Investigating the mode of action and characterisation of active principles -
*A Salvia officinalis* tincture for the amelioration of hot flushes

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This thesis describes research conducted in the School of Pharmacy, University of London between November 2007 and July 2011 under the supervision of Dr. Deniz Tasdemir and Professor Andreas Kortenkamp. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date
The life of most women is at some point affected by side effects of their menopausal transition. The most common menopausal side effects are the hot flushes, which influence the quality of life severely. The physiology behind the hot flushes and the connection between hormones, neurotransmitters and hot flush occurrence is still unclear. However, *in vivo* studies suggest a correlation between the hormone 17-β-estradiol (E2), the neurotransmitter serotonin (5-HT) and the regulation of the core body temperature.

Menosan® (a *Salvia officinalis* tincture) is an herbal medicinal product (HMP), which has proven to reduce hot flushes, but the mechanism underlying this effect has remained unknown. In order to elucidate the anti-hot flush mechanism of action, the *Salvia officinalis* tincture as well as three subextracts (*n*-hexane, chloroform and aqueous-ethanolic (aq-EtOH) subextract (SE)), which were obtained by solvent-solvent partitioning of the tincture, were tested for relevant *in vitro* activities. In a multi-target approach, the influence of the tincture and SE on the serotonergic system was assessed in a selective serotonin re-uptake inhibition (SSRI) assay and estrogenicity was investigated in an estrogen receptor luciferase reporter gene (ERLUX) assay and an estrogen screen (ESCREEN) assay. None of the extracts showed activity in the SSRI assay. The aq-EtOH SE was found to be estrogenic in the ERLUX assay (EC₅₀ 64 pg/ml) and the ESCREEN assay (EC₅₀ 130 µg/ml), but none of the other extract exhibited an estrogenic response.

In a second approach the *Salvia officinalis* tincture was investigated for a possible multi-beneficial effect for menopausal women and whether the active principles underlying the anti-hot flush activity and treatment of Alzheimer disease could be linked. The *Salvia officinalis* tincture and SEs were therefore tested for acetylcholinesterase inhibition (AChEI) activity. The neurotransmitter acetylcholine (ACh) has been linked with Alzheimer diseases (AD) and as it has been suggested that menopausal women may have a higher risk than men to develop AD, hence the effect on ACh levels would be of interest. No activity in the AChEI assay was found for the *Salvia officinalis* tincture or the SEs.

HMPs need to meet high safety standards. Thus, for the safety assessment the inhibitory potential of the *Salvia officinalis* tincture and its SEs against CYP3A4 was evaluated in an *in vitro* assay. Cytochrome P450 (CYP) enzyme 3A4 is as key enzyme in human drug metabolism and all four extracts inhibited the CYP3A4 enzyme. While the activity of the aq-EtOH SE was very low, the activity of the *Salvia officinalis* tincture (EC₅₀ 6.8 µg/ml), the chloroform SE (EC₅₀ 3.1 µg/ml) and the *n*-hexane SE (EC₅₀ 1.8 µg/ml)
Abstract

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were considerably stronger and the inhibitory principle of these extracts warrant further investigation.

In the initial screening the aq-EtOH SE was the only SE, which displayed an anti-hot flush activity. Hence, an activity-guided fractionation of the aq-EtOH SE was performed in order to isolate and identify the active constituent(s). Following the estrogenicity in the ERLUX assay resulted in identification of luteolin-7-O-β-glucuronide as the active principle in fraction AQ 4. The estrogenic potential of the isolated luteolin-7-O-β-glucuronide was weak (EC$_{50}$ 129 μg/ml). Luteolin-7-O-glucoside was identified as the major component in the most active fraction (AQ 7.6.7.6, EC$_{50}$ 0.7 μg/ml). The compounds were identified in the estrogenic fractions by nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS), ultra-high-pressure liquid chromatography at elevated temperature coupled to time-of-flight mass spectrometry (UHPLC-TOF-MS) and capillary (cap) NMR. The estrogenic activity of pure commercially available luteolin-7-O-glucoside (EC$_{50}$ 0.44 μg/ml) was similar to that of fraction AQ 7.6.7.6. Quantitative analysis of luteolin-7-O-glucoside in the aq-EtOH SE and the Salvia officinalis tincture explained the estrogenic activity of the aq-EtOH SE, but failed to explain the absence of estrogenic effects by the Salvia officinalis tincture.

