-3,4-quinone in vitro and in female ACI rat mammary gland in vivo. *Carcinogenesis, 25*(2), 289-297. doi:10.1093/carcin/bgg191

Roumeliotis, G. A., Dostaler, G., & Boyd, K. U. (2017). Complementary and Alternative Medicines and Patients With Breast Cancer: A Case of Mortality and Systematic Review of Patterns of Use in Patients With Breast Cancer. *Plast Surg (Oakv), 25*(4), 275-283. doi:10.1177/2292550317716126

Russo, J., Ao, X., Grill, C., & Russo, I. H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res Treat, 53*(3), 217-227.

Russo, J., & Russo, I. H. (2006). The role of estrogen in the initiation of breast cancer. *J Steroid Biochem Mol Biol, 102*(1-5), 89-96. doi:10.1016/j.jsbmb.2006.09.004

Saha Roy, S., & Vadlamudi, R. K. (2012). Role of estrogen receptor signaling in breast cancer metastasis. *Int J Breast Cancer, 2012*, 654698. doi:10.1155/2012/654698

Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., & Gustafsson, J. A. (2000). Estrogen receptors alpha and beta in the rodent mammary gland. *Proceedings of the National Academy of Sciences of the United States of America, 97*(1), 337-342.

Saljoughian, S., Roohinejad, S., Bekhit, A. E.-D. A., Greiner, R., Omidizadeh, A., Nikmaram, N., & Mousavi Khaneghah, A. (2018). The effects of food essential oils on cardiovascular diseases: A review. *Critical Reviews in Food Science and Nutrition, 58*(10), 1688-1705. doi:10.1080/10408398.2017.1279121

Samandari, E., Kempna, P., Nuoffer, J. M., Hofer, G., Mullis, P. E., & Fluck, C. E. (2007). Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295A cells and differ in 3beta-hydroxysteroid dehydrogenase type 2 and 17,20 lyase activities. *J Endocrinol, 195*(3), 459-472. doi:10.1677/joe-07-0166

Samojlik, I., Đaković-Švajcer, K., Božin, B., & Mikov, M. (2012a). Herb-drug interactions: the influence of essential oil of caraway (Carum carviL.) on the pharmacokinetics of paracetamol. *BMC Pharmacology and Toxicology, 13*(1), A27. doi:10.1186/2050-6511-13-s1-a27

Samojlik, I., Mijatović, V., Petković, S., Škrbić, B., & Božin, B. (2012b). The influence of essential oil of aniseed (Pimpinella anisum, L.) on drug effects on the central nervous system. *Fitoterapia, 83*(8), 1466-1473. doi:https://doi.org/10.1016/j.fitote.2012.08.012

Samojlik, I., Petković, S., Stilinović, N., Vukmirović, S., Mijatović, V., & Božin, B. (2016). Pharmacokinetic Herb–Drug Interaction between Essential Oil of Aniseed (Pimpinella anisum L., Apiaceae) and Acetaminophen and Caffeine: A Potential Risk for Clinical Practice. *Phytotherapy Research, 30*(2), 253-259. doi:10.1002/ptr.5523

Sanderson, J. T. (2006). The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals. *Toxicological Sciences, 94*(1), 3-21. doi:10.1093/toxsci/kfl051

Sanderson, J. T. (2009). Placental and Fetal Steroidogenesis. In C. Vaillancourt & J. Lafond (Eds.), *Human Embryogenesis: Methods and Protocols* (pp. 127-136). Totowa, NJ: Humana Press.

Santos, B. C. S., Pires, A. S., Yamamoto, C. H., Couri, M. R. C., Taranto, A. G., Alves, M. S., . . . de Sousa, O. V. (2018). Methyl Chavicol and Its Synthetic Analogue as Possible Antioxidant and Antilipase Agents Based on the In Vitro and In Silico Assays. *Oxidative medicine and cellular longevity, 2018*, 2189348-2189348. doi:10.1155/2018/2189348

Sasson, S., & Notides, A. C. (1983). Estriol and estrone interaction with the estrogen receptor. II. Estriol and estrone-induced inhibition of the cooperative binding of [3H]estradiol to the estrogen receptor. *J Biol Chem, 258*(13), 8118-8122.

Savino, F., Cresi, F., Castagno, E., Silvestro, L., & Oggero, R. (2005). A randomized double-blind placebo-controlled trial of a standardized extract of Matricariae recutita, Foeniculum vulgare and Melissa officinalis (ColiMil) in the treatment of breastfed colicky infants. *Phytother Res, 19*(4), 335-340. doi:10.1002/ptr.1668

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., . . . Lichtensteiger, W. (2004). Endocrine activity and developmental toxicity of cosmetic UV filters--an update. *Toxicology, 205*(1-2), 113-122. doi:10.1016/j.tox.2004.06.043

Schwarzenbach, H., Manna, P. R., Stocco, D. M., Chakrabarti, G., & Mukhopadhyay, A. K. (2003). Stimulatory effect of progesterone on the expression of steroidogenic acute regulatory protein in MA-10 Leydig cells. *Biol Reprod, 68*(3), 1054-1063.

Sebastian, S., Takayama, K., Shozu, M., & Bulun, S. E. (2002). Cloning and Characterization of a Novel Endothelial Promoter of the Human CYP19 (Aromatase P450) Gene that Is Up-Regulated in Breast Cancer Tissue. *Molecular Endocrinology, 16*(10), 2243-2254. doi:10.1210/me.2002-0123

Shenhav, S., Gemer, O., Volodarsky, M., Zohav, E., & Segal, S. (2003). Midtrimester triple test levels in women with severe preeclampsia and HELLP syndrome. *Acta Obstet Gynecol Scand, 82*(10), 912-915.

Shinohara, K., Doi, H., Kumagai, C., Sawano, E., & Tarumi, W. (2017). Effects of essential oil exposure on salivary estrogen concentration in perimenopausal women. *Neuro Endocrinol Lett, 37*(8), 567-572.

Shirazi, M., Mohebitabar, S., Bioos, S., Yekaninejad, M. S., Rahimi, R., Shahpiri, Z., . . . Nejatbakhsh, F. (2017). The Effect of Topical Rosa damascena (Rose) Oil on Pregnancy-Related Low Back Pain:A Randomized Controlled Clinical Trial. *Journal of Evidence-Based Complementary & Alternative Medicine, 22*(1), 120-126. doi:10.1177/2156587216654601

Simard, J., Ricketts, M.-L., Gingras, S. b., Soucy, P., Feltus, F. A., & Melner, M. H. (2005). Molecular Biology of the 3β-Hydroxysteroid Dehydrogenase/Δ5-Δ4 Isomerase Gene Family. *Endocrine Reviews, 26*(4), 525-582. doi:10.1210/er.2002-0050

Simões, B. M., Kohler, B., Clarke, R. B., Stringer, J., Novak-Frazer, L., Young, K., . . . Howell, S. J. (2018). Estrogenicity of essential oils is not required to relieve symptoms of

urogenital atrophy in breast cancer survivors. *Therapeutic Advances in Medical Oncology, 10*, 1758835918766189. doi:10.1177/1758835918766189
Simpson, E. R. (2003a). Sources of estrogen and their importance. *J Steroid Biochem Mol Biol, 86*.
Simpson, E. R. (2003b). Sources of estrogen and their importance. *The Journal of Steroid Biochemistry and Molecular Biology, 86*(3-5), 225-230. doi:10.1016/s0960-0760(03)00360-1
Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., . . . Jones, M. (2002). Aromatase-a brief overview. *Annu Rev Physiol, 64*.
Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Corbin, C. J., & Mendelson, C. R. (1993). Tissue-specific promoters regulate aromatase cytochrome P450 expression. *Clin Chem, 39*(2), 317-324.
Skinner, M. K., Manikkam, M., & Guerrero-Bosagna, C. (2011). Epigenetic transgenerational actions of endocrine disruptors. *Reproductive Toxicology, 31*(3), 337-343. doi:http://dx.doi.org/10.1016/j.reprotox.2010.10.012
Standen, M. D., & Myers, S. P. (2004). The roles of essential oils in the modulation of immune function and inflammation: survey of aromatherapy educators. *International Journal of Aromatherapy, 14*(4), 150-161. doi:https://doi.org/10.1016/j.ijat.2004.09.004
Stanley, E. L., Hume, R., Visser, T. J., & Coughtrie, M. W. (2001). Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development. *J Clin Endocrinol Metab, 86*(12), 5944-5955. doi:10.1210/jcem.86.12.8081
Sugawara, T., Kiriakidou, M., McAllister, J. M., Holt, J. A., Arakane, F., & Strauss, J. F. (1997). Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: A central role for steroidogenic factor 1. *Steroids, 62*(1), 5-9. doi:http://dx.doi.org/10.1016/S0039-128X(96)00152-3
Sumit, K., Swarnkar, V., S, S., & Baldi, A. (2012). *Herbal Cosmetics: Used for Skin and Hair* (Vol. 2012).
Sun, T., Zhao, Y., Mangelsdorf, D. J., & Simpson, E. R. (1998). Characterization of a region upstream of exon I.1 of the human CYP19 (aromatase) gene that mediates regulation by retinoids in human choriocarcinoma cells. *Endocrinology, 139*(4), 1684-1691. doi:10.1210/endo.139.4.5959
Svoboda, K. P., & Greenaway, R. I. (2003). Lemon scented plants. *International Journal of Aromatherapy, 13*(1), 23-32. doi:http://dx.doi.org/10.1016/S0962-4562(03)00048-1
Tascilar, M., de Jong, F. A., Verweij, J., & Mathijssen, R. H. (2006). Complementary and alternative medicine during cancer treatment: beyond innocence. *Oncologist, 11*(7), 732-741. doi:10.1634/theoncologist.11-7-732
Thibeault, A. A., Deroy, K., Vaillancourt, C., & Sanderson, J. T. (2014). A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals. *Environ Health Perspect, 122*(4), 371-377. doi:10.1289/ehp.1307518
Tillett, J., & Ames, D. (2010). The uses of aromatherapy in women's health. *J Perinat Neonatal Nurs, 24*(3), 238-245. doi:10.1097/JPN.0b013e3181ece75d
Tisserand, R., & Balacs, T. (2014). In R. Tisserand & R. Young (Eds.), *Essential Oil Safety (Second Edition)* (pp. xi-xii). St. Louis: Churchill Livingstone.

Torres-Martínez, R., García-Rodríguez, Y. M., Ríos-Chávez, P., Saavedra-Molina, A., López-Meza, J. E., Ochoa-Zarzosa, A., & Garciglia, R. S. (2018). Antioxidant Activity of the Essential Oil and its Major Terpenes of Satureja macrostema (Moc. and Sessé ex Benth.) Briq. *Pharmacognosy magazine, 13*(Suppl 4), S875-S880. doi:10.4103/pm.pm_316_17

Truhaut, R., Le Bourhis, B., Attia, M., Glomot, R., Newman, J., & Caldwell, J. (1989). Chronic toxicity/carcinogenicity study of trans-anethole in rats. *Food Chem Toxicol, 27*(1), 11-20.

Tuckey, R. C., Bose, H. S., Czerwionka, I., & Miller, W. L. (2004). Molten Globule Structure and Steroidogenic Activity of N-218 MLN64 in Human Placental Mitochondria. *Endocrinology, 145*(4), 1700-1707. doi:10.1210/en.2003-1034

Uribe, A., Strauss, I. I. I. J. F., & Martínez, F. (2003). Contact sites from human placental mitochondria: Characterization and role in progesterone synthesis. *Archives of Biochemistry and Biophysics, 413*(2), 172-181. doi:https://doi.org/10.1016/S0003-9861(03)00097-3

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Jr., Lee, D.-H., . . . Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine reviews, 33*(3), 378-455. doi:10.1210/er.2011-1050

von Krogh, K., Harjen, H., Almas, C., Zimmer, K. E., Dahl, E., Olsaker, I., . . . Verhaegen, S. (2010). The effect of valproate and levetiracetam on steroidogenesis in forskolin-stimulated H295R cells. *Epilepsia, 51*(11), 2280-2288. doi:10.1111/j.1528-1167.2010.02702.x

Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S., & Marc, J. (2014). The many faces of estrogen signaling. *Biochemia medica, 24*(3), 329-342. doi:10.11613/BM.2014.035

Wada, T., Tsuneki, H., & Sasaoka, T. (2013). New Insights Into Metabolic Regulation via Bifurcated Function of Estrogen Receptor α. *Diabetes, 62*(12), 3996-3998. doi:10.2337/db13-1325

Wang, X., Sang, X., Diorio, C., Lin, S. X., & Doillon, C. J. (2015). In vitro interactions between mammary fibroblasts (Hs 578Bst) and cancer epithelial cells (MCF-7) modulate aromatase, steroid sulfatase and 17beta-hydroxysteroid dehydrogenases. *Mol Cell Endocrinol, 412*, 339-348. doi:10.1016/j.mce.2015.05.032

Watanabe, M., & Nakajin, S. (2004). Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. *J Endocrinol, 180*(1), 125-133.

Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., . . . Strauss, J. F., 3rd. (1997). MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc Natl Acad Sci U S A, 94*(16), 8462-8467.

Watson, P. H., Pon, R. T., & Shiu, R. P. (1991). Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. *Cancer Res, 51*(15), 3996-4000.

Weizman, Z., Alkrinawi, S., Goldfarb, D., & Bitran, C. (1993). Efficacy of herbal tea preparation in infantile colic. *J Pediatr, 122*(4), 650-652.

Wilde, J., Erdmann, M., Mertens, M., Eiselt, G., & Schmidt, M. (2013). Aromatase activity induction in human adipose fibroblasts by retinoic acids via retinoic acid receptor α. *Journal of molecular endocrinology, 51*(2), 247-260. doi:10.1530/jme-12-0129

Worldwide cancer data: Global cancer statistics for the most common cancers. (2018). *Worlwide Cancer Research Fund.* Retrieved from https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data

Xiao, Y., Yang, F.-Q., Li, S.-P., Hu, G., Lee, S.-M.-Y., & Wang, Y.-T. (2008). Essential oil of Curcuma wenyujin induces apoptosis in human hepatoma cells. *World journal of gastroenterology, 14*(27), 4309-4318. doi:10.3748/wjg.14.4309

Xu, Y., Yu, R. M., Zhang, X., Murphy, M. B., Giesy, J. P., Lam, M. H., . . . Yu, H. (2006). Effects of PCBs and MeSO2-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. *Chemosphere, 63*(5), 772-784. doi:10.1016/j.chemosphere.2005.08.013

Yang, O., Kim, H. L., Weon, J. I., & Seo, Y. R. (2015). Endocrine-disrupting Chemicals: Review of Toxicological Mechanisms Using Molecular Pathway Analysis. *J Cancer Prev, 20*(1), 12-24. doi:10.15430/JCP.2015.20.1.12

Yaşar, P., Ayaz, G., User, S. D., Güpür, G., & Muyan, M. (2016). Molecular mechanism of estrogen-estrogen receptor signaling. *Reproductive medicine and biology, 16*(1), 4-20. doi:10.1002/rmb2.12006

Zeng, H., Chen, X., & Liang, J. (2015). In vitro antifungal activity and mechanism of essential oil from fennel (Foeniculum vulgare L.) on dermatophyte species. *J Med Microbiol, 64*(Pt 1), 93-103. doi:10.1099/jmm.0.077768-0

Zhang, M., Liu, P., Dwyer, N. K., Christenson, L. K., Fujimoto, T., Martinez, F., . . . Strauss, J. F., 3rd. (2002). MLN64 mediates mobilization of lysosomal cholesterol to steroidogenic mitochondria. *J Biol Chem, 277*(36), 33300-33310. doi:10.1074/jbc.M200003200

Zhao, H., Zhou, L., Shangguan, A. J., & Bulun, S. E. (2016). Aromatase expression and regulation in breast and endometrial cancer. *Journal of molecular endocrinology, 57*(1), R19-R33. doi:10.1530/JME-15-0310

Zhao, Y., Nichols, J. E., Bulun, S. E., Mendelson, C. R., & Simpson, E. R. (1995). Aromatase P450 Gene Expression in Human Adipose Tissue. ROLE OF A Jak/STAT PATHWAY IN REGULATION OF THE ADIPOSE-SPECIFIC PROMOTER. *Journal of Biological Chemistry, 270*(27), 16449-16457. doi:10.1074/jbc.270.27.16449

Zhou, J., Gurates, B., Yang, S., Sebastian, S., & Bulun, S. E. (2001). Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. *Cancer Res, 61*.

Zu, Y., Yu, H., Liang, L., Fu, Y., Efferth, T., Liu, X., & Wu, N. (2010). Activities of ten essential oils towards Propionibacterium acnes and PC-3, A-549 and MCF-7 cancer cells. *Molecules (Basel, Switzerland), 15*(5), 3200-3210. doi:10.3390/molecules15053200

SUPPLEMENTARY INFORMATION

Basil essential oil GC/MS report

Date : June 25, 2015

SAMPLE IDENTIFICATION

Internal code : 15F03-RMO13-1-AS
Customer identification : Basil (NEW RMO) - 1508
Type : Huile essentielle
Source : *Ocimum basilicum*
Customer : Rocky Mountain Oils

ANALYSIS

Method : PC-PA-001-14G14, "Analysis of the composition of a liquid essential oil by GC-FID" (in French). Identification counter-checked with GC-MS.
Analyst : Alexis St-Gelais, M. Sc.
Analysis date : 2015-06-17

IDENTIFIED COMPOUND

Identification	Colonne: BP5			Colonne: WAX			Molecular Class
	R.T.	R.I.	%	%	R.I.	R.T.	
Sabinene	3.62	962	0.02	0.04	1060	1.98	Monoterpene
β-Pinene	3.67	965	0.06	0.06	1041	1.80	Monoterpene
Octen-3-ol	3.96	984	0.08	0.06	1405	6.94	Aliphatic alcohol
6-Methyl-5-hepten-2-one	4.04	989	0.15	0.13	1280	4.88	Aliphatic ketone
Octan-3-ol	4.24	1001	0.03	0.03	1344	5.90	Aliphatic alcohol
α-Phellandrene	4.34	1007	0.05	0.06	1115	2.57	Monoterpene
cis-Hex-3-en-1-yl acetate	4.37	1009	0.02	0.02	1264	4.64	Aliphatic ester
para-Cymene	4.57	1020	0.01	0.02	1209	3.82	Monoterpene
Limonene	4.60	1022	0.07	0.06	1138	2.87	Monoterpene
1,8-Cineole	4.67	1026	0.39	0.40	1144	2.94	Monoterp. ether
cis-β-Ocimene	4.80	1033	0.03	0.03	1187	3.51	Monoterpene
trans-β-Ocimene	4.98	1043	0.23	0.24	1202	3.71	Monoterpene
cis-Sabinene hydrate	5.42	1069	0.03	0.04	1419	7.28	Monoterp. alcohol
Terpinolene	5.62	1080	0.05	0.04	1234	4.19	Monoterpene
trans-Linalool oxide (fur.)	5.73	1086	0.05	0.05	1385	6.56	Monoterp. alcohol
Linalool	6.12	1106	19.55	19.72	1500	9.30	Monoterp. alcohol
Terpinen-4-ol?	7.74	1176	0.32				Monoterp. alcohol
Methylchavicol	8.65	1209	74.61	75.03	1592	12.83	Phenylpropanoid
Neral	9.75	1236	0.59	0.54	1598	13.06	Monoterp. aldehyde
Geranial	11.02	1268	0.78	0.78	1642	15.41	Monoterp. aldehyde

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α-Copaene	15.29	1351	0.04	0.03	1422	7.35	Sesquiterpene
β-Caryophyllene	17.82	1393	0.52	0.40	1512	9.76	Sesquiterpene
trans-α-Bergamotene	19.34	1414	0.50	0.49	1518	9.98	Sesquiterpene
cis-β-Farnesene	20.03	1423	0.09	0.29	1569	11.93*	Sesquiterpene
α-Humulene	20.32	1426	0.13	[0.29]	1569	11.93*	Sesquiterpene
trans-β-Farnesene	21.57	1442	0.11	0.08	1606	13.48	Sesquiterpene
Germacrene D	22.35	1452	0.18	0.09	1609	13.63	Sesquiterpene
trans-α-Bisabolene	28.42	1528	0.37	0.42	1691	18.07	Sesquiterpene
Total identified			**99.06%**	**99.15%**			

*: Two or more compounds are coeluting on this column

[xx]: Duplicate percentage due to coelutions, not taken account in the identified total

Note: no correction factor was applied

Other Data

Physical aspect : Clear liquid

Refractive Index : 1.5050 ± 0.0003 (20.0 °C)

Conclusion

No adulterant, contaminant or diluent were detected using this method.

Checked and approved by :

Alexis St-Gelais, M. Sc., chimiste 2013-174

Note: This report is a shortened version of the original.

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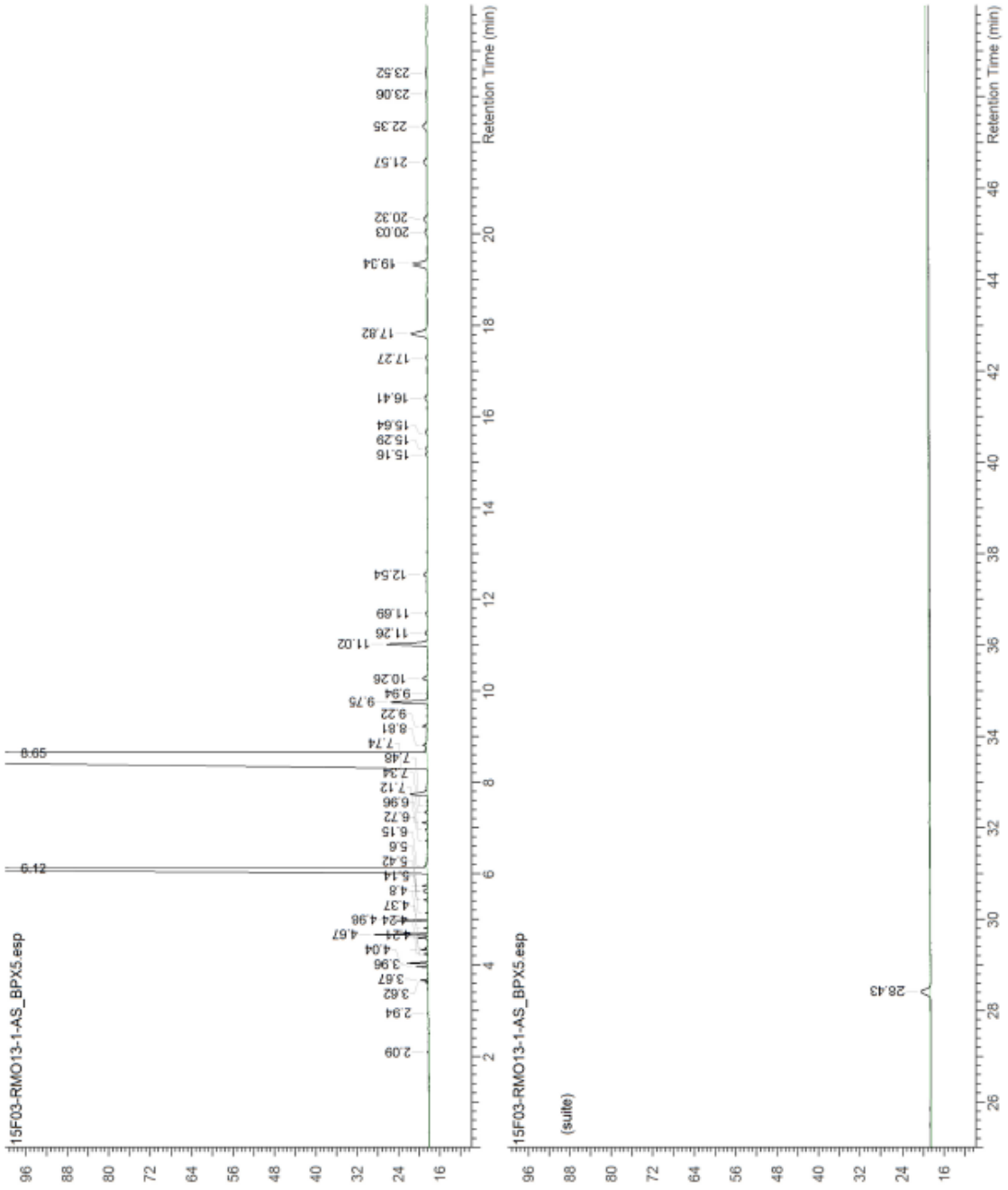
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23.06
22.35
21.57
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20.03
19.34
17.82
17.27
16.41
15.64
15.29
15.16
12.54
11.89
11.26
11.02
10.26
9.94
9.75
9.22
8.81
7.74
7.48
7.34
7.12
6.96
6.72
6.15
5.6
5.42
5.14
4.8
4.37
4.24
4.98
4.21
4.67
4.04
3.96
3.87
3.62
2.94
2.09
15F03-RMO13-1-AS_BPX5.esp
(suite)
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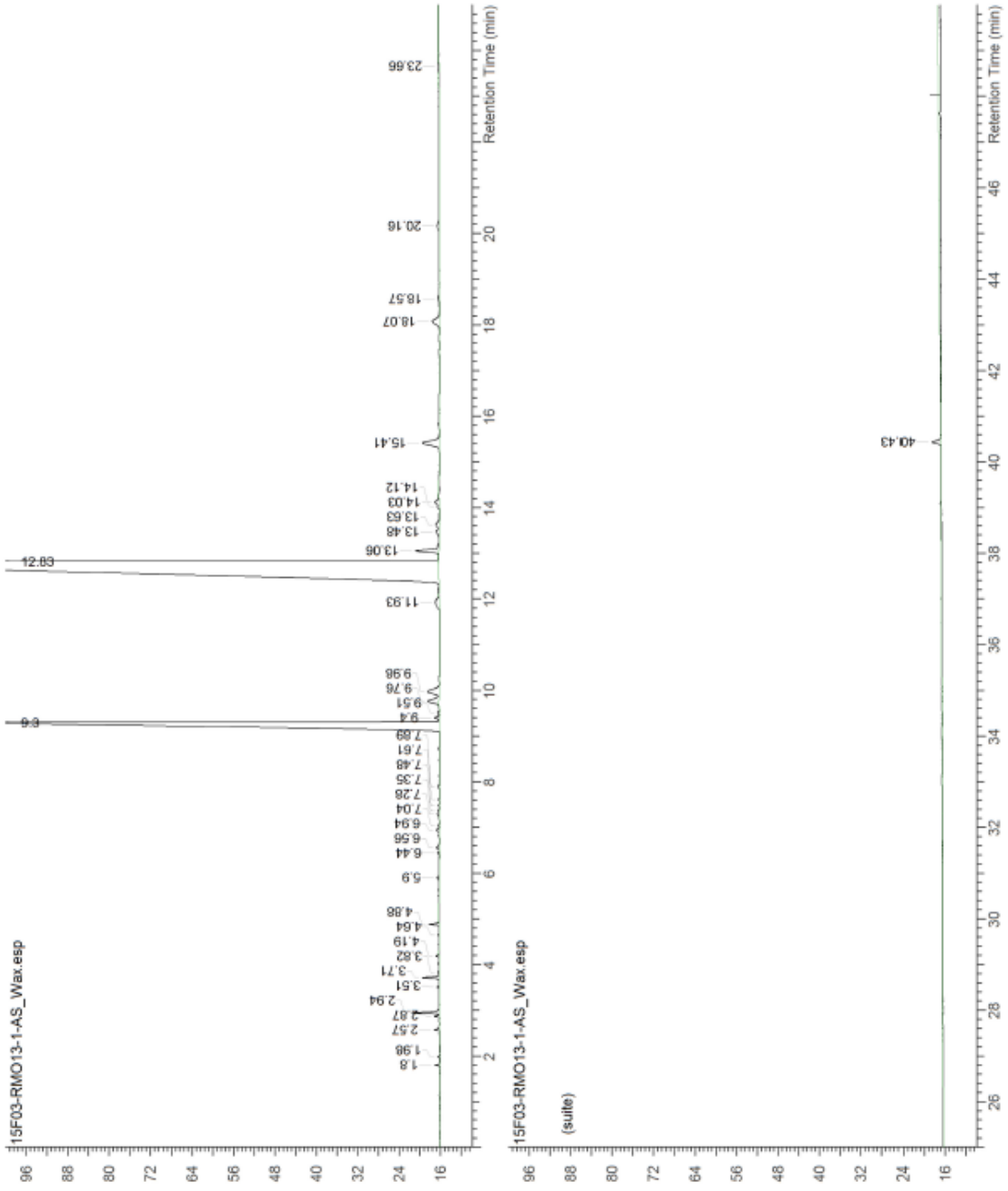
Essential Oil, Ocimum basilicum
Internal Code: 15F03-RMO13-1-AS Customer Identification: Basil (NEW RMO) - 1508

Report prepared for
Rocky Mountain Oils



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Plus que des analyses... des conseils

Fennel seed essential oil GC/MS report

Date : August 26, 2015

SAMPLE IDENTIFICATION

Internal code : 15H11-RMO4-1-AS
Customer identification : Fennel Seed - TU0123
Type : Essential Oil
Source : *Foeniculum vulgare*
Customer : Rocky Mountain Oils

ANALYSIS

Method : PC-PA-001-15E06, "Analysis of the composition of a liquid essential oil by GC-FID" (in French).
Analyst : Alexis St-Gelais, M. Sc.
Analysis date : 2015-08-22

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IDENTIFIED COMPOUNDS

Identification	Colonne: BP5			Colonne: WAX			Molecular Class
	R.T.	R.I.	%	%	R.I.	R.T.	
Tricyclene	2.63	914	0.03	0.02	963	0.96	Monoterpene
α-Thujene	2.72	920	0.01	0.02	996	1.11	Monoterpene
α-Pinene	2.82	927	6.57	6.46	987	1.08	Monoterpene
Camphene	3.07*	942	0.06	0.06	1024	1.35	Monoterpene
α-Fenchene	3.07*	942	[0.06]	0.01	1017	1.29	Monoterpene
Sabinene	3.48	969	0.02	0.02	1083	1.87	Monoterpene
β-Pinene	3.52	972	0.05	0.05	1063	1.69	Monoterpene
Myrcene	3.83	991	0.17	0.17	1134	2.45	Monoterpene
α-Phellandrene	4.06*	1005	0.08	0.06	1125	2.34	Monoterpene
Δ3-Carene	4.06*	1005	[0.08]	0.01	1110	2.14	Monoterpene
Limonene	4.50*	1030	8.61	8.24	1160	2.77	Monoterpene
para-Cymene	4.50*	1030	[8.61]	0.23	1228	3.68	Monoterpene
γ-Terpinene	5.00	1059	0.12	0.13	1206	3.36	Monoterpene
Terpinolene	5.46	1085	0.01	0.02	1240	3.85	Monoterpene
Fenchone	5.59	1093	3.39	3.36	1342	5.35	Monoterp. ketone
Linalool	5.92	1109	0.01	0.01	1517	8.84	Monoterp. alcohol
Camphor	6.70	1145	0.06	0.07	1442	7.09	Monoterp. ketone
Methylchavicol	8.17	1207	3.51	3.48	1610	12.02	Phenylpropanoid
Carvone	9.63	1247	0.13	0.12	1649	13.87	Monoterp. ketone
cis-Anethole	10.00	1257	0.08	0.09	1688	15.75	Phenylpropanoid
para-Anisaldehyde	10.56	1272	0.22	0.40	1921	30.98	Simple phenolic
trans-Anethole	12.04	1307	76.53	76.50	1760	20.02	Phenylpropanoid
Total identified			**99.66%**	**99.53%**			

*: Two or more compounds are coeluting on this column

[xx]: Duplicate percentage due to coelutions, not taken account in the identified total

Note: no correction factor was applied

Other Data

Physical aspect : Clear liquid
Refractive index : 1.5430 ± 0.0003 (20 °C)

Conclusion

No adulterant, contaminant or diluent were detected using this method.

Checked and approved by :

Alexis St-Gelais, M. Sc., chimiste 2013-174

Note: This report is a shortened version of the original.

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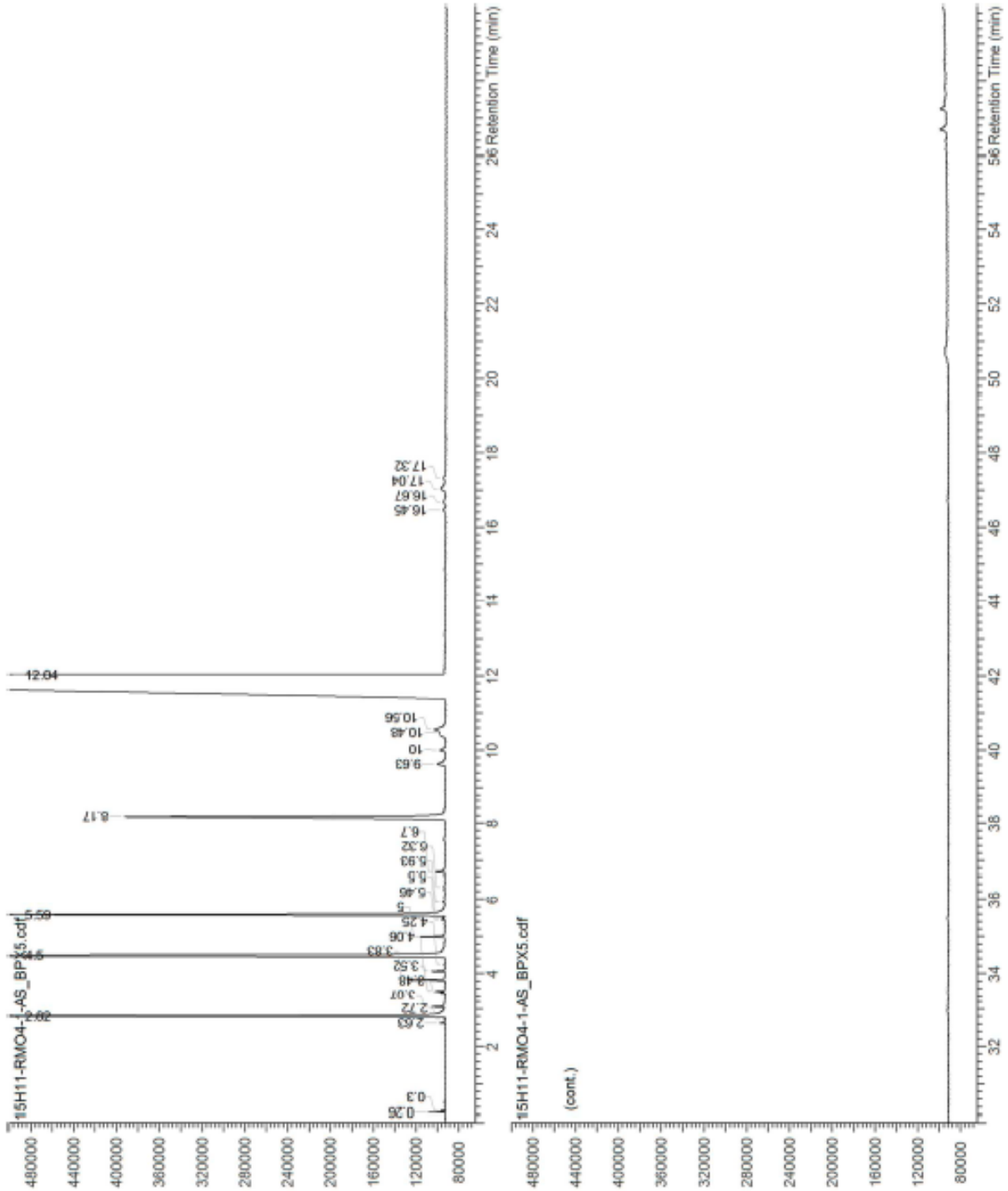
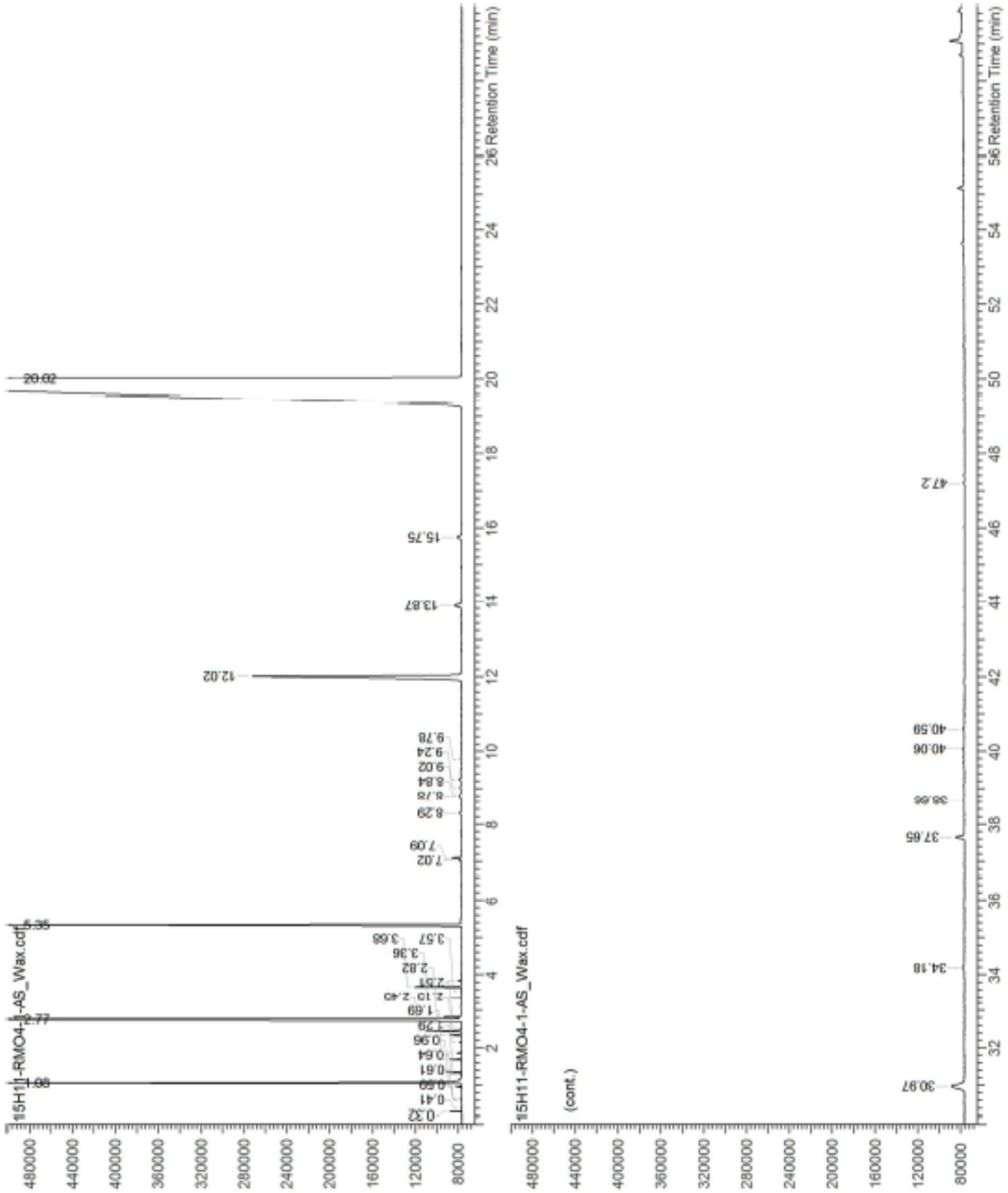