In conclusion the investigation revealed noteworthy CYP3A4 inhibitory activity by the Salvia officinalis tincture, luteolin-7-O-β-glucuronide has been identified as the estrogenic principle in fraction AQ 4 and luteolin-7-O-glucoside as the putative estrogenic principle of fraction AQ 7.6.7.6.
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<td>Figure 32</td>
<td>ERLUX results of fractions AQ 7.6.1, AQ 7.6.2, AQ 7.6.3, AQ 7.6.4 and AQ 7.6.5</td>
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<td>Figure 42</td>
<td>Gradient elution of AQ 7 UHPLC-TOF-MS</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>$A_\beta$</td>
<td>beta-amyloid peptides</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>$A_{max}$</td>
<td>absorbance maximum</td>
</tr>
<tr>
<td>aq-EtOH</td>
<td>aqueous-ethanolic</td>
</tr>
<tr>
<td>Bf</td>
<td>Bungarus fasciatus</td>
</tr>
<tr>
<td>BuChE</td>
<td>butyrylcholinesterase</td>
</tr>
<tr>
<td>CD</td>
<td>charcoal-dextran</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChAT</td>
<td>cholin acetyltransferase</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>chloroform</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DM</td>
<td>dosing media</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dr.</td>
<td>drop(s)</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-2-nitrobenzoate ion</td>
</tr>
<tr>
<td>E2</td>
<td>17-β-estradiol</td>
</tr>
<tr>
<td>$EC_{50}$</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EPT</td>
<td>estrogen-progesterone therapy</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERLUX</td>
<td>estrogen receptor reporter gene</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ESCREEN</td>
<td>estrogen screen</td>
</tr>
<tr>
<td>ET</td>
<td>estrogen therapy</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FMP</td>
<td>final menstrual period</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GluA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin Releasing Hormone</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HERS</td>
<td>Heart and Estrogen / Progestin Replacement study</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond coherence</td>
</tr>
<tr>
<td>HMP</td>
<td>herbal medicinal product</td>
</tr>
<tr>
<td>HMQC</td>
<td>heteronuclear multiple quantum coherence</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>hSERT</td>
<td>human serotonin transporter</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin (5-hydroxytryptamine)</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Sinikka Rahte</td>
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<tr>
<td>5-HTT</td>
<td>serotonin transporter (5-hydroxytryptamine transporter)</td>
</tr>
<tr>
<td>HuBChE</td>
<td>human butyrylcholinesterase</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IMS</td>
<td>International Menopause society</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectroscopy</td>
</tr>
<tr>
<td>LH</td>
<td>luteinising hormone</td>
</tr>
<tr>
<td>max</td>
<td>maximal</td>
</tr>
<tr>
<td>MEM-NEAA</td>
<td>MEM-non-essential aminoacids</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MTT</td>
<td>thiazolyl blue</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NST-PEG</td>
<td>Naturstoffreagenz—polyethylenglycol</td>
</tr>
<tr>
<td>PAM</td>
<td>pre-assay medium</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>SE</td>
<td>subextract</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SRB</td>
<td>sulforhodamine B</td>
</tr>
<tr>
<td>SSNRI</td>
<td>selective serotonin and noradrenalin re-uptake inhibitor</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin re-uptake inhibitor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TPH</td>
<td>tryptophan hydroxylase</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>WHI</td>
<td>Woman's Health Initiative</td>
</tr>
<tr>
<td>WHIMS</td>
<td>Woman's Health Initiative Memory study</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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First I would like to thank my supervisor Dr. Deniz Tasdemir for giving me the chance to work on this multidisciplinary project and for introducing me to the challenging world of natural product research.

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1. Introduction

One of the challenges for researchers today is finding a treatment for menopausal women, which alleviates the life affecting symptoms that accompany the reproductive ageing and also complies with the desire for safe treatment options.

The first part of this introduction briefly reviews the menopausal stages, the most bothersome menopausal symptom of hot flushes, the worryingly increased risk for Alzheimer's disease (AD) amongst postmenopausal women, and the currently available treatment options.

The focus of the second part of this introduction is on the plant which was chosen for this study. *Salvia officinalis* may possibly be a safe and effective alternative treatment option for menopause related symptoms. It has a reputation as a medicinal plant throughout the Ancient and Middle Ages and is nowadays commonly used as a herb in food preparation, for inflammations and infections of the mouth and throat, and hyperhidrosis. Since the 11th century Salvia's importance as a medicinal plant is described in sayings such as *Cur moriantur homo cui Salvia crescit in horto?* ("Why should a man die whilst sage grows in his garden?") (Grieve, 1980). The long history of *Salvia* as a medicinal plant has often attracted the attention of researchers and the chemistry and biological activity of *Salvia officinalis* and very briefly a few other *Salvia* species will be reviewed in the second part of this introduction.

1.1. The menopause

Menopause describes the cessation of menses, with the Greek words for *men* (month) and *pausis* (to cease) (Mosby, 2002). The term menopause is commonly used, but defining the different stages around the menopause still seems to be a difficult task.

The menopause is a normal, natural event, which occurs during the ageing process of women. The hypothalamic-pituitary-ovarian axis and the paramesonephric structures (Muellerian structures, e.g. uterus) are essentially what is described as the female reproductive axis. Normal ageing will lead to a nonfunctional state of this reproductive axis independently of the other organs or the health situation of the woman. Upon reaching that stage, the reproductive phase of a woman's life ends. This moment is the menopause. On average women experience the menopause between 42–58 years of age (Soules et al., 2001). The menopause can also be induced by medical interventions such as surgery, chemotherapy, or pelvic radiation therapy. Numerous attempts have been made to describe the different stages of reproductive ageing in women. This was
addressed by expert groups from the World Health Organization (WHO, 1996), the International Menopause Society (IMS) (Utian, 1999), and the Stages of Reproductive Aging Workshop (STRAW) (Soules et al., 2001). The results of the most recent attempt to address the problem of the confusing nomenclature for the menopausal stages are the definitions by STRAW, which are briefly summarised here (Fig. 1) and used throughout this thesis.

The ovarian hormone production decreases and results in amenorrhea. With the final menstrual period (FMP) it becomes apparent that the reproductive axis ceases to function. Twelve months of amenorrhea define the point when the reproductive axis is considered as non-functional, hence the menopause.

Figure 1.1: Development of menopausal stages defined according to STRAW (adapted from (Soules et al., 2001))

The time before the FMP is described as the menopausal transition. It covers roughly the last two years before the menopause. The term perimenopause encompasses the menopausal transition and the twelve months of amenorrhea. It includes the period where most of the endocrine changes including the shut-down of the ovaries, the menopause, occur. The postmenopause can be subdivided into an early and a late stage. The first 5 years after the FMP are accompanied by final leveling of the ovarian hormone function and certain menopause-related health developments (e.g. osteoporosis) and this period was therefore termed early postmenopause. The time beyond these five years is the postmenopause (Soules et al., 2001).

In 2006 it was estimated that approximately 35 million women in the United States were older than 50 years of age and that each day around 3000 women reach the age of 50 years. Their reproductive axis is ceasing to function or has already ceased. Many women go beyond the time of the perimenopause without hormone related symptoms and with little disruption to their normal life and well-being. Unfortunately, many women
experience symptoms that considerably affect their quality of life, well-being and general health (Cobin et al., 2006).

1.1.1. The female reproductive system and its ageing

Reproductive ageing is a continuous process which occurs naturally. It is accompanied by a decrease in the number of primary oocytes. Women are born with around 1 million oocytes, of which roughly only 400 are ovulated, and the majority of them degenerate during the reproductive years.

The ovaries and the uterus follow cyclic changes, which are described as the menstrual cycle. This cycle is regulated by endocrine hormones, which are secreted by the hypothalamus [Gonadotropin-releasing hormone (GnRH)], the pituitary [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], and the ovaries themselves (estrogen and progesterone) (Fig. 1.2). During the early cycle phase, the follicular phase, LH and FSH encourage maturation of the follicle, which consists of a primary oocyte and surrounding granulosa cells. The follicle grows and secretes estrogens. The increase in estrogens results in a decrease of the synthesis of FSH via a negative feedback. By a positive feedback the estrogens trigger a surge of LH. High LH levels are crucial for follicle rupture and ovulation, which then allow the follicle to develop into an endocrine gland, the corpus luteum. During this luteal phase large quantities of progesterone are released. This inhibits, via a negative feedback loop, the release of GnRH, FSH and LH and prevents the development of a new primary follicle. If fertilisation does not occur, the corpus luteum will start to degenerate and estrogen and progesterone levels will drop. This leads to the menstruation and the beginning of a new cycle (Franks and Webber, 2010). In the fertile years the ovaries secrete the majority of estrogens. Small amounts are produced in adrenal glands or obtained by conversion of androgens. The development of a corpus luteum from a primary oocyte continues throughout a woman's life from the menarche (first menstrual period and sign of the possibility for fertility) until the end of her reproductive phase. Since the number of oocytes is fixed in women a depletion of the oocytes occurs during reproductive ageing (Pavelka et al., 1991).
When, finally, the number of oocytes diminishes, the reproductive cycle becomes more irregular, either shorter or longer. Even anovulatory cycles, where ovaries are inactive, start to occur and become more common during the menopausal transition. When no corpus luteum is developed the usual release of estrogens and progesterone is absent. In the absence of estrogens and progesterone, no negative feedback reaches the hypothalamus and the anterior pituitary, which results in rising GnRH, LH and FSH levels. The FSH can be so high that an abnormal follicle development is triggered, which raises the estrogen levels. These cycle irregularities during the menopausal transition can result in exceptionally high levels of estrogens, which often induce headaches, mood swings and hot flushes (Hale and Burger, 2009).