Laboratoire
PhytoChemia

Plus que des analyses... des conseils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13.87
12.02
9.78
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7.09
7.02
5.35
3.57
3.66
3.36
2.82
2.57
2.15
2.45
1.89
1.79
1.77
0.95
0.84
0.81
0.69
0.68
0.41
0.32
47.2
40.59
40.06
38.66
37.65
34.18
30.97

Orange essential oil GC/MS report

Date : June 9, 2015

SAMPLE IDENTIFICATION

Internal code : 15E06-RMO27-1-HM
Customer identification : Orange - MD46020
Type : Essential oil
Source : *Citrus sinensis*
Customer : Rocky Mountain Oils

ANALYSIS

Method : PC-PA-001-15E06, "Analysis of the composition of a liquid essential oil by GC-FID" (in French)
Analyst : Alexis St-Gelais, M. Sc.
Analysis date : May 21, 2015

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Identified Compounds

Identification	BPX-5 Column			SolgelWax Column			Molecular Class
	R. T.	R. I.	%	%	R. I.	R. T.	
α-Pinene	2.98	927	0.50	0.52	986	1.17	Monoterpene
Sabinene	3.66	969	0.34	0.37	1084	2.03	Monoterpene
β-Pinene	3.71	973	0.03	0.02	1064	1.84	Monoterpene
Myrcene	4.01	991	1.78	1.87	1135	2.63	Monoterpene
Octanal	4.25	1006	0.17	0.20	1253	4.26	Aliphatic aldehyde
Δ3-Carene	4.38	1013	0.21	0.14	1111	2.32	Monoterpene
1,8-Cineole	4.80*	1037	94.72	0.25	1172	3.11	Monoterp. ether
Limonene	4.80*	1037	[94.72]	94.48	1171	3.09	Monoterpene
Linalool	6.06	1107	0.37	0.41	1516	9.31	Monoterp. alcohol
Nonanal	6.17	1111	0.05	0.04	1356	5.81	Aliphatic aldehyde
α-Terpineol	8.30	1202	0.05	0.06	1641	14.32	Monoterp. alcohol
Decanal	8.68	1212	0.30	0.21	1455	7.72	Aliphatic aldehyde
Neral	9.83	1242	0.04	0.05	1618	13.15	Monoterp. aldehyde
Geranial	11.12	1275	0.07	0.09	1667	15.63	Monoterp. aldehyde
β-Caryophyllene	17.95	1399	0.03	0.02	1528	9.73	Sesquiterpene
α-Selinene	23.45*	1470	0.14	0.07	1638	14.17	Sesquiterpene
Valencene	23.45*	1470	[0.14]	0.06	1660	15.24	Sesquiterpene
β-Sinensal	38.17	1693	0.03	0.02	2185	41.97	Sesquiterp. aldehyde
α-Sinensal	40.23	1752	0.02	0.02	2247	43.48	Sesquiterp. aldehyde
Total identified			**98.85 %**	**98.90 %**			

*Two or more compounds are coeluting on this column.
[xx] Duplicate percentage due to coelutions, not taken into account for identified total

Note : No correction factor was applied.

Other Data

Physical aspect : Dark yellow liquid
Refractive index : 1.4728 ± 0.0003 (21 °C)

Conclusion

No adulterant, contaminant or diluent were detected using this method.

Checked and approved by :

Alexis St-Gelais, M. Sc., chimiste 2013-174

Note: This report is a shortened version of the original.

This report is digitally signed, it is only considered valid if the digital signature is intact.

Laboratoire
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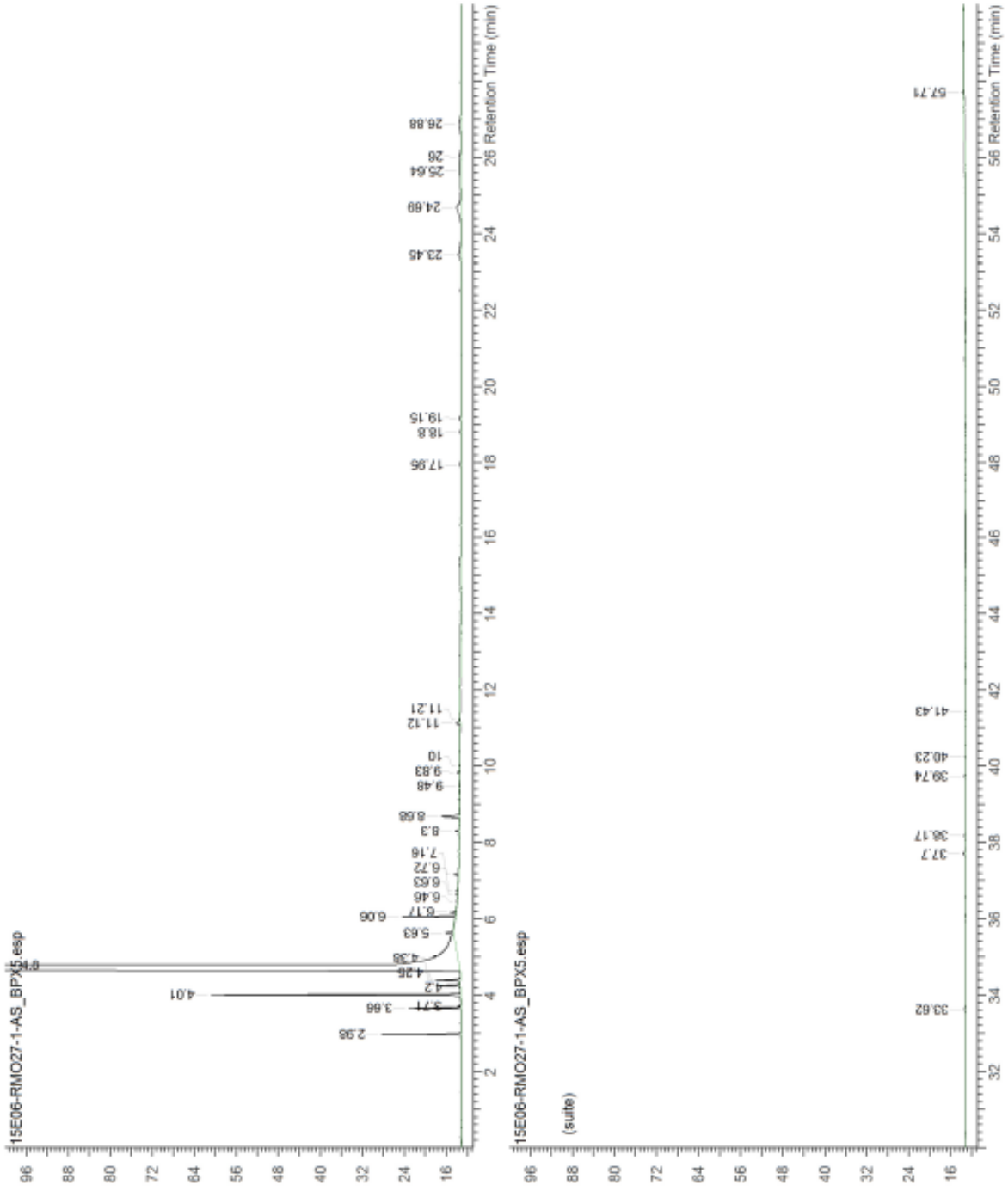
Essential oil, *Citrus sinensis*
Internal code : 15E06-RMO27-1-HM Customer identification : Orange - MD46020

Report prepared for
Rocky Mountain Oils



Laboratoire
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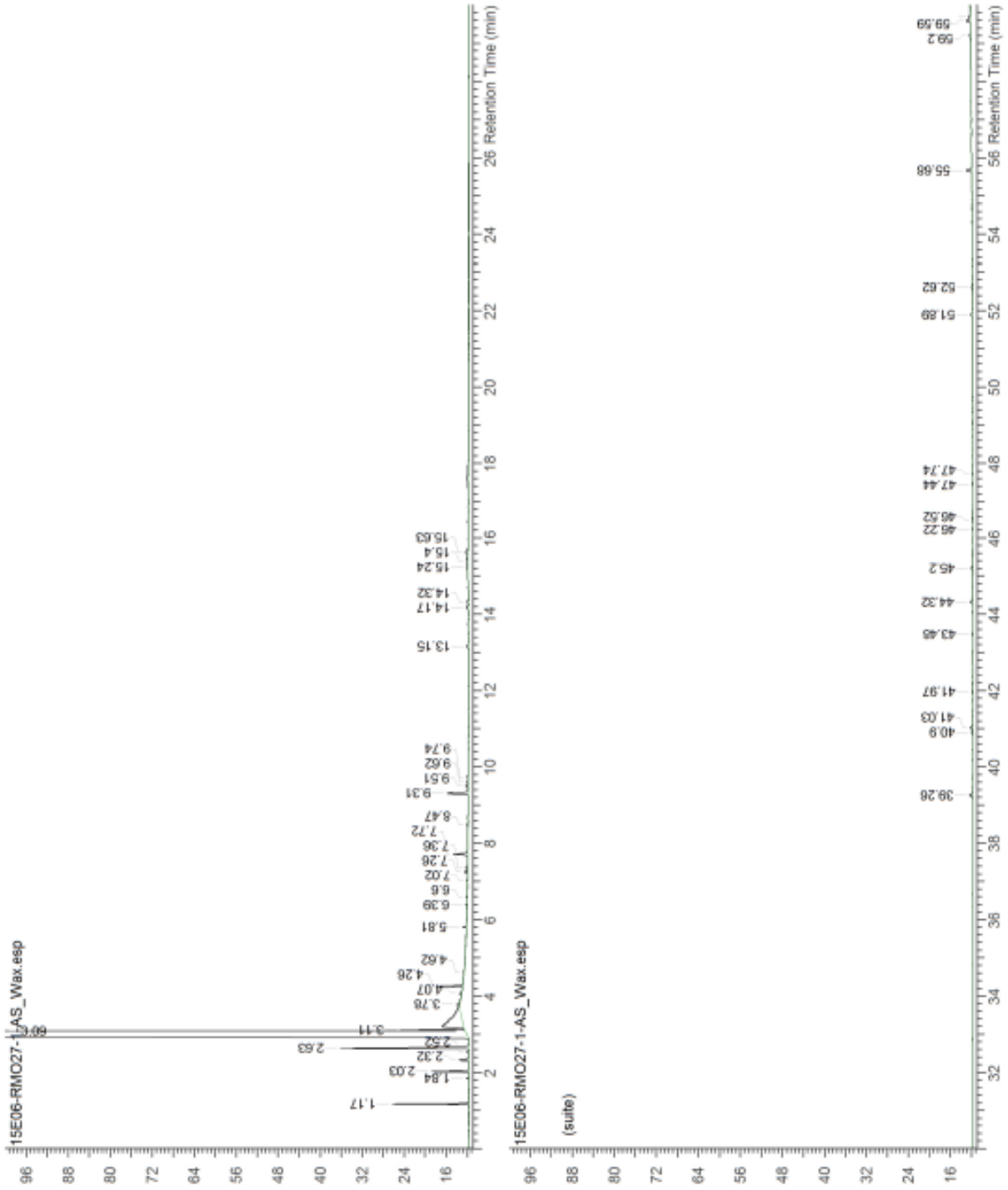
Plus que des analyses... des conseils

Essential oil, *Citrus sinensis*
Internal code : 15E06-RMO27-1-HM Customer identification : Orange - MD46020

Report prepared for
Rocky Mountain Oils



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PhytoChemia

Plus que des analyses... des conseils

Sage essential oil GC/MS report

Date : November 13, 2015

SAMPLE IDENTIFICATION

Internal code : 15K04-RMO2-1-LC
Customer identification : Sage - Turkey - TU0319
Type : Essential oil
Source : *Salvia officinalis*
Customer : Rocky Mountain Oils

ANALYSIS

Method : PC-PA-001-15E06, "Analysis of the composition of a liquid essential oil by GC-FID" (in French).
Analyst : Alexis St-Gelais, M. Sc.
Analysis date : 2015-11-05

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IDENTIFIED COMPOUNDS

Identification	Column: BP5			Column: WAX			Molecular Class
	R.T.	R.I.	%	%	R.I.	R.T.	
cis-Salvene	1.73	841	0.05	0.05	910	0.71	Normonoterpene
cis-Hex-3-en-1-ol	1.97	862	0.01	0.01	1347	5.42	Aliphatic alcohol
Tricyclene	2.60	913	0.45	0.42	964	0.97	Monoterpene
α-Thujene	2.70	919	0.35	0.45	997	1.12	Monoterpene
α-Pinene	2.79	925	4.72	4.45	988	1.08	Monoterpene
Camphene	3.05*	942	5.89	5.56	1026	1.37	Monoterpene
α-Fenchene	3.05*	942	[5.89]	0.08	1018	1.30	Monoterpene
Sabinene	3.45	967	0.06	0.08	1083	1.87	Monoterpene
β-Pinene	3.50	970	2.17	2.12	1065	1.71	Monoterpene
Myrcene	3.80	989	0.96	0.89	1135	2.45	Monoterpene
Pseudolimonene	3.98	1001	0.03	0.02	1129	2.38	Monoterpene
α-Phellandrene	4.04	1004	0.09	0.07	1126	2.34	Monoterpene
α-Terpinene	4.22	1014	0.06	0.07	1140	2.51	Monoterpene
Limonene	4.45*	1027	1.97	1.61	1159	2.76	Monoterpene
para-Cymene	4.45*	1027	[1.97]	0.49	1228	3.67	Monoterpene
1,8-Cineole	4.52*	1031	9.74	9.41	1166	2.85*	Monoterp. ether
β-Phellandrene	4.52*	1031	[9.74]	[9.41]	1166	2.85*	Monoterpene
cis-β-Ocimene	4.75	1045	0.01	1.66	1207	3.38*	Monoterpene
trans-β-Ocimene	4.81	1048	0.02	0.03	1221	3.57	Monoterpene
γ-Terpinene	4.98	1057	1.60	[1.66]	1207	3.38*	Monoterpene
cis-Sabinene hydrate	5.33	1077	0.03	0.04	1424	6.72	Monoterp. alcohol
Terpinolene	5.42	1083	0.59	0.61	1241	3.86	Monoterpene
α-Thujone	5.98*	1112	25.77	25.68	1372	5.81	Monoterp. ketone
Linalool	5.98*	1112	[25.77]	0.42	1515	8.79	Monoterp. alcohol
β-Thujone	6.15	1119	6.12	6.02	1388	6.05	Monoterp. ketone
Camphor	6.76	1148	20.43	20.85	1446	7.18	Monoterp. ketone
Borneol	7.41	1177	4.17	4.40	1636	13.29*	Monoterp. alcohol
Terpinen-4-ol	7.51	1182	0.59	0.44	1545	9.75	Monoterp. alcohol
Myrtenal	8.00	1203	0.01	0.03	1548	9.87	Monoterp. aldehyde
Bornyl acetate	10.79	1278	1.72	1.67	1521	8.99	Monoterp. ester
β-Caryophyllene	17.25	1399	5.22	5.17	1534	9.40	Sesquiterpene
α-Humulene	19.75	1432	6.11	6.05	1601	11.63	Sesquiterpene
Germacrene D	21.15	1450	0.07	[4.40]	1636	13.29*	Sesquiterpene
Caryophyllene oxide	29.71	1557	0.06	0.05	1858	26.75	Sesquiterp. ether
Viridiflorol	31.24	1575	0.24	0.24	1989	34.94	Sesquiterp. alcohol
Humulene epoxide II	32.17	1586	0.06	0.05	1915	30.64	Sesquiterp. ether
Total identified			**99.37%**	**99.19%**			

*: Two or more compounds are coeluting on this column

[xx]: Duplicate percentage due to coelutions, not taken account in the identified total

Note: no correction factor was applied

Other Data

Physical aspect : Clear liquid
Refractive index : 1.4665 ± 0.0003 (20 °C)

Conclusion

No adulterant, contaminant or diluent were detected using this method.

Checked and approved by :

Alexis St-Gelais, M. Sc., chimiste 2013-174

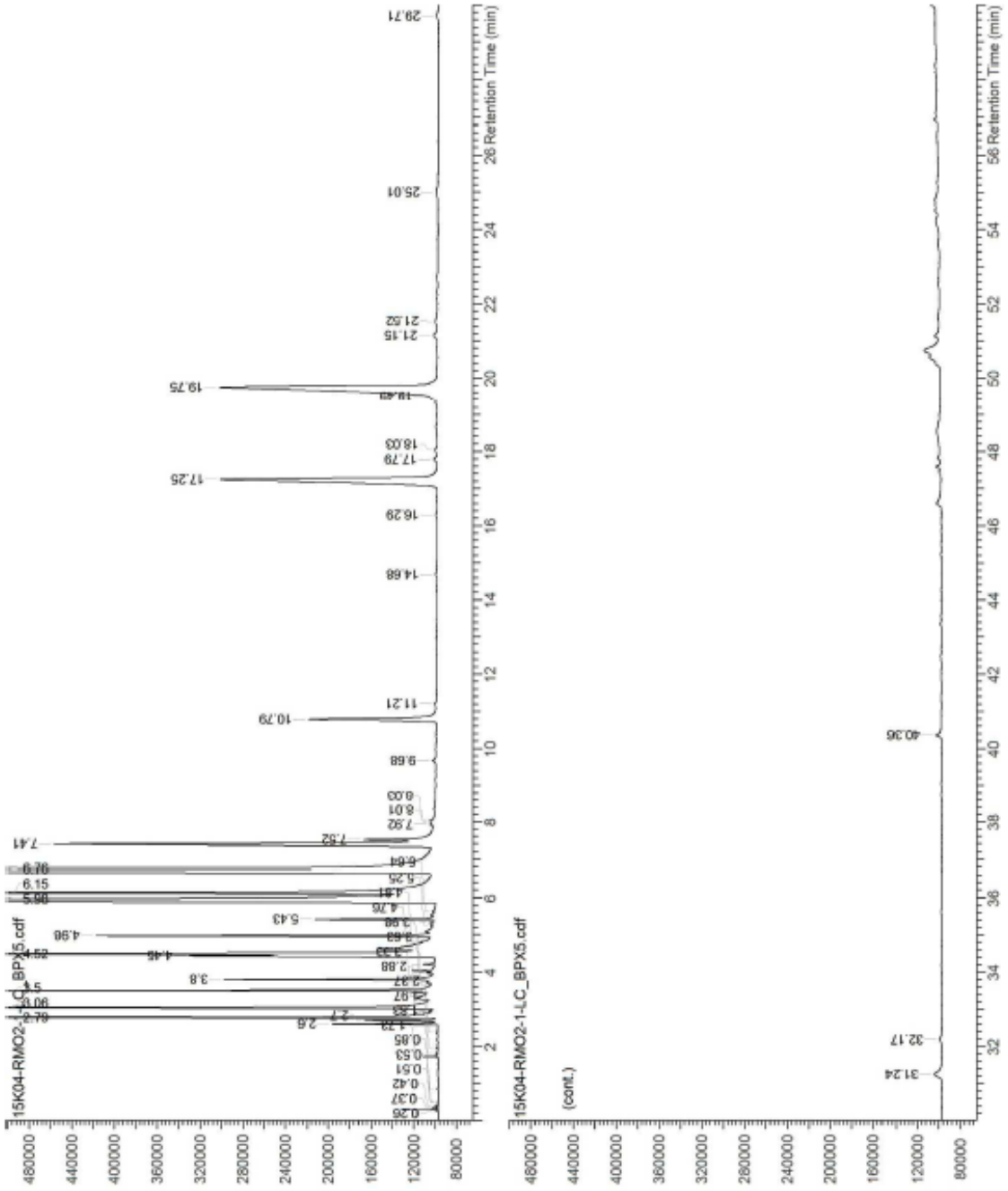
Note: This report is a shortened version of the original.