During the year before the menopause the FSH and LH levels are high and stable, whereas the estrogen levels start to lower, but they are still highly variable. In the postmenopausal phase, where estrogens settle at low levels and FSH and LH levels remain high and stable, the complete depletion of ovaries and therefore the cessation of ovulation mark the end of the reproductive phase (Hale and Burger, 2009).
1.2. Health issues accompanying the menopausal phases

In the same way as one cannot predict the onset or the progression of a woman’s menopausal stages, it is impossible to predict how a woman will experience this time period. However, for most of the women, the end and the time beyond their reproductive phase is accompanied by more or less severe menopausal symptoms. These menopausal symptoms usually continue for about 5 years after onset and many women feel that they greatly influence their life quality (Cobin et al., 2006). The severity of the symptoms can, for some women, be attenuated by certain life style choices (food, alcohol, nicotine, physical activities, stress-management (Reinhard-Hennch et al., 2006), but additional medical treatment of menopausal symptoms is common. Menopausal symptoms can already occur during the late stages of the reproductive phase, before the menopausal transition starts. The decrease and fluctuation of the ovarian estrogen secretion, before their complete failure, is the main cause of menopausal symptoms. It seems that every woman has her own estrogen threshold, as the occurrence of the menopausal symptoms is not related to a certain estrogen blood level. Symptoms may appear gradually or within a short period of time, more or less severe, and last a few months or several years. The extent to which the symptoms develop determines how dramatic a woman’s life is affected. Menopausal symptoms can have influence on daily activities and the life quality in general or, in more severe cases, might even result in an inability to work.

The menopausal symptoms can be divided into categories, including menstrual bleeding disorders (e.g. hypo- or hypermenorrhea), vasomotor symptoms (e.g. hot flushes), psychosomatic symptoms (e.g. mood instabilities), somatotrophic changes (e.g. atrophic skin and breast changes) and metabolic changes (e.g. osteoporosis) (Schindler, 2006). Commonly reported symptoms are sweating, hot flushes, insomnia, breast tenderness, migraines and problems with sexual function (Dennerstein et al., 2000). Most prominent among the long term risks, which are often associated with the menopause, are depression (Cohen et al., 2006), a higher risk of cognitive dysfunction and AD (Aloysi et al., 2006), and cardiovascular events (IMS, 2009), as well as osteoporosis (Clarke and Khosla, 2010). Quantitative assessment of the severity and intensity of these symptoms is difficult, as the perception of them can be subjective and their personal impact may vary between individuals. The symptoms experienced and the way they are rated also vary among ethnic groups, cultures, socioeconomic groups, and climates (Cobin et al., 2006).
Overall, the menopause, with its different stages, appearances and the varying influence on a woman’s life, is a very complex subject. In order to focus on the symptoms and the physiological and biological correlations that serve the overall idea of the project, the following sections will concentrate on the current theories and the research about hot flushes and the higher incidence for AD and review these more comprehensively.

1.2.1. Hot flushes

The most frequent menopausal symptoms are the hot flushes. Women experience them as the menopausal symptom that influences their life quality most severely. Hot flushes are also referred to as vasomotor symptoms and are experienced as an episode of flushing, sweating and the sensation of heat. They have a sudden onset, last between 3-10 minutes, and can be accompanied by palpitations and anxiety. The sudden onset especially causes problems for many women during their everyday life, which can lead to anxiety attacks or depression. Insomnia, due to awakenings during the night, may also be associated with hot flushes. Hence, hot flushes can disturb daily functioning, work performance, general well-being, sleep and social relations (Kronenberg, 1994).

Hot flushes appear most frequently in peri- and postmenopausal women, but women can also experience severe hot flushes before their reproductive axis fails, in which case their estrogen levels drop rapidly. This radical drop is usually caused by medical interventions like removal of ovaries or breast cancer treatment with selective estrogen receptor modulators such as tamoxifen. However, hot flushes are usually an early sign of approaching menopause. During the menopausal transition the frequency of the hot flushes varies. They can occur monthly, weekly, daily or even hourly. The incidence rate varies from woman to woman and also between different populations (Loprinzi and Reame, 2004), but it has been estimated that about 75% of women over 50 years of age experience hot flushes (Utian, 2005). The results depend largely on the design of the study, especially as many studies of hot flushes rely on self-reported measures of symptom frequency and severity by using questionnaires or diaries. Measurement of the skin conductance is used as an objective measure of hot flushes. Laboratory studies showed a high correlation between sternal skin conductance measures and self-reported hot flushes. Unfortunately, the long time periods, which need to be monitored, and the portable monitors connected by wires to the areas where the hot flush is apparent, are cumbersome to wear and therefore there are not many studies based on accurate, objective and long term observations (Kronenberg, 2010). The trend that the prevalence for hot flushes is higher in Western countries than e.g. in Asian countries, caught the
attention of many researchers during the last few years (Freeman and Sherif, 2007). Evaluating whether these differences are due to genetic, environmental, cultural factors or lifestyle choices was the aim of extensive studies. For example, Brown et al. (2009) showed that Japanese-American women reported fewer hot flushes than the European-American women, when the number of objectively measured hot flushes (measuring of skin conductance) was the same. He assumed that it was the cultural background of the Japanese-Americans, which led them to appraise what is acceptable to report and discuss differently to the European-American women. However, the differences between populations and cultures cannot be discussed at length here and despite variations in reported frequency of hot flushes, all studies highlight that hot flushes are bothersome or even extremely distressing symptoms. They are especially common in the period of the menopausal transition and the most common reasons why women seek treatment (Kronenberg, 1994).

1.2.1.1. Physiology of hot flushes

Despite intensive research efforts in the last two decades, the physiology of hot flushes is still poorly understood. Many studies have been aimed to understand the endocrinology, which correlates with this common menopausal symptom, and various treatment options were developed based on the results. Nevertheless, researchers are still not certain about the exact connection between hormones, neurotransmitters and hot flush occurrence (Kronenberg, 2010). It is certain that the decrease in estrogen levels correlates with hot flushes (Weiss et al., 2004), and it has also been shown many times that treatment with estrogens is highly effective against hot flushes (MacLennan et al., 2001). However, estradiol (E2) levels in symptomatic and asymptomatic menopausal women were found to be very similar. Interestingly, women reported to experience hot flushes even before changes in their serum estrogen level were detected (Loprinzi and Reame, 2004), and hot flushes were also reported in the last trimester of pregnancy, when estrogen levels are especially high. All this indicates that the occurrence of hot flushes cannot solely be explained by estrogen levels (Miller and Li, 2004). Other studies confirmed that the decrease of the hormone levels (estrogens, LH or FSH) in postmenopausal women does not correlate with the occurrence of hot flushes directly (Freedman, 2001) and it may well be that the hot flushes arise due to a decrease in the estrogen sensitivity of the hypothalamic-pituitary-axis (Weiss et al., 2004). *In vivo* studies, investigating the role of the hypothalamus-regulated hormones (e.g. FSH), estrogens and serotonin (5-hydroxytryptamine, 5-HT) in relation to the hot flush occurrence, use the
rat hot flush model. The rats are ovariectomised (deficient of estrogens) and dependent on morphine. Withdrawal of morphine results in a rise of the tail skin temperature and the heart rate, but the core body temperature drops. These symptoms are similar to the symptoms observed in menopausal women during a hot flush. In this model estrogen receptor agonists proved to play an important role in the regulation of the core body temperature. Additionally, it was shown in the rat model that 5-HT<sub>2A</sub> receptor agonists were able to prevent hyperthermia, which led to the hypothesis that 5-HT<sub>2A</sub> receptors could be downstream mediators of estrogen effects on the core body temperature, hence basic thermoregulation (Merchenthaler et al., 1998; Sipe et al., 2004).

All hot flush-related studies and hypotheses are based on the general understanding of the female reproductive axis (see 1.1.1, The female reproductive system and its ageing). The hormones of the hypothalamus and the anterior pituitary, GnRH, FSH and LH, stimulate the maturation of an ovarian follicle, which secretes E2 and enables a negative feedback to the hypothalamus (Fig. 1.3). At the end of the reproductive phase of a woman, when the supply of ova ends, the secretion of E2 and the negative feedback to the hypothalamus is absent (Franks and Webber, 2010).