This report is digitally signed, it is only considered valid if the digital signature is intact.

Essential oil, *Salvia officinalis*
Internal Code: 15K04-RMO2-1-LC

Customer Identification:
Sage - Turkey - TU0319

Report prepared for
Rocky Mountain Oils



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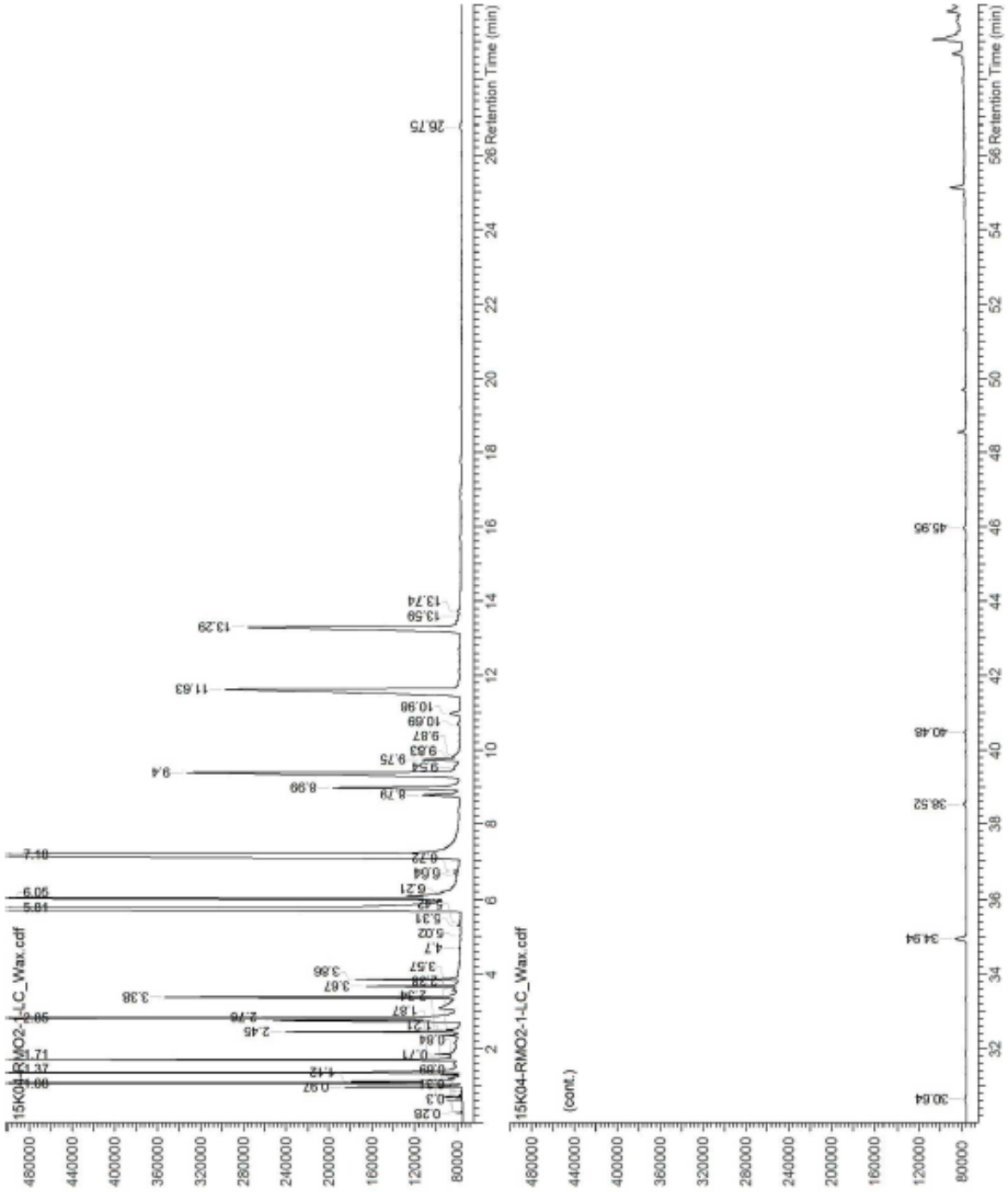
Essential oil, *Salvia officinalis*
Internal Code: 15K04-RMO2-1-LC

Customer Identification:
Sage - Turkey - TU0319

Report prepared for
Rocky Mountain Oils



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Black pepper essential oil GC/MS report

Date : January 8, 2016

SAMPLE IDENTIFICATION

Internal code : 16A04-RMO13-1-LC
Customer identification : Black Pepper - India - TU0410
Type : Essential oil
Source : *Piper nigrum*
Customer : Rocky Mountain Oils

ANALYSIS

Method : PC-PA-001-15E06, "Analysis of the composition of a liquid essential oil by GC-FID" (in French). Identifications double-checked by GC-MS
Analyst : Alexis St-Gelais, M. Sc., chimiste
Analysis date : 2016-01-05

Checked and approved by :

Alexis St-Gelais, M. Sc., chimiste 2013-174

Note: This report is a shortened version of the original.

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IDENTIFIED COMPOUNDS

Identification	Column: BP5			Column: WAX			Molecular Class
	R.T.	R.I.	%	%	R.I.	R.T.	
α-Thujene	2.68	915	0.74	6.54	958	1.03††	Monoterpene
α-Pinene	2.78	921	5.78	[6.54]	949	0.99††	Monoterpene
Camphene	3.02*	937	0.15	0.14	1003	1.25	Monoterpene
α-Fenchene	3.02*	937	[0.15]	tr	994	1.19	Monoterpene
Sabinene	3.46†	966	14.00	6.80	1066	1.76	Monoterpene
β-Pinene	3.51†	969	[14.00]	7.22	1045	1.59	Monoterpene
Myrcene	3.79	987	1.31	1.29	1121	2.30	Monoterpene
Δ3-Carene	4.04*	1003	5.54	4.57	1097	2.03	Monoterpene
α-Phellandrene	4.04*	1003	[5.54]	0.98	1112	2.19	Monoterpene
α-Terpinene	4.20	1013	0.16	0.16	1125	2.35	Monoterpene
Limonene	4.46*	1028	14.04	12.31	1147	2.61	Monoterpene
para-Cymene	4.46*	1028	[14.04]	0.30	1216	3.47	Monoterpene
1,8-Cineole	4.46*	1028	[14.04]	1.54	1152	2.67*	Monoterp. ether
β-Phellandrene	4.46*	1028	[14.04]	[1.54]	1152	2.67*	Monoterpene
cis-β-Ocimene	4.62	1037	0.02	0.30	1193	3.16*	Monoterpene
trans-β-Ocimene	4.79	1047	0.15	0.16	1209	3.37	Monoterpene
γ-Terpinene	4.95	1056	0.29	[0.30]	1193	3.16*	Monoterpene
cis-Sabinene hydrate	5.27	1075	0.14	0.10	1415	6.45	Monoterp. alcohol
Isoterpinolene	5.33	1078	0.08	0.08	1223	3.57	Monoterpene
Terpinolene	5.40	1082	0.34	0.33	1227	3.63	Monoterpene
para-Cymenene	5.61	1094	0.02	0.02	1380	5.84	Monoterpene
trans-Sabinene hydrate	5.83	1106	0.07	0.10	1498	8.13	Monoterp. alcohol
Linalool	5.87	1108	0.41	0.44	1511	8.50	Monoterp. alcohol
endo-Fenchol	6.24	1125	0.03	0.76	1516	8.68*	Monoterp. alcohol
Terpinen-4-ol	7.45	1181	0.43	0.42	1538	9.37	Monoterp. alcohol
Bicycloelemene	12.48	1316	0.05	0.01	1423	6.60	Sesquiterpene
δ-Elemene	12.70	1320	1.99	2.15	1420	6.55	Sesquiterpene
α-Cubebene	13.21	1329	0.24	0.24	1408	6.30	Sesquiterpene
Cycloisosativene	13.99	1343	0.07	0.08	1413	6.39	Sesquiterpene
α-Copaene	14.68	1355	3.31	3.28	1431	6.76	Sesquiterpene
β-Cubebene	15.48	1369	0.28	0.29	1476	7.68	Sesquiterpene
β-Elemene	15.74	1374	0.81	1.67	1528	9.05*	Sesquiterpene
α-Gurjunene	16.28	1383	0.11	0.12	1460	7.35	Sesquiterpene
β-Caryophyllene	17.42*	1402	29.25	28.63	1527	9.02	Sesquiterpene
cis-α-Bergamotene	17.54	1404	0.24	[0.76]	1516	8.68*	Sesquiterpene
β-Copaene	17.92	1409	0.17	[0.76]	1516	8.68*	Sesquiterpene
trans-α-Bergamotene	18.55*	1417	1.02	[1.67]	1528	9.05*	Sesquiterpene
α-Guaiene	18.55*	1417	[1.02]	0.03	1529	9.09	Sesquiterpene
cis-β-Farnesene	19.20	1426	0.10	1.78	1584	10.83*	Sesquiterpene
α-Humulene	19.57	1431	1.64	[1.78]	1584	10.83*	Sesquiterpene
trans-β-Farnesene	20.75	1446	0.87	1.14	1624	12.46*	Sesquiterpene

γ-Muurolene	21.53*	1457	0.68	0.46	1611	11.85	Sesquiterpene
Germacrene D	21.53*	1457	[0.68]	1.53	1619	12.25*	Sesquiterpene
Unknown (m/z = 93, 119 (94), 91 (63), 77 (41), 105 (39), 41 (33)... 204 (21))	22.08	1464	1.07	[1.53]	1619	12.25*	
β-Selinene	22.15	1465	0.54	[1.14]	1624	12.46*	Sesquiterpene
α-Selinene	22.66*	1472	1.17	0.71	1630	12.75	Sesquiterpene
Bicyclogermacrene	22.66*	1472	[1.17]	0.36	1639	13.16	Sesquiterpene
α-Muurolene	23.34	1481	0.33	0.34	1642	13.28	Sesquiterpene
4-epi-Cubebol	23.69*	1485	1.22	0.12	1808	22.66	Sesquiterp. alcohol
Valencene	23.69*	1485	[1.22]	1.17	1653	13.80	Sesquiterpene
γ-Cadinene	24.22	1492	0.18	0.18	1662	14.23	Sesquiterpene
β-Bisabolene	24.71	1499	3.84	3.80	1659	14.12	Sesquiterpene
δ-Cadinene	25.06*	1503	2.05	1.81	1674	14.77	Sesquiterpene
Cubebol	25.06*	1503	[2.05]	0.17	1855	26.07	Sesquiterp. alcohol
trans-Calamenene	25.36	1506	0.05	0.06	1733	18.03	Sesquiterpene
trans-γ-Bisabolene	25.89*	1513	1.51	1.20	1684	15.28	Sesquiterpene
trans-Cadina-1,4-diene	25.89*	1513	[1.51]	0.32	1694	15.74	Sesquiterpene
trans-α-Bisabolene	27.43	1531	0.32	0.27	1705	16.32	Sesquiterpene
α-Elemol	28.32	1542	0.41	0.44	1998	35.19	Sesquiterp. alcohol
Caryophyllene oxide	29.62	1557	0.70	0.57	1848	25.52	Sesquiterp. ether
(*E*)-Nerolidol	30.03	1562	0.18	0.15	1992	34.87	Sesquiterp. alcohol
Isospathulenol	34.30	1622	0.09	0.12	2140	39.95*	Sesquiterp. alcohol
τ-Muurolol	35.03	1637	0.05	0.06	2089	38.45	Sesquiterp. alcohol
α-Muurolol	35.20	1641	0.27	0.24	2104	38.96	Sesquiterp. alcohol
α-Bisabolol	37.35	1685	0.15	[0.12]	2140	39.95*	Sesquiterp. alcohol
Total identified			**97.59%**	**98.06%**			

*: Two or more compounds are coeluting on this column

[xx]: Duplicate percentage due to coelutions, not taken account in the identified total

†: Peaks apexes were resolved, but peaks overlapped and were summed for analysis

Note: no correction factor was applied

Other Data

Physical aspect : Clear liquid

Refractive index : 1.4860 ± 0.0003 (20 °C)

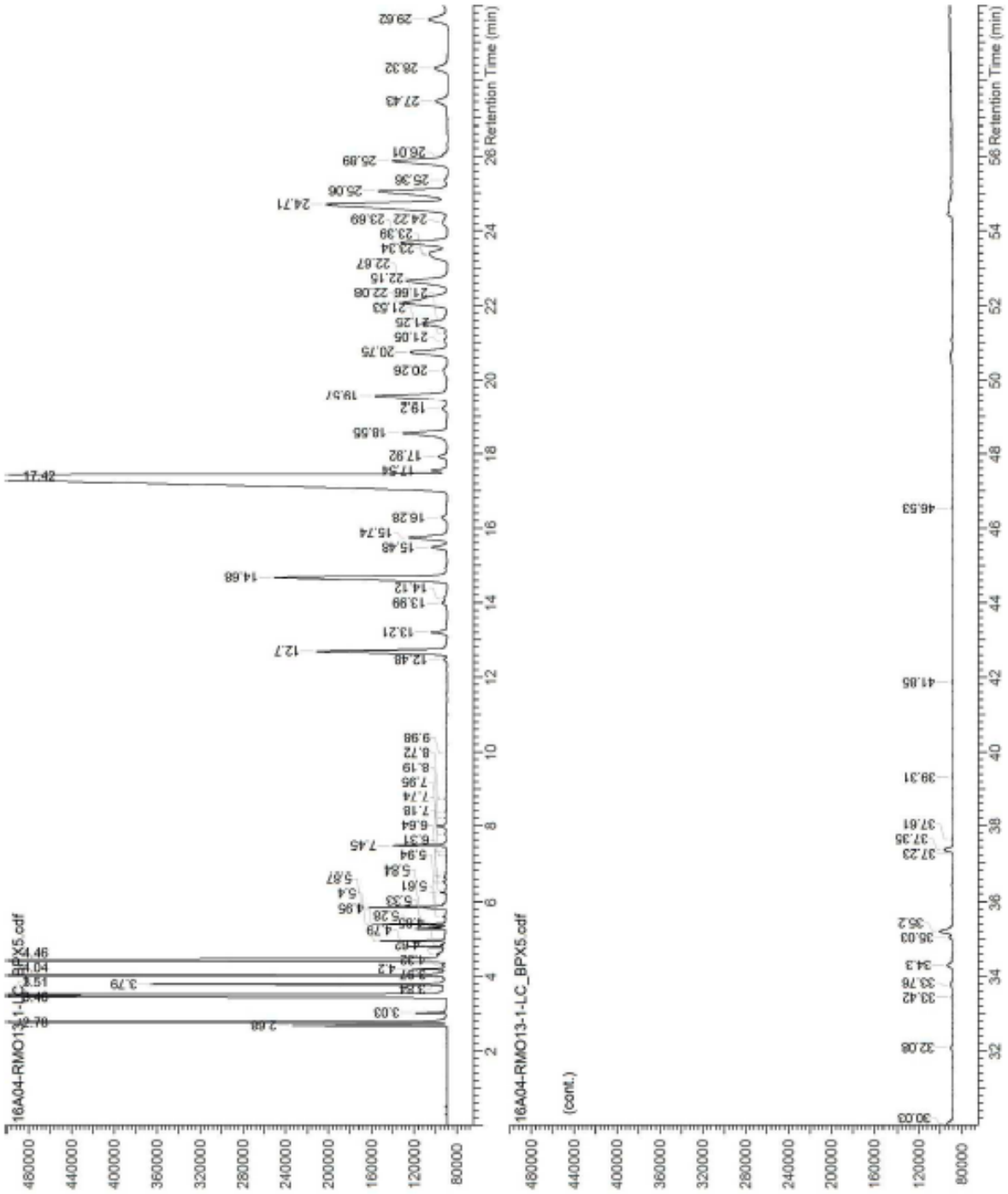
Conclusion

No adulterant, contaminant or diluent were detected using this method.

Essential oil, *Piper nigrum*
Internal Code: 16A04-RMO13-1-LC

Customer Identification:
Black Pepper - India - TU0410

Report prepared for
Rocky Mountain Oils



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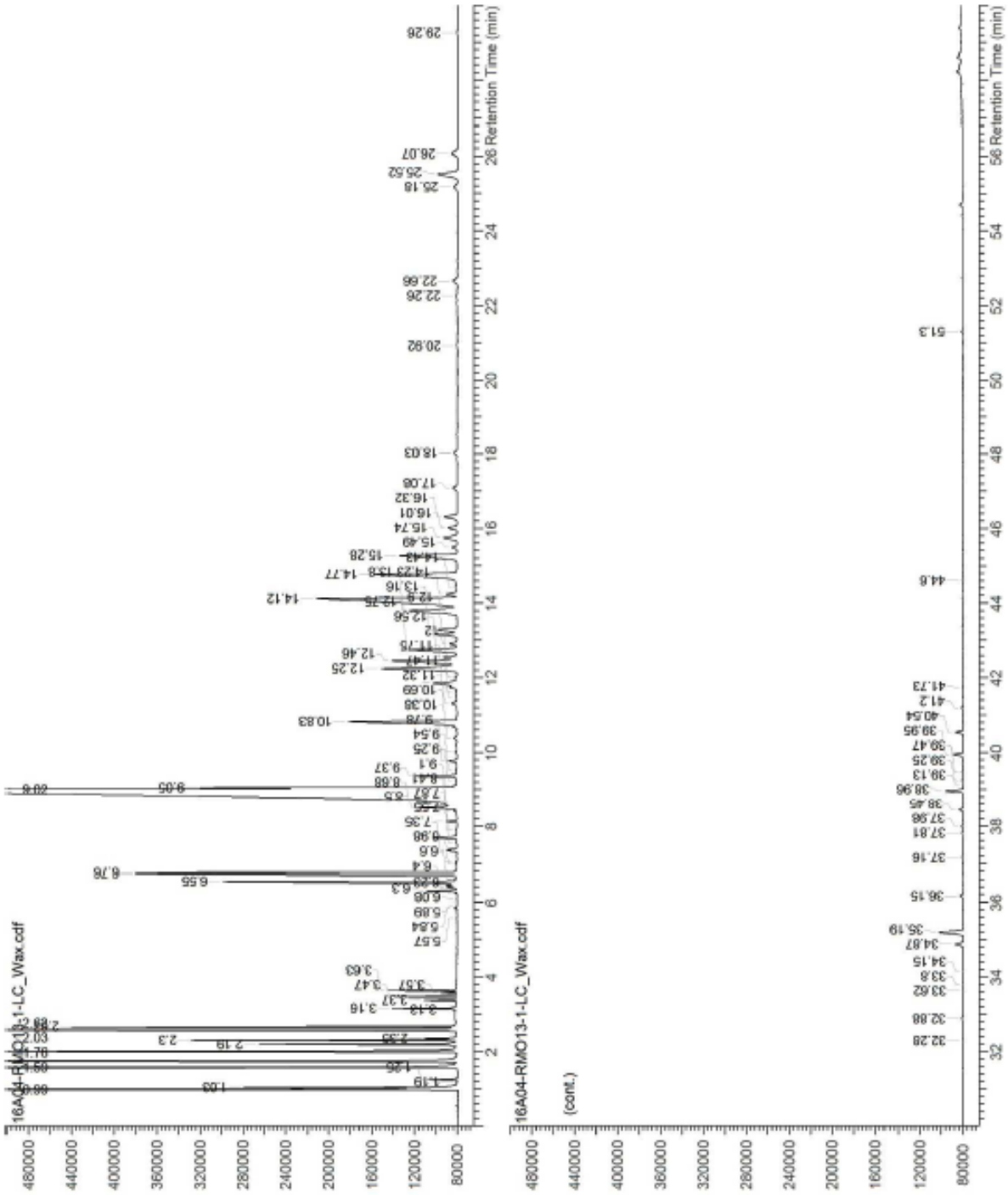
Essential oil, *Piper nigrum*
Internal Code: 16A04-RMO13-1-LC

Customer Identification:
Black Pepper - India - TU0410

Report prepared for
Rocky Mountain Oils



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ANNEXE 1

ORGANOMETALLICS

Article

pubs.acs.org/Organometallics

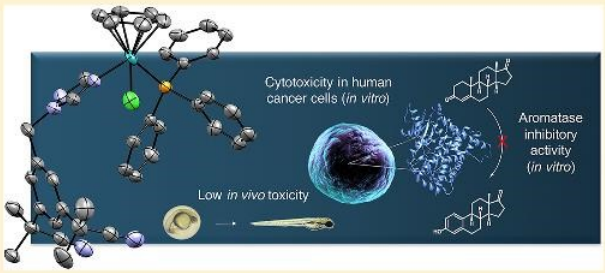
Organoruthenium(II) Complexes Bearing an Aromatase Inhibitor: Synthesis, Characterization, in Vitro Biological Activity, and in Vivo Toxicity in Zebrafish Embryos

Golara Golbaghi, Mohammad Mehdi Haghdoost, Debbie Yancu, Yossef López de los Santos, Nicolas Doucet, Shunmoogum A. Patten, J. Thomas Sanderson, and Annie Castonguay*

INRS - Institut Armand-Frappier, Université du Québec, 531 boul. des Prairies, Laval, Quebec H7 V 1B7, Canada

Supporting Information

ABSTRACT: Third-generation aromatase inhibitors such as anastrozole (ATZ) and letrozole (LTZ) are widely used to treat estrogen receptor-positive (ER+) breast cancers in postmenopausal women. Investigating their ability to coordinate metals could lead to the emergence of a new category of anticancer drug candidates with a broader spectrum of pharmacological activities. In this study, a series of ruthenium(II) arene complexes bearing the aromatase inhibitor anastrozole was synthesized and characterized. Among these complexes, $Ru(C_6H_6)(PPh_3)(ATZ)Cl$ (**3**) was found to be the most stable in cell culture media, to lead to the highest cellular uptake and in vitro cytotoxicity in two ER+ human breast cancer cell lines (MCF7 and T47D), and to induce a decrease in aromatase activity in H295R cells. Exposure of zebrafish embryos to complex **3** (12.5 μM) did not lead to noticeable signs of toxicity over 96 h, making it a suitable candidate for further in vivo investigations.



INTRODUCTION

The coordination of biologically active molecules to metals is a promising strategy for the development of agents with a broader range of anticancer properties. Because metal complexes are widely studied for their ability to reduce cancer cells' viability, the introduction of biologically active ligands within their structure can result in multitargeting drug candidates that could limit the emergence of cancer cell resistance mechanisms.[1−3] For instance, numerous enzyme inhibitors are used to treat and/or prevent several types of cancers,[4−6] making them auspicious ligands for the design of metal-based therapeutics. In addition, metal complexation greatly increases the structural possibilities to form enzyme inhibitors relative to purely organic molecules. Because they can adopt geometries other than linear, trigonal, or tetrahedral, metals can allow organic ligands (or enzyme inhibitors) to occupy a specific position in the active site of enzymes.[7,8] A number of anticancer metal complexes including an enzyme inhibitor in their structure have been reported previously.[9−11] However, metal complexes bearing aromatase inhibitors have so far been overlooked. Aromatase is the enzyme that catalyzes the final, rate-limiting step in estrogen synthesis from androgens.[12] More than two-thirds of breast tumors are estrogen receptor positive (ER+),[13] and estrogens play a key role in initiating and promoting this type of hormone-dependent cancer.[14−17] Currently, third-generation aromatase inhibitors such as the nonsteroidal triazole derivatives anastrozole (Arimidex) and letrozole (Femara) are found to inhibit the aromatase activity in breast tissues. They are widely used to treat ER+ breast cancer, particularly in postmenopausal women who no longer produce ovarian estrogens and derive their estrogens mainly from adrenal androgens in extra ovarian tissues that have aromatase activity such as (breast) adipose.[18] However, in about one-third of patients with metastatic ER+ breast cancer, endocrine therapies that involve aromatase inhibitors (or tamoxifen, known to inhibit ER+ cancer growth by blocking estrogen receptors) lead to the emergence of tumor cells that grow even in the absence of estrogens, resulting in a treatment-resistant cancer that is often incurable.[19] Depriving ER+ cells of estrogens was also previously shown to sensitize them to cytotoxic agents.[20,21] Thus, investigating the anticancer properties arising from the coordination of aromatase inhibitors to metals could lead to the development of efficient drug/prodrug candidates that display more than one mode of action, which could potentially circumvent the emergence of drug resistance mechanisms, a common cause of mortality in ER+ breast cancer patients. It was recently reported that the coordination of hydroquinoline, aminoquinoline, and uracyl ligands to copper can lead to cytotoxic complexes with an aromatase inhibitory activity.[22,23] To our knowledge, only a few investigations from other groups involved the preparation of clinically approved letrozole (Cu, Co, and Ni)[24,25] or anastrozole (Pt)[26] metal complexes, and

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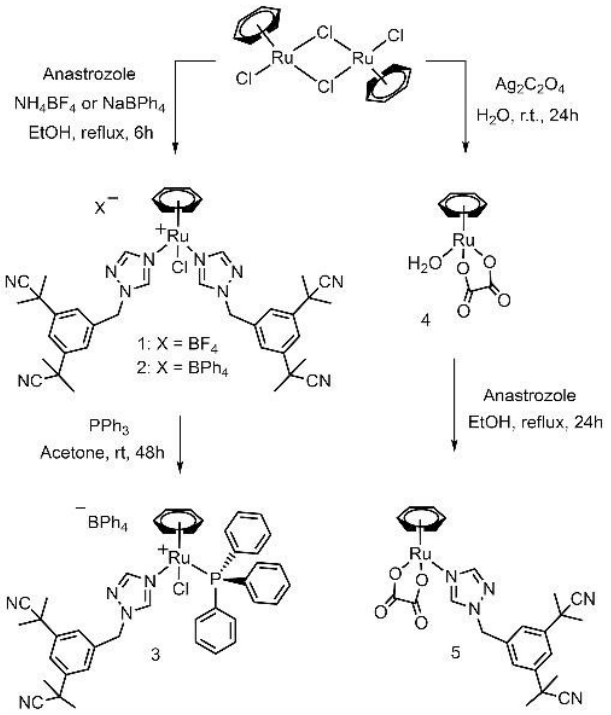
Organometallics XXXX, XXX, XXX−XXX

none of these studies reported an assessment of their aromatase inhibitory activity. Organoruthenium complexes are of particular interest for their activity against numerous types of cancer cells via multiple mechanisms and are often considered as interesting alternatives to currently used therapeutics.[27−32] Maysinger et al. previously reported a preliminary account of the cytotoxicity of a series of ruthenium complexes bearing letrozole ligands.[33] Here, we report the synthesis, characterization, and biological activity of a similar class of ruthenium(II) complexes bearing the aromatase inhibitor anastrozole.

RESULTS AND DISCUSSION

Cationic complex **1** was obtained from a previously reported procedure[33] that led to its letrozole analogue $[Ru(C_6H_6)(LTZ)_2Cl]BF_4$ (Ru-LTZ) by refluxing an ethanol solution of $[Ru(C_6H_6)Cl_2]_2$ and anastrozole (4 equiv) with an excess of NH_4BF_4 (68% yield) (Scheme 1). The same synthetic strategy

Scheme 1. Synthetic Route to Complexes 1−5



was used to prepare complex **2**, using $NaBPh_4$ (60% yield). As the ability of the triphenylphosphine ligand to enhance the cytotoxicity of complexes by improving their lipophilicity and by providing them with a mitochondrial targeting ability is well-precedented,[34−37] the synthesis of compound **3** was undertaken by allowing an acetone solution of **2** to react with an excess of PPh_3 (74% yield). During the preparation of this 18-electron complex, only one of its anastrozole ligands underwent a substitution reaction, most likely via a dissociative mechanism. Complexes **1**−**3** are air-stable; soluble in acetone, in chlorinated solvents, and DMSO; but poorly soluble in water. In alcohols, complex **1** is freely soluble whereas complexes **2** and **3** have a relatively lower solubility. In addition to obtaining the right balance between lipophilicity and hydrophilicity, improving the poor water solubility of drug candidates remains an essential challenge in drug design.[38] It was previously reported that oxalate ligands could significantly enhance the water solubility of ruthenium arene complexes when included in their coordination sphere.[39] Complex **5**, bearing both an oxalate and an anastrozole ligand, was therefore prepared by refluxing an ethanol solution of anastrozole and the ruthenium oxalate precursor **4** (52% yield). The identity of complexes **1**−**5** was confirmed by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), elemental analysis, and NMR spectroscopy. As expected, in the ^1H NMR spectrum of complexes **1**−**3** and **5**, resonances corresponding to the triazole protons of anastrozole are observed at downfield chemical shifts compared to the corresponding resonances in the spectrum of the free ligand. In the case of compound **3**, the presence of a singlet at 35 ppm (acetone-d_6) observed by $^{31}P\{^1H\}$ NMR confirms the coordination of triphenylphosphine to the ruthenium.[33,40−42]

Solid-state structures of complexes **2**−**5** were obtained from single-crystal X-ray diffraction analyses (Table S1 in the Supporting Information). ORTEP views of the complexes are shown in Figure 1. As expected, they revealed a piano-stool

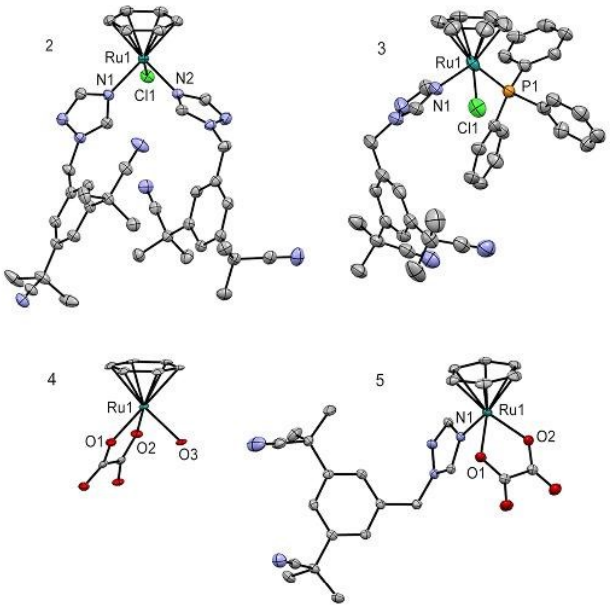


Figure 1. ORTEP diagrams (showing thermal ellipsoids at the 50% probability level) of complexes **2**−**5**. Note that in the case of **3**, only one site is shown for the disordered benzene, Cl and CN. **2**: Ru1−N1, 2.1024(12) Å; Ru1−N2, 2.1104(12) Å. **3**: Ru1−N1, 2.114(4); Ru1′−N1′, 2.091(5). **5**: Ru1−N1, 2.1130(15) Å.

configuration, characteristic of ruthenium arene complexes.[43] Notably, similar Ru−N (anastrozole) bond lengths were noted for complexes **2**, **3**, and **5**, reflecting their comparable bond strength (Figure 1 and Table S2).

As metal-based drug candidates often display a limited solubility in cell culture media, DMSO is commonly used for the preparation of metal complex stock solutions for biological screenings. However, their solubility once diluted in culture medium and the lability of their ligand(s) in the presence of DMSO are often overlooked.[44] All complexes reported here were found to be soluble in cell culture medium (0.5% DMSO) at concentrations typically used for cytotoxicity screenings, as assessed by ultraviolet−visible (UV−vis)

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absorbance measurements. Their stability was assessed in three different conditions relevant to the biological experiments performed for this study: (i) in DMSO-d_6, (ii) in water (0.5% DMSO), and (iii) in DMEM-F12 medium (0.1% DMSO). ^{1}H NMR analysis of all complexes in DMSO-d_6 revealed a high stability for all the complexes for which less than 5% of anastrozole dissociation was observed, except for **5**, for which a 15% anastrozole release was noted. Moreover, a limited anastrozole release (3−15%) was observed for all complexes when 150 μM aqueous solutions (0.5% DMSO) were separately incubated at 37 °C for 48 h (**3** being the most stable) (Table S3), as assessed by ^{1}H NMR spectroscopy. The stability of each complex was also evaluated under conditions similar to that of the aromatase assay. To this aim, a liquid/liquid extraction/HPLC-UV method was developed (Figure S1) to measure the amount of released anastrozole (or letrozole) when 10 μM solutions of complexes **1**−**3**, **5**, and Ru-LTZ (previously reported letrozole analogue of **1**)[33] were incubated in DMEM/F-12 medium for 1.5 h (the duration of the aromatase assay). The release of anastrozole (or letrozole) from most complexes was found to be considerable, except for complex **3**, for which only 4% of its aromatase inhibitor ligand was released (Table S4).

Because all ruthenium complexes reported in this study (except **4**) include at least one anastrozole ligand, their cytotoxicity was evaluated after 48 h in two ER+ human breast cancer cell lines, T47D and MCF7, using the SRB assay, and compared to clinically approved anticancer drugs *cis*-platin and anastrozole. As previously reported,[33] the cell growth inhibitor anastrozole did not display any noticeable cytotoxicity in MCF7 or T47D cells (results not shown). However, complexes **1**−**3** were each found to be active to various extents (Figure 2 and Table 1), whereas complexes **4** and **5** did not display any significant cytotoxicity (results not shown). It is noteworthy that because of the significant contribution of the tumor microenvironment to the implementation of the antitumor activity of similar types of Ru(II) species, the observed in vitro cytotoxicities of the complexes reported in this study are not necessarily indicative of their potential in vivo antitumor activities.[29,45] Although complexes **1** and **2** were less cytotoxic than *cis*-platin in MCF7 cancer cells, compounds **2** and **3** had a similar or even more effective cytotoxicity than that of the clinically approved drug in T47D cancer cells, which are known for their *cis*-platin resistance.[46] These results highlight the importance of developing such alternative complexes for breast cancer therapy. At concentrations below 12.5 μM, compound **3** was found to be the most cytotoxic of all complexes, reducing T47D and MCF7 cell viability by almost half at 4 μM. Cancer cells exposed to higher concentrations of the various complexes were especially susceptible to compound **2**, more significantly in the case of T47D cells, where the cell viability was inhibited by almost half at 50 μM. Interestingly, a significant difference was observed between the cytotoxicity of complexes **1** and **2** on both cell lines, which differ only in the nature of their counterion (BF_4^- vs BPh_4^-) (Table 1). The higher cytotoxicity of complexes **2** and **3** is not likely due to the sole contribution of the BPh_4^- counterion as both complexes induced a significantly higher cytotoxicity than $NaBPh_4$ in T47D cells at 25 μM (Figure S2).

Table 1. Estimated IC_{50} Values Illustrating the Effect of Complexes 1−3 and *cis*-Platin on the Viability of MCF7 and T47D Cancer Cells[a]

	IC_{50} (μM)[b]	
	MCF7	T47D
1	>150	>150
2	139.4 (±14.3)	53.5 (±9.1)
3[c]	≥4	≥4
cis-platin	37.0 (±2.4)	>150

[a]Data extracted from Figure 2. [b]Cytotoxicity was determined by exposure of cell lines to each complex for 48 h and expressed as the concentration required to inhibit cell viability by 50% (IC_{50}). Values in parentheses correspond to the standard deviation of 3 independent experiments. [c]As seen in Figure 2, cell viability reached a plateau at concentrations above 4 μM.

Cellular levels of ruthenium were measured by inductively coupled plasma mass spectrometry (ICP-MS) after MCF7 cells were exposed to 4 μM solutions of all complexes for 48 h (Figure 3A). Compared to nontreated cells (control), a significant amount of ruthenium was observed in cells treated with each complex. Cellular ruthenium levels of each complex appeared to depend on their respective lipophilicity, in agreement with previous reports demonstrating that more

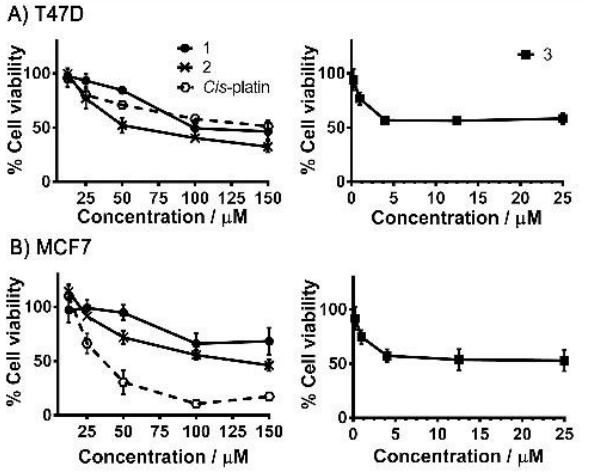

Figure 2. Cell viability determined by the SRB assay (48 h) in ER(+) breast cancer cells: T47D (A) and MCF7 (B), treated with **1**, **2**, and *cis*-platin (black dashed line) at the concentrations 12.5, 25, 50, 100, and 150 μM (left) and **3** at the concentrations 0.25, 1, 4, 12.5, and 25 μM (right). All values are expressed as means (from three independent experiments) ± SD relative to the carrier.

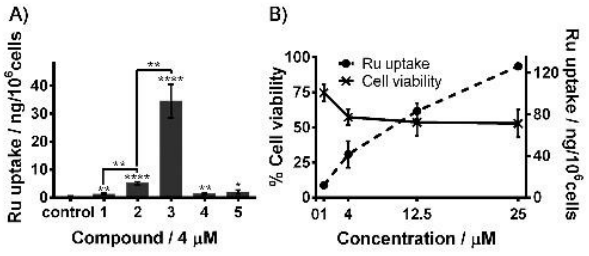

Figure 3. (A) Ruthenium cellular uptake (determined by ICP-MS) after exposure of MCF7 cells to 4 μM solutions of **1**−**5** (48 h); (B) ruthenium cellular uptake (determined by ICP-MS) and cell viability after exposure of MCF7 cells to 1, 4, 12.5, and 25 μM solutions of **3** (48 h). Error bars in the graph represent the standard deviation. Significant differences: *p < 0.05; **p < 0.01; ****p < 0.0001.