![Figure 1.3: Influence of E2 on the regulation of the female reproductive axis. Solid arrows are direct stimulation of hormone secretion, dotted arrows indicate for an inhibiting effect on the secretion of hormones (adapted from (Franks and Webber, 2010)).](image)

The core body temperature, which is usually maintained within a specific thermoregulatory zone by vasodilatation and perspiration, is narrowed during the menopausal transition. Hence, the body becomes less tolerant to changes in core body temperature and the normal temperature regulation is then often accompanied by sweating (Fitzpatrick, 2004). *In vivo* studies found correlations of E2 and 5-HT with the
regulation of the core body temperature, which are now briefly reviewed in the following section.

E2 alters the available concentration of 5-HT in the synaptic cleft through two mechanisms and furthermore, indirectly regulates 5-HT receptor expression (Fig. 1.4). First, E2 increases the production of tryptophan hydroxylase (TPH) (Bethea et al., 2000), which is the rate-limiting step in the 5-HT synthesis from tryptophan. Up-regulation of TPH indirectly increases the 5-HT concentration (Cheng et al., 2005). Secondly, E2 inhibits the gene expression for the 5-HT re-uptake transporter (5-HTT), which increases the time that the 5-HT remains in the synaptic cleft (Rybaczyk et al., 2005). Additionally, the expression of estrogen receptors (ER) is directly regulated by E2 and in the presence of progesterone it comes to a down-regulation of ERα and an up-regulation of ERβ (Cheng et al., 2005).

This modulation of the ER expression leads to an up-regulation of 5-HT_{2A} receptors and a down-regulation of 5-HT_{1A} receptors in the central nervous system. The increased density of 5-HT_{2A} receptors has an impact on the temperature regulation, as agonists on the 5-HT_{2A} receptors have been reported to prevent hyperthermia in in vivo models, hence linking E2 with the thermoregulation (Sipe et al., 2004). 5-HT itself influences subtypes of the human 5-HT receptor (Fig. 1.5). Activation of 5-HT_{2A} by 5-HT also un couples the 5HT_{1A} auto-receptor and decreases 5-HT effects at this receptor subtype. 5HT_{1A} receptors usually trigger negative feedback, which then fails to appear and 5-HT concentrations increase.
negative feedback

5-HT influence on 5-HT receptor subtypes involved in thermoregulation. + represents agonistic effect, - represents inhibitory effects. Tc = core body temperature.

The same effect can also be achieved by E2, which indirectly inhibits the expression of 5HT1A (Fig. 1.4). Several in vivo studies investigated the involvement of 5-HT receptors on induced hypothermia in rodents (Hagan et al., 2000; Guscott et al., 2003; Hedlund et al., 2003). The receptor subtypes 5-HT1A, 5-HT2A and 5-HT7 were found to play a role in maintaining the core body temperature in animal models. Agonists on these 5-HT1A and 5-HT7 receptors can induce hypothermia or rather, prevent hyperthermia, by lowering the thermoregulatory set point (Fig. 1.5). Agonists of 5-HT2A were found to restore the temperature dysfunction, which occurred after estrogen deprivation (Hedlund et al., 2004; Sipe et al., 2004). Accordingly, the thermoregulation system could be positively influenced when the thermoregulatory zone was narrowed during the menopausal stages, by either an increase in estrogen levels, 5HT2A receptor agonists or inhibitors of the 5-HTT.

When, during the menopause, the estrogen levels deplete, the 5-HT levels follow. Consequently, this results in a suppression of 5-HT2A receptors (Rybaczyk et al., 2005) and an over-expression of 5-HTT, and finally a decrease of the 5-HT concentrations in the synaptic cleft. The dysfunction of the regulation of the core body temperature results in the lower tolerance for changes in temperature and increases the likelihood of hot flushes. Another theory involves the interaction of estrogens with the adrenergic system. It was observed that norepinephrine (NE) levels increased prior to a hot flush, suggesting that α2-adrenergic receptors might play a role in the occurrence of hot flushes. Clonidine, an α2-adrenergic agonist, was found to reduce the number of hot
flushes in symptomatic menopausal women (Freedman, 2001). However, there are only a few studies, which show contradictory results, and further studies on the mechanisms are needed to clarify the correlation of NE and hot flushes. All these correlations and hypotheses were the basis for many of the pharmacological treatment options for the amelioration of hot flushes at hand today. These options are reviewed in section 1.3 ‘Treatment of menopausal symptoms’ with the focus on hot flushes.