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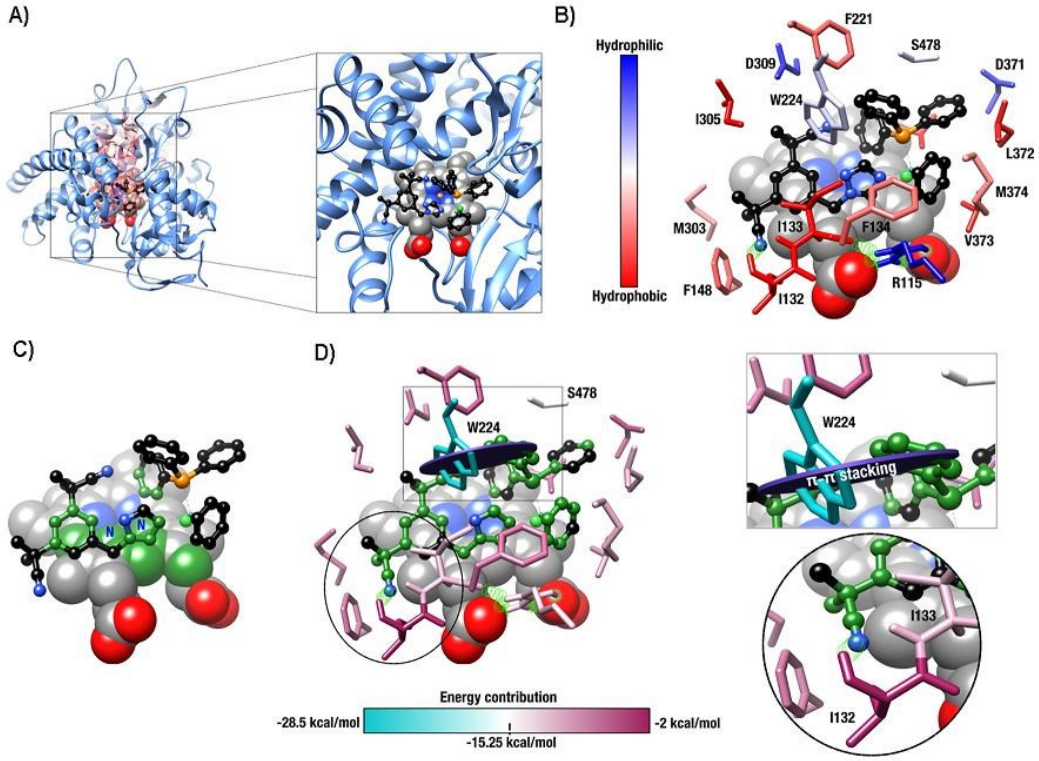

Figure 4. Ternary complex formation between compound **3**, human aromatase, and a heme group as enzymatic cofactor. (A) Active-site pocket of aromatase[61] highlighted by a red transparent surface with a zoomed view of the ligand interacting with the cofactor. (B) Ternary complex showing the most important stabilizing interactions between enzyme residues and the ruthenium complex. Amino acid (stick representation) identity is shown with single-letter code, and their hydropathic profile is illustrated using the Kyte−Doolittle scale.[62] Cofactor atoms (sphere representation) are color-coded as follows: carbon (gray), nitrogen (blue), iron (orange), and oxygen (red). Only hydrogen atoms participating in H-bonding interactions are shown (green strings and dotted lines). The ruthenium complex atoms (ball-and-stick representation) are shown using the same color coding, except for carbon (black) and phosphorus atoms (orange). The ruthenium and chlorine atoms are depicted in dark and light green, respectively. (C) Atomic interactions exhibiting higher energy values between the ruthenium-based ligand and cofactor in the ternary complex are highlighted in dark green. (D) Energy contributions for amino acids that stabilize the ternary complex are shown on a scale ranging from −2 to −28.5 kcal/mol. Trp224 is the most important energy contributor to this interaction, exhibiting a potential orthogonal $\pi-\pi$ stacking energy value of −28.5 kcal/mol (black circle in the rectangle inset). Two alternative hydrogen bonding interactions were identified for Ile132 and Ile133 (circle inset). All panels show the same atomic orientation.

hydrophobic systems have a greater affinity for the cell membrane.[47] For instance, lipophilic counterion-containing compounds **2** and **3** (BPh_4^-) displayed the highest ruthenium cellular uptake (**3** > **2** > **1**, **4**, **5**). As previously suggested,[48] hydrophobic interactions between the arene ligands of organoruthenium cations and the phenyl groups of their BPh_4^- counterion might lead to strong ion-pairing, which might modulate drug uptake and consequently have an impact on their cytotoxicity. More specifically, complex **3** produced 7-fold higher cellular ruthenium levels than complex **2** (at 4 μM), most likely because of the high lipophilicity of its PPh_3 ligand. Whereas complex **3** was found to display the highest ruthenium cellular uptake, complexes **4** and **5** resulted in a very low ruthenium cellular uptake. Interestingly, we found that the steady cytotoxicity of complex **3** at concentrations greater than 1 μM was not a consequence of its limited complex cellular uptake at those concentrations (Figure 3B), as these concentrations did result in higher ruthenium levels in MCF7 cells. A solubility assessment by UV−vis absorbance measurements also revealed that solutions of the complexes were not saturated at the tested concentrations. This suggests that complex **3** might act via a distinct mechanism of action. It is noteworthy that further studies revealed that **4** and **5** can inhibit the migration of MCF7 breast cancer cells (Figure S3), for which extracellular modes of action are often known to take place.[49]

Because complex **3** showed negligible anastrozole ligand lability in DMSO and DMSO/media, we evaluated, theoretically and experimentally, the potential of this compound to act as an aromatase inhibitor. Docking simulations have been previously used to study the plausible interactions between transition-metal complexes and proteins or DNA.[50−52] Here, we report a theoretical investigation of the potential interaction between a ruthenium complex (compound **3**) and the aromatase enzyme using a docking simulation, based on the crystal structure of human placental aromatase cytochrome P450 (CYP19A1). Indeed, unlike the conclusion suggested from previous docking studies for free anastrozole,[53,54] the binding of anastrozole to the heme iron of CYP19 via the N-4 is not possible in this system because of the involvement of this triazole nitrogen atom in the ruthenium coordination sphere. Nevertheless, results from this docking calculation (Figure 4) suggest that the interaction between the aromatase protein and

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the inhibitor-containing ruthenium complex is energetically highly favorable.

The tritiated water assay was then selected to measure aromatase activity because it is a rapid and simple technique with high sensitivity and reproducibility.[55,56] Given the low levels of aromatase in MCF7 cells,[57] human H295R adrenocortical carcinoma cells were selected for this study. Notably, H295R cells express numerous steroidogenic enzymes, including aromatase, making them very useful to examine compounds for their potential to interfere with the activity and/or expression of several key cytochrome P450 (CYP) enzymes involved in the biosynthesis of steroid hormones.[58−60] Moreover, H295R cells were found to be less sensitive than MCF7 cells to the cytotoxicity of the ruthenium complexes we report here, allowing their use for this assay (Figure S4).

To determine the level of inhibition of the catalytic activity of aromatase in the presence of each complex (all complexes were studied for comparison purposes), H295R cells were coincubated with 1β-^{3}H-androstenedione and each ruthenium complex for 1.5 h. Aromatase activity was assessed by quantifying the radioactivity of the tritium oxide produced from the aromatization reaction of the labeled androstenedione. Exposure of H295R cells to all complexes at concentrations greater than 1 nM resulted in a statistically significant, concentration-dependent reduction of aromatase activity (Figure 5), except for complex **4** (data not shown),

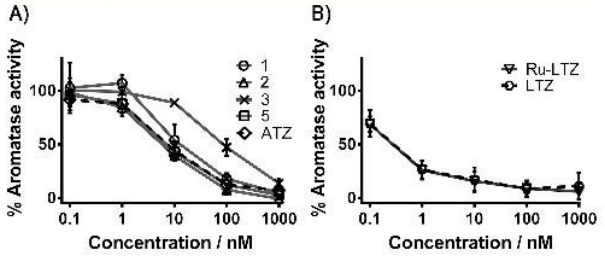

Figure 5. Effects of the exposure of H295R cells with (A) anastrozole (ATZ) (black dashed line), **1**, **2**, **3**, and **5** and (B) letrozole (LTZ) (black dashed line) and Ru-LTZ on aromatase activity. Cells were treated for 1.5 h with the indicated concentrations of the compounds. Values represent the mean ± SD. Significant differences for aromatase activity are reported relative to the controls. Significant differences ($p < 0.001$): 14 ± 4% for **3** at 1000 nM vs 34 ± 7% for ATZ at 40 nM; 47 ± 3% for **3** at 100 nM vs 74 ± 3% for ATZ at 4 nM (ATZ results obtained from interpolation).

confirming the crucial role of anastrozole in the structure of the ruthenium complex. The aromatase inhibitory activity observed for complexes **1**, **2**, **5**, and Ru-LTZ was not significantly different from that of the corresponding aromatase inhibitor alone, an observation which is consistent with the considerable lability of the enzyme inhibitor ligands under these conditions (vide supra). Interestingly, complex **3** was found to be a less potent inhibitor of aromatase activity than anastrozole alone but to have a significantly higher activity than the free anastrozole levels expected from stability studies (Figure 5A). The observed activity for **3** suggests a supplementary contribution from the intact complex, as supported by docking studies (Figure 4), and/or an intracellular substitution of the anastrozole ligand. Notably, during this short period of incubation (1.5 h), a relatively high level of ruthenium in H295R cells treated with **3** was revealed by ICP-MS (Figure S5), which confirms the internalization of the complex.

Over the past few years, the development of zebrafish embryos has become a prominent in vivo model as a tool for drug discovery and toxicity assessment because of a high degree of conservation between the human and zebrafish genomes, rapid embryogenesis, short reproductive cycle, high transparency, and low cost.[63,64] Also, the zebrafish embryo assay has previously been reported to be a suitable phenotype-based screening method to assess the in vivo toxicity of ruthenium complexes.[47,65−67] Because of their greater in vitro cytotoxicity in T47D cells compared to that of the currently used chemotherapeutic agent *cis*-platin, complexes **2** and **3** were selected for an in vivo toxicity assessment using the zebrafish embryo assay. Hatching rates, survival rates, and phenotype changes of the zebrafish embryos treated with 12.5 μM of each compound were determined at 24, 48, 72, and 96 h post fertilization (hpf) (Figure 6). Given the poor solubility of

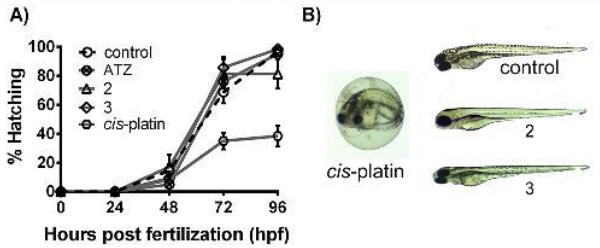

Figure 6. (A) Effect of **2**, **3**, anastrozole (ATZ), and *cis*-platin on the hatching rate of developing zebrafish embryos. Hatching rates were assessed at 12.5 μM over 4 days postfertilization (96 hpf). Control is shown as a black dashed line. (B) Gross morphological phenotypes in zebrafish embryos: untreated (control) and treated with 12.5 μM **2**, **3**, and *cis*-platin. Data are expressed as means ± standard deviation from three independent experiments (a total of 60 embryos).

the complexes in fish medium, concentrations higher than 12.5 μM were not tested. Specifically, *cis*-platin was more toxic to the embryos than the other compounds. Until 96 hpf, no significant mortality of zebrafish embryos was observed for any of the ruthenium complexes. However, a 72 h exposure to *cis*-platin dramatically decreased hatching rates, whereas no significant difference was observed in the hatching rate of embryos treated with complexes **2**, **3**, and anastrozole to that of nontreated embryos. Moreover, at 96 hpf, a significant number of phenotype abnormalities such as edema (25% ± 5%) was detected in *cis*-platin treated embryos.

■ CONCLUSION

A series of ruthenium complexes bearing anastrozole ligands were prepared and characterized. Among these complexes, **3** was found to be the most stable in cell culture media and to lead to the highest cellular uptake in ER+ human breast cancer cells. In contrast to anastrozole alone, considerable in vitro cytotoxicity was observed in two ER+ human breast cancer cell lines (MCF7 and T47D) treated with **3**. In addition, complex **3** was found to induce a decrease in aromatase activity in H295R cells. Exposure of zebrafish embryos to complex **3** (12.5 μM) did not lead to noticeable signs of toxicity over 96 h, making it a suitable candidate for further in vivo investigations. This study opens the door to the development of a novel class of anastrozole-containing organometallic

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anticancer drug candidates with a broader spectrum of pharmacological activities.

EXPERIMENTAL SECTION

General Comments. All chemicals were obtained from commercial sources and were used as received. Anastrozole and letrozole were purchased from Triplebond and AK scientific, respectively. $RuCl_3 \cdot xH_2O$, ammonium tetrafluoroborate, sodium tetraphenylborate, triphenylphosphine, silver nitrate, hydrocortisone, and oxalic acid were purchased from Sigma-Aldrich. $[Ru(benzene)Cl_2]_2$ dimer, silver oxalate, and Ru-LTZ were prepared from previously reported procedures.[33,68,69] Experiments were performed under a nitrogen atmosphere, and solvents were dried on activated molecular sieve columns using a solvent purification system. All NMR spectra were recorded at room temperature on a 400 MHz (or 600 MHz) Varian spectrometer and were referenced to solvent resonances. Chemical shifts and coupling constants are reported in parts per million and and Hertz, respectively. Mass spectral data was obtained from high-resolution and high accuracy mass analysis (HR-ESI-MS) using an Exactive Orbitrap spectrometer from Thermo-Fisher Scientific (Department of Chemistry, McGill University). A PerkinElmer Nexion 300X ICP mass spectrometer was used for the determination of ruthenium in biological samples (Department of Chemistry, Université de Montreal). The purity of all ruthenium complexes (>95%) was assessed by elemental analyses (Laboratoire d'Analyze ElÉmentaire, Department of Chemistry, UniversitÉ de MontrEal) and HPLC-MS, using a Waters 2795 separation module coupled to a Waters Micromass Quattro Premier XE tandem quadrupole mass spectrometer.

Complex Synthesis and Characterization. *$[Ru(C_6H_6)(\eta^1\text{-}Anas)_2Cl]BF_4$, 1.* Anastrozole (0.235 g, 0.80 mmol), $[Ru(benzene)Cl_2]_2$ (0.100 g, 0.20 mmol), and NH_4BF_4 (0.104 g, 1.00 mmol) were dissolved in degassed ethanol (20 mL). The mixture was heated under reflux for 6 h, allowed to cool to room temperature, and filtered, and the precipitate was washed with a minimum of ethanol. The filtrate was then collected and dried under vacuum. The residue was dissolved in dichloromethane (10 mL), filtered, and concentrated to a minimum. Compound **1** was precipitated with diethyl ether, washed with hexane, and dried under vacuum. The final product was obtained as a yellow powder (0.240 g, 68%). ^1H NMR ($CDCl_3$, 400 MHz): δ 1.75 (d, J = 4.9 Hz, CH_3, 24H), 5.40 (m, CH_2, 4H), 5.9 (s, C_6H_6, 6H), 7.43 (d, J = 1.6 Hz, ArH, 4H), 7.54 (t, J = 1.5 Hz, ArH, 2H), 8.27(s, $H_{triazole}$, 2H), 9.35 (s, $H_{triazole}$, 2H). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 100 MHz): δ 28.8 (CH_3, 8C), 37.3 (CCN, 4C), 54.2 (CH_2, 2C), 85.5 (C_6H_6, 6C), 122.3 (CHC_{Ar}, 2C), 124.0 (CN, 4C), 125.0 (CHC_{Ar}, 4C), 135.4 (C_{Ar}, 2C), 143.4 (C_{Ar}, 4C), 146.7 ($C_{triazole}$, 2C), 151.6 ($C_{triazole}$, 2C). Found (%): C, 54.65; H, 5.18; N, 16.05. $C_{40}H_{44}B_1Cl_1F_4N_{10}Ru_1 \cdot 1/16C_6H_{14}$ requires C, 54.28; H, 5.06; N, 15.67. HR-ESI-MS m/z (+): found 801.25 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-}ATZ)_2Cl]^+$) (calcd 801.25), 508.08 $[M^+\text{-}ATZ]^+$ (calcd 508.08).

$[Ru(C_6H_6)(\eta^1\text{-}ATZ)_2Cl]BPh_4$, 2. Degassed ethanol (20 mL) was added to anastrozole (0.235 g, 0.80 mmol), $[Ru(benzene)Cl_2]_2$ (0.100 g, 0.20 mmol), and $NaBPh_4$ (0.342 g, 1.00 mmol). The mixture was heated under reflux for 6 h until a yellow precipitate appeared and then cooled to room temperature and filtered. The precipitate was washed with a minimum amount of ethanol before being dissolved in acetone (5 mL). The resulting solution was filtered on a Celite pad, and the filtrate was concentrated under reduced pressure. Compound **2** was precipitated with diethyl ether, washed with hexane, and dried under vacuum. The final product was obtained as a yellow powder (0.270 g, 60%). ^1H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.73 (s, CH_3, 24H), 5.60 (m, CH_2, 4H), 6.07 (s, C_6H_6, 6H), 6.77 (t, J = 7.2 Hz, H_{BPh4}, 4H), 6.91 (t, J = 7.4 Hz, H_{BPh4}, 8H), 7.33 (m, H_{BPh4}, 8H), 7.45 (d, J = 1.7 Hz, ArH, 4H), 7.67 (t, J = 1.8 Hz, ArH, 2H), 8.50 (s, $H_{triazole}$, 2H), 9.08 (s, $H_{triazole}$, 2H). $^{13}C\{^1H\}$ NMR ($(CD_3)_2CO$, 100 MHz): δ 28.1 (CH_3, 8C), 37.2 (CCN, 4C), 53.6 (CH_2, 2C), 85.3 (C_6H_6, 6C), 121.3 ($CBPh_4$, 4C), 122.0 (CHC_{Ar}, 2C), 123.8 (CN, 4C), 124.7 (CHC_{Ar}, 4C), 125.1 ($CBPh_4$, 8C), 136.1 ($CBPh_4$, 8C), 136.4 (C_{Ar}, 2C), 143.5 (C_{Ar}, 4C), 146.4 ($C_{triazole}$, 2C), 153.0 ($C_{triazole}$, 2C), 163.3 ($CBPh_4$, 4C). Found (%): C, 69.27; H, 6.13; N, 11.70. $C_{64}H_{64}B_1Cl_1N_{10}Ru_1 \cdot 1/2C_6H_{14}$ requires C, 69.16; H, 6.15; N, 12.03. HR-ESI-MS m/z (+): 801.25 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-}ATZ)_2Cl]^+$) (calcd 801.25), 508.08 $[M^+\text{-}ATZ]^+$ (calcd 508.08), 1922.66 $[2M^+ + BPh_4]^+$ (calcd 1922.66).

$[Ru(C_6H_6)(\eta^1\text{-}ATZ)(PPh_3)Cl]BPh_4$, 3. Triphenylphosphine (0.032 g, 0.12 mmol) was added to a solution of **2** (0.112 g, 0.10 mmol) in acetone (8 mL), and the mixture was stirred at ambient temperature for 48 h. The solvent was then concentrated, and the product precipitated with diethyl ether. After the powder was washed with diethyl ether, compound **3** was collected as a yellow powder (0.081 g, 74%). ^1H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.77 (s, CH_3, 12H), 5.44 (m, CH_2, 2H), 6.01 (s, C_6H_6, 6H), 6.77 (t, J = 7.2 Hz, H_{BPh4}, 4H), 6.91 (t, J = 7.4 Hz, H_{BPh4}, 8H), 7.33 (m, H_{BPh4}, 8H), 7.39−7.51 (m, H_{PPh3}, 15H), 7.62 (d, J = 1.8 Hz, ArH, 2H), 7.75 (t, J = 1.8 Hz, ArH, 1H), 8.35 (s, $H_{triazole}$, 1H), 9.03 (s, $H_{triazole}$, 1H). $^{13}C\{^1H\}$ NMR ($(CD_3)_2CO$, 100 MHz): δ 28.2 (CH_3, 4C), 37.2 (CCN, 2C), 53.5 (CH_2, 1C), 90.7 (C_6H_6, 6C), 121.3 ($CBPh_4$, 4C), 122.3 (CHC_{Ar}, 1C), 123.9 (CN, 2C), 125.1 (C_{BPh4}, 8C), 125.5 (CHC_{Ar}, 2C), 128.5 ($CPPh_3$), 128.6 ($CPPh_3$), 131.1 (C_{PPh3}), 133.7 ($CPPh_3$), 133.8 (C_{PPh3}), 135.9 (C_{Ar}, 1C), 136.1 ($CBPh_4$, 8C), 143.5 (C_{Ar}, 2C), 146.4 ($C_{triazole}$, 1C), 154.2 ($C_{triazole}$, 1C), 164.2 ($CBPh_4$, 4C). $^{31}P\{^1H\}$ NMR ($(CD_3)_2CO$, 200 MHz): δ 35.4. Found (%): C, 70.45; H, 5.45; N, 7.16. $C_{65}H_{60}B_1Cl_1N_5P_1Ru_1 \cdot H_2O$ requires C, 70.49; H, 5.64; N, 6.32. [Although the elemental analysis value of N is outside the range viewed as establishing analytical purity, it is provided to illustrate the best value obtained to date. NMR spectra are provided in the Supporting Information as evidence of bulk purity (Figures S6−S8).] HR-ESI-MS m/z (+): 770.17 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-}ATZ)(PPh_3)Cl]^+$) (calcd 770.17).

$Ru(C_6H_6)oxalate(H_2O)$, 4. A mixture of silver oxalate (0.303 g, 1.00 mmol) and $[Ru(benzene)Cl_2]_2$ (0.200 g, 0.40 mmol) was stirred in degassed water (60 mL) at ambient temperature for 24 h. The reaction mixture was filtered on a Celite pad and washed with water (30 mL), and the filtrate was dried under reduced pressure. After adding dichloromethane, the product precipitated as an orange powder (0.157g, 65%).^1H NMR (D_2O, 400 MHz): δ 5.75 (s, C_6H_6, 6H). $^{13}C\{^1H\}$ NMR (D_2O, 100 MHz): δ 77.9 (C_6H_6, 6C), 163.8 ($C_{oxalate}$, 2C). Found (%): C, 32.40; H, 2.58. $C_8H_8O_5Ru_1 \cdot 1/4CH_2Cl_2$ requires C, 32.34; H, 2.80. HR-ESI-MS m/z (−): 284.93 $[M\text{-}H]^-$ (calcd 284.93), 552.87 $[2M\text{-}H_2O\text{-}H]^-$ (calcd 552.86), 820.80 $[3M\text{-}2H_2O\text{-}H]^-$ (calcd 820.80).

$Ru(C_6H_6)oxalate(\eta^1\text{-}ATZ)$, 5. Silver oxalate (0.303 g, 1.00 mmol) was added to a solution of $[Ru(benzene)Cl_2]_2$ (0.200 g, 0.40 mmol) in degassed water (60 mL), and the suspension was stirred at ambient temperature for 24 h. The reaction mixture was filtered on a Celite pad and washed with water (30 mL), and the filtrate was dried under reduced pressure. The resulting intermediate was dissolved in ethanol (60 mL), and anastrozole (0.469 g, 1.60 mmol) was added to the solution. The mixture was then heated under reflux for 24 h. The reaction mixture was concentrated under reduced pressure to dryness, and the product was purified by column chromatography (silica gel) using dichloromethane/methanol (7,1). Compound **5** was obtained as a light yellow powder (0.235 g, 52%). ^1H NMR (CD_3OD, 400 MHz): δ 1.71 (s, CH_3, 12H), 5.54 (s, CH_2, 2H), 5.88 (s, C_6H_6, 6H), 7.44 (d, J = 1.8 Hz, ArH, 2H), 7.64 (t, J = 1.8 Hz, ArH, 1H), 8.21(s, $H_{triazole}$, 1H), 8.88 (s, $H_{triazole}$, 1H). $^{13}C\{^1H\}$ NMR ($(CD_3)_2CO$, 100 MHz): δ 27.7 (CH_3, 4C), 37.2 (CCN, 2C), 53.4 (CH_2, 1C), 82.5 (C_6H_6, 6C), 122.1 (CHC_{Ar}, 1C), 123.7 (CN, 2C), 124.3 (CHC_{Ar}, 2C), 136.2 (C_{Ar}, 1C), 143.5 (C_{Ar}, 2C), 145.2 ($C_{triazole}$, 1C), 151.7 ($C_{triazole}$, 1C), 165.7 ($C_{oxalate}$, 2C). Found (%): C, 52.76; H, 4.62; N, 12.16. $C_{25}H_{25}N_5O_4Ru_1 \cdot 1/8CH_2Cl_2$ requires C, 52.84; H, 4.46; N, 12.26. HR-ESI-MS m/z (+): 584.08 $[M + Na]^+$ (calcd 584.08), 852.01 $[2M\text{-}ATZ+Na]^+$ (calcd 852.01), 1145.18 $[2M+Na]^+$ (calcd 1145.18).

Solubility in DMSO/Media. UV−vis spectroscopy was used to evaluate the solubility of the ruthenium complexes. Accordingly, a 2 mL solution of each complex was prepared in full RMPI growth media (phenol red free) at the concentrations used for cellular proliferation studies (DMSO final concentration: 0.5%). After incubation for 48 h

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at 37 °C, the solution was filtered on a Celite pad, and the absorbance was recorded using a Cary 300 Bio UV−vis spectrometer. The concentration of saturation of the complexes in growth media was assessed by determining the concentration at which a maximum intensity in UV absorbance (274−278 nm) was observed.

X-ray Diffraction. Four ruthenium(II) complexes were structurally characterized by single-crystal X-ray analysis. Suitable crystals were obtained by slow evaporation of the solutions of compounds **2**, **3**, **4**, and **5** in ethanol/acetone, acetone/diethyl ether, water, and methanol, respectively. Data were collected at 100 K using a Bruker Smart APEX diffractometer. Structures were solved with the XT structure solution program and refined with the XL refinement package using least squares minimizations.[70−72]

Cell Culture. Protocols used for biological studies were approved by the Institutional Research Ethics Committee of INRS - Institut Armand-Frappier. Estrogen receptor positive (ER+) breast cancer cells MCF7 and T47D were kindly provided by Prof. Chatenet (INRS). The H295R human adrenocortical carcinoma cell line that expresses CYP19 (aromatase)[59] were obtained from Prof. Sanderson's collection (INRS). The human MCF7 breast cancer cells were grown in RPMI 1640 containing fetal bovine serum (10%). The growth medium for T47D breast cancer cells was RPMI 1640 supplemented with HEPES (2.38 g/L), sodium pyruvate (0.11 g/L), glucose (2.5 g/L), insulin bovine (10 μg/mL), and fetal bovine serum (10%). H295R cells were cultured in DMEM/F-12 supplemented with Nu Serum (2.5%) and ITS (1%). All growth media were supplemented with penicillin/streptomycin. All cell culture products were obtained from commercial sources, including Gibco, Sigma-Aldrich, Corning, and Invitrogen.

Cytotoxicity. Cell viability was examined by slightly modified standard methods using the Sulforhodamine B (SRB) colorimetric assay described by Vichai and Kirtikara.[73] Briefly, for all experiments, cells were seeded in 96-well plates (Sarstedt) at a density of 1×10^4 cells/well (MCF7 and T47D) or 3×10^4 cells/well (H295R) maintained at 37 °C, 5% CO_2 in a humidified atmosphere and were grown in serum-containing media for 24 h before treatment. Stock solutions of the compounds were prepared in DMSO, and the final concentration of DMSO was kept constant (noncytotoxic concentration) depending on the cell line: 0.5% for MFC-7 and T47D and 0.25% for H295R. To reach final concentrations of 1, 4, 12.5, 25, 50, 100, and 150 μM, 1 μL (or 0.5 μL) of each stock solution (200 or 400 times more concentrated than the corresponding final concentration) was added to each well containing 200 μL of fresh and complete growth medium. Also, cancer cells were exposed to complete growth medium alone, growth medium containing 0.5% or 0.25% DMSO (negative control), and growth medium containing 25% DMSO (positive control). After incubation for 48 h, without removing the cell culture supernatant, cells in each well were fixed with 100 μL of cold trichloroacetic acid (TCA) 10% w/v at 4°C for 1 h. After fixation, TCA was discarded, and wells were washed four times with slow-running tap water and then air-dried. An SRB solution (0.057% w/v) was added to the wells, and plates were kept for 30 min at room temperature. Unbound SRB was removed by washing the wells four times with 1% acetic acid. Plates were air-dried. To dissolve the protein-bound dye, cells from each well were exposed to 200 μL of 10 mM Tris base solution (pH 10.5) for 30 min. Absorbance was measured at 510 nm using a microplate reader. The viability of the cancer cells versus different concentrations of each complex was reported. This assay was carried out in two or three independent sets of experiments, each experiment with four or five replicates per concentration level.

Stability in DMSO/Water. The 150 μM solutions of all complexes were prepared in DMSO (200 μL) and distilled water (40 mL), for which final concentrations in DMSO were 0.5% (which was the highest concentration used for cytotoxicity studies). After incubation of the samples for 48 h at 37 °C, solutions were dried by vacuum. An NMR sample of each compound was prepared in an appropriate deuterated solvent (the same solvents as the ones used for ^{1}H NMR characterizations) in which both the complex and the aromatase inhibitor were freely soluble. For each complex, the percentage of released aromatase inhibitor was calculated by comparing, in their ^{1}H NMR spectrum, the signal intensity of one of the protons of anastrozole or letrozole with that of corresponding signal in the ^{1}H NMR spectrum of their complex. This experiment was done in triplicate.

Stability in DMSO/Media. A HPLC-UV method was developed and consisted of a simple liquid/liquid extraction after incubation of the complexes in media. The method was found to be reproducible and linear over the range of concentrations used for the aromatase activity assessment. *Preparation of standards.* Solutions of anastrozole (or letrozole) in 20 mL of phenol red free DMEM/F-12 at 0.1, 1, 5, 10, and 15 μM (DMSO final concentration: 0.1%) were incubated at 37 °C for 1.5 h (conditions used for the tritiated water assay). After incubation, anastrozole or letrozole was retrieved from the media solution by liquid/liquid extraction using diethyl ether (3 × 10 mL), which has been previously been reported as an adequate solvent to recover anastrozole from human plasma.[74] The diethyl ether solution was then dried under vacuum, and the residue was dissolved in 2 mL of acetone and loaded on a thin layer of silica. Acetone (20 mL) was used as a mobile phase to completely recover anastrozole (or letrozole) from silica and minimize the amount of media residue in the final HPLC samples. Final HPLC samples were prepared by evaporating the acetone solution to dryness and dissolving the residue in 1 mL of HPLC grade acetone containing 100 μM hydrocortisone as an internal standard. Standard curves of anastrozole and letrozole are shown in Figure S1. *HPLC-UV method.* The chromatography was performed on an Agilent UHPLC system (1260 Infinity GPC/SEC). An Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm) was used, and the column temperature was maintained at room temperature. Chromatographic separation was obtained through a 13 min gradient delivery of a mixture of acetonitrile and water at a flow rate of 2 mL/min: (a) 0−1 min, water, 100%; (b) 1−4 min, acetonitrile, 0−30%; (c) 4−10 min, acetonitrile, 30%; (d) 10−11 min, acetonitrile, 30%−100%; (e) 11−13 min, acetonitrile, 100%. UV absorbance at both 254 and 215 nm was recorded, but only the data acquired at 215 nm was used. *Sample preparation.* Stock solutions (10^4 μM) of anastrozole, letrozole and all complexes (except **4**, which has no aromatase inhibitor ligand) were prepared in DMSO. The 10 μM solutions of the compounds were prepared by adding 20 μL of a stock solution to 19 980 μL of DMEM/F12 (DMSO final concentration: 0.1%). Solutions were incubated at 37 °C for 1.5 h, and further steps were as described for the standards' preparation. The experiment was carried out in triplicate.

Antimigratory Activity. MCF7 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. 100 000 cels/well were seeded in 24 well plates (Sarstedt) and incubated at 37 °C and 5% CO_2 to reach confluency. Scratches were created using a pipet tip on the confluent monolayer and washed with full growth medium (1 × 500 μL) to remove cellular debris. The fresh medium (500 μL) supplemented with 0.5% FBS containing 10 μM of the synthesized complexes (except compound **3** which was cytotoxic at 10 μM) was individually added into each well and incubated for 48 h to allow the wound closure. NAMI-A was also evaluated under identical conditions as an established ruthenium-containing reference drug candidate. The RPMI-1640 medium containing 0.5% FBS and 0.1% DMSO (vehicle) was used as a control. The migration of the treated and untreated cells (vehicle) into the wound area at incubation 0 and 48 h were compared by capturing the images with a Nikon Eclipse Ti microscope (equipped with a Nikon DS-Ri2 camera) at 10× magnification. Cell migration was analyzed using ImageJ software and the MRI wound healing tool plugin (NIH, Bethesda, MD) and computed into a percentage of control (means ± SEM; $n = 8$) using untreated wells at 100%.

Cellular Uptake. MCF7 cells were grown in 6-well plates (200 000 cells/well) and incubated for 24 h. Stock solutions (20 mM) of the complexes in DMSO were freshly prepared and diluted with cell culture medium to the desired concentration, for which no cytotoxicity was expected to maintain nearly complete cell survival (final complex concentration was 4 μM, except compound **3**, which was tested at four concentrations: 1, 4, 12.5, and 25 μM). The cell

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culture medium of each well of the 6-well plates was replaced with 2 mL of the cell culture medium solutions containing the complexes, and the plates were incubated at 37 °C under 5% CO_2 for 48 h. The culture medium was removed, and the cell layer of each well was washed with 2 mL of phosphate buffered saline (PBS). Then cells in each well were trypsinized (300 μL) and resuspended in 1700 μL of growth media. The number of cells in each well was counted, and cell pellets were isolated by centrifugation (3000*g* for 10 min at room temperature). Each pellet was digested for 3 days at room temperature in HNO_3 (70%, Sigma-Aldrich), and the resulting solutions were diluted to 25 mL using Milli-Q water (final concentration of 2.8% nitric acid). The amount of Ru cellular uptake was evaluated by ICP-MS. The experiment was carried out in triplicate.

Aromatase Inhibition. The H295R cell line was selected for this study because it expresses CYP19 enzyme, making it a suitable model for the study of aromatase activity.[58] Aromatase activity was measured using a tritiated water-release assay as described previously.[59,75] Briefly, H295R cells were cultured in 24-well plates (100 000 cells/well) containing 1 mL of the appropriate culture medium. After 24 h, the medium was removed and cells were washed once with 500 μL PBS. Then, a volume of 250 μL of culture medium containing 54 nM 1β-^{3}H-androstenedione and different concentrations of anastrozole, letrozole. or each complex (0.1, 1, 10, 100, and 1000 nM) was added to each well, and cells were incubated for 1.5 h at 37°C (5% CO_2). Further steps were as described previously.[59] Tritiated water was counted in 24-well plates containing a liquid scintillation cocktail using a Microbeta Trilux (PerkinElmer, Waltham, MA). Incubations in the absence of cells (blanks) and in the presence of DMSO 0.1% (which was the concentration used to dissolve the complexes in the growth media for this study) were included as negative and positive controls, respectively.

*Virtual Docking of Compound **3** with the Human Aromatase Enzyme.* Formation of the ternary complex between human aromatase, compound **3**, and the heme group was simulated using a ligand-imprinted docking procedure.[12] A Nelder−Mead simplex iteration was applied during the energy minimization steps, and the steric interactions, hydrogen bonding, and electrostatic forces were calculated by piecewise linear potentials and Coulomb potentials, respectively.[13] The crystal structure of **3** was generated with the XT structure solution program and refined with the XL refinement package using least squares minimization against the crystallographically resolved aromatase template (PDB entry 5JI6).[61] The MolDock scoring function was used in combination with a cavity prediction algorithm using the Molegro Virtual Docker 6.0 suite without the inclusion of water molecules.[13] Twenty rounds of docking simulations were performed to maintain search robustness, with the best conformations emerging from a group of up to 4 000 000 combinations.

Zebrafish Embryo Assay. Wild-type zebrafish (*Danio rerio*) embryos were raised at 28.5 °C and staged as previously described.[76] Embryos at the 4-cell stage were separately exposed to **2**, **3**, anastrozole, and *cis*-platin solutions (12.5 μM), prepared by diluting the DMSO stock solution of each compound in the fish medium (DMSO final concentration = 0.1%). The medium (containing the compound to be tested) was refreshed after 48 h for each experiment. A no-treatment control was also included. Experiments were performed in triplicate, and a total of 60 embryos from the pooling of three different crosses have been used per each treatment. The mortality, gross morphology, and hatching rates of the embryos in each system were observed every 24 h for a period of 96 h under a stereo microscope (Leika S6E). Zebrafish experiments were performed following a protocol approved by the Canadian Council for Animal Care (CCAC) and our local animal care committee.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.organomet.8b00897.

(PDF)

Accession Codes

CCDC 1840870−1840873 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

■ AUTHOR INFORMATION

ORCID

Annie Castonguay: 0000-0001-5705-6353

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Vessières, A.; Top, S.; Beck, W.; Hillard, E.; Jaouen, G. Metal complex SERMs (selective oestrogen receptor modulators). The influence of different metal units on breast cancer cell antiproliferative effects. *Dalton Trans.* **2006**, *4*, 529−541.

(2) Mandal, P.; Kundu, B. K.; Vyas, K.; Sabu, V.; Helen, A.; Dhankhar, S. S.; Nagaraja, C. M.; Bhattacherjee, D.; Bhabak, K. P.; Mukhopadhyay, S. Ruthenium(ii) arene NSAID complexes: inhibition of cyclooxygenase and antiproliferative activity against cancer cell lines. *Dalton Trans.* **2018**, *47*, 517−527.

(3) Li, P.; Su, W.; Lei, X.; Xiao, Q.; Huang, S. Synthesis, characterization and anticancer activity of a series of curcuminoids and their half-sandwich ruthenium(II) complexes. *Appl. Organomet. Chem.* **2017**, *31*, No. e3685.

(4) Vasaitis, T. S.; Bruno, R. D.; Njar, V. C. O. CYP17 inhibitors for prostate cancer therapy. *J. Steroid Biochem. Mol. Biol.* **2011**, *125*, 23−31.

(5) Carpenter, R.; Miller, W. R. Role of aromatase inhibitors in breast cancer. *Br. J. Cancer* **2005**, *93*, S1−S5.

(6) Dikmen, Z. G.; Gellert, G. C.; Jackson, S.; Gryaznov, S.; Tressler, R.; Dogan, P.; Wright, W. E.; Shay, J. W. In vivo Inhibition of Lung Cancer by GRN163L: A Novel Human Telomerase Inhibitor. *Cancer Res.* **2005**, *65*, 7866−7873.

(7) Meggers, E.; Atilla-Gokcumen, G. E.; Bregman, H.; Maksimoska, J.; Mulcahy, S. P.; Pagano, N.; Williams, D. S. Exploring Chemical Space with Organometallics: Ruthenium Complexes as Protein Kinase Inhibitors. *Synlett* **2007**, *2007* (8), 1177−1189.