1.2.2. Alzheimer's disease

A natural part of ageing is a decline in memory. Often, in particular in later years in life, a specific form of dementia, Alzheimer’s disease (AD), develops. Women may have a higher risk than men to develop AD (Launer et al., 1999). AD is generally a disease of the elderly and the risk of developing AD increases steadily after the age of 65 and finally about 50% of the over 85 year olds are affected. However, apart from age, the aetiology of AD may be related to genetics (e.g. first degree relatives have a higher lifetime incidence), long-term treatment with anti-inflammatory agents (e.g. including nonsteroidal anti-inflammatory drugs for rheumatoid arthritis), head trauma (neurological deterioration e.g. caused by boxing), low education level, vascular disease, environmental factors and gender (Launer et al., 1999; Castellani et al., 2010).

It is true that a larger proportion of patients suffering from AD are women and that may be indicative of an influence of gender on the AD risk. On the other hand, it is also known that women live longer than men (WHO, 2009). It has been suggested that the influence of age overlaps with the gender influence and distorts the importance the gender has on the likelihood to develop AD. However, it also has been shown that the withdrawal of estrogens, which all women experience after the menopause, has consequences on the brain (Alberca et al., 2002) and this may increase the risk of postmenopausal women to develop AD.

AD is a progressive neurodegenerative disease. The term neurodegenerative disease is used to classify various conditions arising from chronic breakdown and deterioration of neurons. This effect occurs especially in the central nervous system (CNS). In addition to the damage to the neurons, aggregated proteins were found to accumulate in the CNS, which cause further dysfunctions. AD is a specific form of dementia and approximately 70% of all dementia cases are classified as AD. It is characterised by cognitive dysfunction, especially memory loss, behaviour or personality changes, and impairments in the performance of activities of daily living (Castellani et al., 2010).
The key features of neuropathology are neuritic plaques, extracellular accumulation of beta-amyloid peptides (Aβ) and neurofibrillary tangles. The neurofibrillary tangles, which are intracellular depositions, resulting from aggregations of the hyperphosphorylated microtubular protein tau, are the major consensus criteria for the diagnosis of AD at autopsy. The consequence of the extracellular accumulation of the polypeptide Aβ is an amyloid tissue, which is often constituted of the highly neurotoxic isoform Aβ42, which forms insoluble plaques. These plaques have been found in patients suffering from AD. Today there are still gaps in the knowledge about the exact neuropathology of AD and identification of risk factors is still not well understood (Alberca et al., 2002; Castellani et al., 2010).

Estrogens can influence brain-related neurons via regulation of transcription of related genes and by altering neuronal functions. The effects of estrogens have been shown to be beneficial on the nervous system by enhancing the cerebral blood flow, protecting neurons against oxidative stress and preventing atrophy of cholinergic neurons (Castellani et al., 2010). Cognition in general is mediated through neurotransmitters, specifically acetylcholine (ACh), 5-HT and estrogens. It has been shown that estrogens influence the cholinergic system in the basal forebrain, which influences the cerebral cortex and the hippocampus. Estrogens modulate the expression of cholin acetyltransferase (ChAT), which regulates the synthesis of ACh. Further, estrogens indirectly influence the release of ACh through 5HT1A receptors in the frontal cortex. This effect has been observed in rats obtaining long-term estrogen replacement. The neurotransmitter 5-HT itself plays a role in regulating functions, which affect the working memory, the attentional set shifting (directing the attention from a particular focus to another) and learning (Aloysi et al., 2006).

The question as to whether the withdrawal of endocrine hormones, especially estrogens after the menopause, might be responsible for an increased risk for women to develop AD, has been addressed indirectly in various studies focusing on the effect of hormone replacement therapy (HRT) on AD risk (Shumaker et al., 1998; Resnick et al., 2004). The influence of HRT on AD risk in menopausal women is described in detail in the following section 1.3.1, Hormone replacement therapy.

In conclusion, the influence of the postmenopausal hormone levels on the neurodegenerative process, which underlies the development of AD, needs further clarification. Also, whether there are more women with AD just because older women outnumber older men, and whether cognition might be affected by other symptoms,
like depression, during the menopausal stages, has to be shown. Overall, it is apparent that postmenopausal women are highly affected by AD. Whether one condition might worsen the other is unclear. However, there is a great need for AD treatment options, which ideally would minimise any gender influence on the development of AD.