(8) Mulcahy, S. P.; Meggers, E. Organometallics as Structural Scaffolds for Enzyme Inhibitor Design. In *Medicinal Organometallic*

DOI: 10.1021/acs.organomet.8b00897
Organometallics XXXX, XXX, XXX−XXX

Chemistry, 1st ed.; Jaouen, G., Metzler-Nolte, N., Eds.; Springer: Heidelberg, 2010; pp 141−153.
(9) Kilpin, K. J.; Dyson, P. J. Enzyme inhibition by metal complexes: concepts, strategies and applications. *Chem. Sci.* **2013**, *4*, 1410−1419.
(10) Anstaett, P.; Gasser, G. Organometallic Complexes as Enzyme Inhibitors: A Conceptual Overview. In *Bioorganometallic Chemistry*, 1st ed.; Jaouen, G., Salmain, M., Eds.; Wiley: Weinheim, 2014; pp 1−42.
(11) Zamora, A.; Denning, C. A.; Heidary, D. K.; Wachter, E.; Nease, L. A.; Ruiz, J.; Glazer, E. C. Ruthenium-containing P450 inhibitors for dual enzyme inhibition and DNA damage. *Dalton Trans.* **2017**, *46*, 2165−2173.
(12) Siiteri, P. K.; Thompson, E. A. Studies of human placental aromatase. *J. Steroid Biochem.* **1975**, *6*, 317−322.
(13) Huang, W.-Y.; Newman, B.; Millikan, R. C.; Schell, M. J.; Hulka, B. S.; Moorman, P. G. Hormone-related Factors and Risk of Breast Cancer in Relation to Estrogen Receptor and Progesterone Receptor Status. *Am. J. Epidemiol.* **2000**, *151*, 703−714.
(14) Osborne, C. K. Steroid hormone receptors in breast cancer management. *Breast Cancer Res. Treat.* **1998**, *51*, 227−238.
(15) Simpson, E.; Rubin, G.; Clyne, C.; Robertson, K.; O'Donnell, L.; Jones, M.; Davis, S. The Role of Local Estrogen Biosynthesis in Males and Females. *Trends Endocrinol. Metab.* **2000**, *11*, 184−188.
(16) Junko, Y.; Takara, Y.; Hiroji, O. Aromatization of androstenedione by normal and neoplastic endometrium of the uterus. *J. Steroid Biochem.* **1985**, *22*, 63−66.
(17) Watanabe, K.; Sasano, H.; Harada, N.; Ozaki, M.; Niikura, H.; Sato, S.; Yajima, A. Aromatase in human endometrial carcinoma and hyperplasia. Immunohistochemical, in situ hybridization, and biochemical studies. *Am. J. Pathol.* **1995**, *146*, 491−500.
(18) Brueggemeier, R. W.; Hackett, J. C.; Diaz-Cruz, E. S. Aromatase Inhibitors in the Treatment of Breast Cancer. *Endocr. Rev.* **2005**, *26*, 331−345.
(19) Jeselsohn, R.; Bergholz, J. S.; Pun, M.; Cornwell, M.; Liu, W.; Nardone, A.; Xiao, T.; Li, W.; Qiu, X.; Buchwalter, G.; Feiglin, A.; Abell-Hart, K.; Fei, T.; Rao, P.; Long, H.; Kwiatkowski, N.; Zhang, T.; Gray, N.; Melchers, D.; Houtman, R.; Liu, X. S.; Cohen, O.; Wagle, N.; Winer, E. P.; Zhao, J.; Brown, M. Allele-Specific Chromatin Recruitment and Therapeutic Vulnerabilities of ESR1 Activating Mutations. *Cancer Cell* **2018**, *33*, 173−186.
(20) O'Neill, M.; Paulin, F. E. M.; Vendrell, J.; Ali, C. W.; Thompson, A. M. The aromatase inhibitor letrozole enhances the effect of doxorubicin and docetaxel in an MCF7 cell line model. *BioDiscovery* **2012**, *6*, No. e8940.
(21) Miranda, A. A.; Limon, J.; Medina, F. L.; Arce, C.; Zinser, J. W.; Rocha, E. B.; Villarreal-Garza, C. M. Combination treatment with aromatase inhibitor and capecitabine as first- or second-line treatment in metastatic breast cancer. *J. Clin. Oncol.* **2012**, *30*, No. e11016.
(22) Prachayasittikul, V.; Pingaew, R.; Nantasenamat, C.; Prachayasittikul, S.; Ruchirawat, S.; Prachayasittikul, V. Investigation of aromatase inhibitory activity of metal complexes of 8-hydroxyquinoline and uracil derivatives. *Drug Des., Dev. Ther.* **2014**, *8*, 1089−1096.
(23) Sinthupoom, N.; Prachayasittikul, V.; Pingaew, R.; Worachartcheewan, A.; Prachayasittikul, S.; Ruchirawat, S.; Prachayasittikul, V. Copper Complexes of 8-Aminoquinoline and Uracils as Novel Aromatase Inhibitors. *Lett. Drug Des. Discovery* **2017**, *14*, 880−884.
(24) Yuan, R.-X.; Xiong, R.-G.; Abrahams, B. F.; Lee, G.-H.; Peng, S.-M.; Che, C.-M.; You, X.-Z. A Cu(I) coordination polymer employing a nonsteroidal aromatase inhibitor letrozole as a building block. *Dalton Trans.* **2001**, *14*, 2071−2073.
(25) Tang, Y.-Z.; Zhou, M.; Huang, J.; Cao, Z.; Qi, T.-T.; Huang, G.-H.; Wen, H.-R. Synthesis, Crystal Structure, and Characterization of three New Letrozole Complexes. *Z. Anorg. Allg. Chem.* **2012**, *638*, 372−376.
(26) Tran, M. T. Q.; Furger, E.; Alberto, R. Two-step activation prodrugs: transplatin mediated binding of chemotherapeutic agents to vitamin B12. *Org. Biomol. Chem.* **2013**, *11*, 3247−3254.
(27) Aird, R. E.; Cummings, J.; Ritchie, A. A.; Muir, M.; Morris, R. E.; Chen, H.; Sadler, P. J.; Jodrell, D. I. In vitro and in vivo activity and cross resistance profiles of novel ruthenium (II) organometallic arene complexes in human ovarian cancer. *Br. J. Cancer* **2002**, *86*, 1652.
(28) Guerriero, A.; Oberhauser, W.; Riedel, T.; Peruzzini, M.; Dyson, P. J.; Gonsalvi, L. New Class of Half-Sandwich Ruthenium(II) Arene Complexes Bearing the Water-Soluble CAP Ligand as an in Vitro Anticancer Agent. *Inorg. Chem.* **2017**, *56*, 5514−5518.
(29) Scolaro, C.; Bergamo, A.; Brescacin, L.; Delfino, R.; Cocchietto, M.; Laurenczy, G.; Geldbach, T. J.; Sava, G.; Dyson, P. J. In Vitro and in Vivo Evaluation of Ruthenium(II)−Arene PTA Complexes. *J. Med. Chem.* **2005**, *48*, 4161−4171.
(30) Gasser, G.; Ott, I.; Metzler-Nolte, N. Organometallic Anticancer Compounds. *J. Med. Chem.* **2011**, *54*, 3−25.
(31) Zhang, P.; Sadler, P. J. Advances in the design of organometallic anticancer complexes. *J. Organomet. Chem.* **2017**, *839*, 5−14.
(32) Gasser, G.; Metzler-Nolte, N. The potential of organometallic complexes in medicinal chemistry. *Curr. Opin. Chem. Biol.* **2012**, *16*, 84−91.
(33) Castonguay, A.; Doucet, C.; Juhas, M.; Maysinger, D. New Ruthenium(II)−Letrozole Complexes as Anticancer Therapeutics. *J. Med. Chem.* **2012**, *55*, 8799−8806.
(34) Zhou, W.; Wang, X.; Hu, M.; Zhu, C.; Guo, Z. A mitochondrion-targeting copper complex exhibits potent cytotoxicity against cisplatin-resistant tumor cells through multiple mechanisms of action. *Chem. Sci.* **2014**, *5*, 2761−2770.
(35) Lee, M. H.; Han, J. H.; Lee, J.-H.; Choi, H. G.; Kang, C.; Kim, J. S. Mitochondrial Thioredoxin-Responding Off−On Fluorescent Probe. *J. Am. Chem. Soc.* **2012**, *134*, 17314−17319.
(36) Han, M.; Vakili, M. R.; Soleymani Abyaneh, H.; Molavi, O.; Lai, R.; Lavasanifar, A. Mitochondrial Delivery of Doxorubicin via Triphenylphosphine Modification for Overcoming Drug Resistance in MDA-MB-435/DOX Cells. *Mol. Pharmaceutics* **2014**, *11*, 2640−2649.
(37) Sáez, R.; Lorenzo, J.; Prieto, M. J.; Font-Bardia, M.; Calvet, T.; Omeñaca, N.; Vilaseca, M.; Moreno, V. Influence of PPh3 moiety in the anticancer activity of new organometallic ruthenium complexes. *J. Inorg. Biochem.* **2014**, *136*, 1−12.
(38) Di, L.; Fish, P. V.; Mano, T. Bridging solubility between drug discovery and development. *Drug Discovery Today* **2012**, *17*, 486−495.
(39) Ang, W. H.; Daldini, E.; Scolaro, C.; Scopelliti, R.; Juillerat-Jeannerat, L.; Dyson, P. J. Development of Organometallic Ruthenium−Arene Anticancer Drugs That Resist Hydrolysis. *Inorg. Chem.* **2006**, *45*, 9006−9013.
(40) Sengupta, P.; Ghosh, S.; Mak, T. C. W. A new route for the synthesis of bis(pyridine dicarboxylato)bis(triphenylphosphine) complexes of ruthenium(II) and X-ray structural characterisation of the biologically active trans-$[Ru(PPh_3)_2(L^1H)2]$ (L^1H_2=pyridine 2,3-dicarboxylic acid). *Polyhedron* **2001**, *20*, 975−980.
(41) Bhalla, R.; Boxwell, C. J.; Duckett, S. B.; Dyson, P. J.; Humphrey, D. G.; Steed, J. W.; Suman, P. Chemical, Electrochemical, and Structural Aspects of the Ruthenium Complexes Ru(η^6-arene)Cl_2(P) (Where Arene = Benzene, [2.2]Paracyclophane and P = Triphenylphosphine, rac-[2.2]Paracyclophanylphosphine). *Organometallics* **2002**, *21*, 924−928.
(42) Dharmaraj, N.; Viswanathamurthi, P.; Natarajan, K. Ruthenium(II) complexes containing bidentate Schiff bases and their antifungal activity. *Transition Met. Chem.* **2001**, *26*, 105−109.
(43) Habtemariam, A.; Melchart, M.; Fernández, R.; Parsons, S.; Oswald, I. D. H.; Parkin, A.; Fabbiani, F. P. A.; Davidson, J. E.; Dawson, A.; Aird, R. E.; Jodrell, D. I.; Sadler, P. J. Structure−Activity Relationships for Cytotoxic Ruthenium(II) Arene Complexes Containing N,N-, N,O-, and O,O-Chelating Ligands. *J. Med. Chem.* **2006**, *49*, 6858−6868.
(44) Patra, M.; Joshi, T.; Pierroz, V.; Ingram, K.; Kaiser, M.; Ferrari, S.; Spingler, B.; Keiser, J.; Gasser, G. DMSO-Mediated Ligand Dissociation: Renaissance for Biological Activity of N-Heterocyclic-

DOI: 10.1021/acs.organomet.8b00897
Organometallics XXXX, XXX, XXX−XXX

[Ru(η^6-arene)Cl_2] Drug Candidates. *Chem. - Eur. J.* **2013**, *19*, 14768−14772.
(45) Renfrew, A. K.; Phillips, A. D.; Egger, A. E.; Hartinger, C. G.; Bosquain, S. S.; Nazarov, A. A.; Keppler, B. K.; Gonsalvi, L.; Peruzzini, M.; Dyson, P. J. Influence of Structural Variation on the Anticancer Activity of RAPTA-Type Complexes: ptn versus pta. *Organometallics* **2009**, *28*, 1165−1172.
(46) Ang, W. H.; Khalaila, I.; Allardyce, C. S.; Juillerat-Jeanneret, L.; Dyson, P. J. Rational Design of Platinum(IV) Compounds to Overcome Glutathione-S-Transferase Mediated Drug Resistance. *J. Am. Chem. Soc.* **2005**, *127*, 1382−1383.
(47) Haghdoost, M.; Golbaghi, G.; Létourneau, M.; Patten, S. A.; Castonguay, A. Lipophilicity-antiproliferative activity relationship study leads to the preparation of a ruthenium(II) arene complex with considerable in vitro cytotoxicity against cancer cells and a lower in vivo toxicity in zebrafish embryos than clinically approved cis-platin. *Eur. J. Med. Chem.* **2017**, *132*, 282−293.
(48) Loughrey, B. T.; Healy, P. C.; Parsons, P. G.; Williams, M. L. Selective Cytotoxic Ru(II) Arene Cp* Complex Salts [R-PhRuCp*]$^+X^-$ for X = BF_4^-, PF_6^-, and BPh_4^-. *Inorg. Chem.* **2008**, *47*, 8589−8591.
(49) Bergamo, A.; Sava, G. Linking the future of anticancer metal-complexes to the therapy of tumour metastases. *Chem. Soc. Rev.* **2015**, *44*, 8818−8835.
(50) Zhao, J.; Zhang, D.; Hua, W.; Li, W.; Xu, G.; Gou, S. Anticancer Activity of Bifunctional Organometallic Ru(II) Arene Complexes Containing a 7-Hydroxycoumarin Group. *Organometallics* **2018**, *37*, 441−447.
(51) Liu, Y.; Agrawal, N. J.; Radhakrishnan, R. A flexible-protein molecular docking study of the binding of ruthenium complex compounds to PIM1, GSK-3β, and CDK2/Cyclin A protein kinases. *J. Mol. Model.* **2013**, *19*, 371−382.
(52) Huang, H.-L.; Tang, B.; Yi, Q.-Y.; Wan, D.; Yang, L.-L.; Liu, Y.-J. Synthesis, DNA-binding, molecular docking and cytotoxic activity in vitro evaluation of ruthenium(II) complexes. *Transition Met. Chem.* **2018**, 1−14.
(53) Hong, Y.; Li, H.; Yuan, Y.-C.; Chen, S. Molecular Characterization of Aromatase. *Ann. N. Y. Acad. Sci.* **2009**, *1155*, 112−120.
(54) Maurelli, S.; Chiesa, M.; Giamello, E.; Di Nardo, G.; V. Ferrero, V. E.; Gilardi, G.; Van Doorslaer, S. Direct spectroscopic evidence for binding of anastrozole to the iron heme of human aromatase. Peering into the mechanism of aromatase inhibition. *Chem. Commun. (Cambridge, U. K.)* **2011**, *47*, 10737−10739.
(55) Weisz, J. In vitro assays of aromatase and their role in studies of estrogen formation in target tissues. *Cancer Res.* **1982**, *42*, 3295s−3298s.
(56) Lephart, E. D.; Simpson, E. R. Assay of aromatase activity. *Methods Enzymol.* **1991**, *206*, 477−483.
(57) Sanderson, J. T.; Letcher, R. J.; Heneweer, M.; Giesy, J. P.; van den Berg, M. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Perspect.* **2001**, *109*, 1027−1031.
(58) Caron-Beaudoin, E.; Denison, M. S.; Sanderson, J. T. Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. *Toxicol. Sci.* **2016**, *149*, 134−44.
(59) Sanderson, J. T.; Seinen, W.; Giesy, J. P.; van den Berg, M. 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? *Toxicol. Sci.* **2000**, *54*, 121−7.
(60) Sanderson, J. T.; Boerma, J.; Lansbergen, G. W. A.; van den Berg, M. Induction and Inhibition of Aromatase (CYP19) Activity by Various Classes of Pesticides in H295R Human Adrenocortical Carcinoma Cells. *Toxicol. Appl. Pharmacol.* **2002**, *182*, 44−54.
(61) Ghosh, D.; Egbuta, C.; Lo, J. Testosterone complex and non-steroidal ligands of human aromatase. *J. Steroid Biochem. Mol. Biol.* **2018**, *181*, 11−19.
(62) Kyte, J.; Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **1982**, *157*, 105−132.
(63) Mandrekar, N.; Thakur, N. L. Significance of the zebrafish model in the discovery of bioactive molecules from nature. *Biotechnol. Lett.* **2009**, *31*, 171.
(64) MacRae, C. A.; Peterson, R. T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discovery* **2015**, *14*, 721.
(65) Wu, Q.; Zheng, K.; Liao, S.; Ding, Y.; Li, Y.; Mei, W. Arene Ruthenium(II) Complexes as Low-Toxicity Inhibitor against the Proliferation, Migration, and Invasion of MDA-MB-231 Cells through Binding and Stabilizing c-myc G-Quadruplex DNA. *Organometallics* **2016**, *35*, 317−326.
(66) Wang, Y.-H.; Cheng, C.-C.; Lee, W.-J.; Chiou, M.-L.; Pai, C.-W.; Wen, C.-C.; Chen, W.-L.; Chen, Y.-H. A novel phenotype-based approach for systematically screening antiproliferation metallodrugs. *Chem.-Biol. Interact.* **2009**, *182*, 84−91.
(67) Lenis-Rojas, O. A.; Fernandes, A. R.; Roma-Rodrigues, C.; Baptista, P. V.; Marques, F.; Pérez-Fernández, D.; Guerra-Varela, J.; Sánchez, L.; Vázquez-García, D.; Torres, M. L.; Fernández, A.; Fernández, J. J. Heteroleptic mononuclear compounds of ruthenium-(II): synthesis, structural analyses, in vitro antitumor activity and in vivo toxicity on zebrafish embryos. *Dalton Trans.* **2016**, *45*, 19127−19140.
(68) Bennett, M. A.; Smith, A. K. Arene ruthenium(II) complexes formed by dehydrogenation of cyclohexadienes with ruthenium(III) trichloride. *J. Chem. Soc., Dalton Trans.* **1974**, 233−241.
(69) Dong, Y.; Li, X.; Liu, S.; Zhu, Q.; Li, J.-G.; Sun, X. Facile synthesis of high silver content MOD ink by using silver oxalate precursor for inkjet printing applications. *Thin Solid Films* **2015**, *589*, 381−387.
(70) Sheldrick, G. M. A short history of SHELX. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **2008**, *64*, 112−22.
(71) Dolomanov, O. V.; Bourhis, L. J.; Gildea, R. J.; Howard, J. A. K.; Puschmann, H. OLEX2: a complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.* **2009**, *42*, 339−341.
(72) Sheldrick, G. SHELXT - Integrated space-group and crystal-structure determination. *Acta Crystallogr., Sect. A: Found. Adv.* **2015**, *71*, 3−8.
(73) Vichai, V.; Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* **2006**, *1*, 1112−1116.
(74) Yu, J.; He, J.; Zhang, Y.; Qin, F.; Xiong, Z.; Li, F. An ultraperformance liquid chromatography−tandem mass spectrometry method for determination of anastrozole in human plasma and its application to a pharmacokinetic study. *Biomed. Chromatogr.* **2011**, *25*, 511−516.
(75) Higley, E. B.; Newsted, J. L.; Zhang, X.; Giesy, J. P.; Hecker, M. Assessment of chemical effects on aromatase activity using the H295R cell line. *Environ. Sci. Pollut. Res.* **2010**, *17*, 1137−48.
(76) Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **1995**, *203*, 253−310.

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