1.3. Treatment of menopausal symptoms with focus on hot flushes

The diversity of the menopausal symptoms, and hence the diverse needs of the patients, makes the treatment of menopausal symptoms a very complex matter. Hot flushes have been rated as the most distressing and bothersome menopausal symptom and hence it is the most common reason why women seek treatment. Other reasons are urogenital atrophy (e.g. vaginal dryness, sexual dysfunction and infections of the urinary tract), mood swings and sleep disturbances. In addition to the symptoms with acute effects, long term health consequences like osteoporosis, AD and cardiovascular diseases are often considered when the treatment is chosen (Davis and Jane, 2010). The general attitude towards the treatment of menopausal symptoms has changed radically during the last few years. The greatest impact on the discussion was from the Woman's Health Initiative (WHI) study, which found no benefits of HRT on coronary heart disease (CHD) development and, on the contrary, suggested an increased risk of developing breast cancer. It was published in 2002 and was one of the reasons why the benefits of HRT are now highly questioned (Rossouw et al., 2002).

The main focus of the treatment shifted from the classical HRT towards alternatives, which include phytoestrogens, selective serotonin re-uptake inhibitors (SSRIs), clonidine, herbal medicinal products (HMPs) and non-pharmacological options. The general measure of a successful treatment is the ratio of the benefits and the risks of the treatment options (Carroll, 2006). This section will briefly review the commonly used treatments for menopausal symptoms and their benefits, especially in respect of hot flushes and AD risk, as well as the less common treatments options. The risks of the different treatment options, on the other hand, will be reviewed separately in passage 1.4, Risks of the treatments of menopausal symptoms.

1.3.1. Hormone replacement therapy

The quality of life of women during the menopausal transition and postmenopausal stages can be severely compromised and an improvement of this quality can be achieved with HRT (NAMS, 2010). HRT involves therapy with estrogens (ET) and combined
estrogen-progesterone therapy (EPT), but where possible, the generalised term HRT will be used in this thesis.

HRT is widely used as a standard therapy for menopausal symptoms (Pines et al., 2008). Replacement of the ceasing endocrine hormones should prevent health risks of ageing women during the different menopausal stages. Initially, prevention of CHD was the desired benefit from HRT. This has changed when, in 1998, the Heart and Estrogen/Progestin Replacement Study (HERS) failed to confirm prevention of CHD (Hulley et al., 1998). Four years later, the WHI study confirmed the lack of this beneficial effect on CHD development (Rossouw et al., 2002). Since then, many more studies have been conducted, reports, statements and guidelines published, and the advantages of HRT have been assessed from various angles.

HRT should be considered in respect of the treatment goals, and benefits and risks for the individual woman. It is still the most effective treatment for hot flushes, vulvar and vaginal atrophy [vaginal dryness and dyspareunia (painful sexual intercourse, due to medical or psychological causes)]. HRT helps to restore sexual function and, by helping with all these issues, the general quality of life and well-being (NAMS, 2010). Some studies showed an increased risk for major and minor depression during peri- and early postmenopause (Halbreich and Kahn, 2001). An improvement of mood and a stabilisation of mood can usually be observed during HRT. However, HRT itself cannot be considered as an antidepressant (Osterlund, 2010) and the mood improvement might be due to amelioration of the other life-quality affecting symptoms experienced during the menopausal stages.

Today HRT is not recommended for prevention of CHD anymore, although studies observed a trend that HRT reduces the CHD risk in younger patients during perimenopause and early postmenopause. HRT has been shown to be effective in the prevention of all osteoporosis-related fractures, but is not approved for osteoporosis treatment. In the same way, a beneficial effect on reduction of the onset of Type 2-Diabetes (non-insulin dependent Diabetes Mellitus) was observed, but whether that might be due to a reduction of centripetal weight gain, reduced insulin resistance or other factors, is unknown.

HRT still remains the gold standard therapy for hot flushes. In 2001 a meta-analysis of 21 randomised clinical trials (RCT) involving 2511 women found that HRT significantly reduced the frequency (by 77%) and severity (by 87%) of hot flushes relative to placebo (MacLennan et al., 2001). Even though a 51% reduction of hot flush frequency, found
in the placebo groups, highlights that the assessment of the efficacy of anti-hot flush treatment options has its difficulties, no other treatment option showed equally good results.

The effect of HRT on cognitive function and the risk to develop AD appears to be negligible. Although comparison of the undertaken studies is difficult because of manifold methodological differences, like cognitive outcome measures, dose of HRT, prior estrogen used and type of menopause (natural or surgical), they all displayed the same trends. ET did not enhance cognitive test performance of young postmenopausal women (< 65 years), who were not encumbered with menopausal symptoms. On the other hand, the performance of women experiencing menopausal symptoms did improve in cognitive tests. Whether the improvement might result from repressing other disturbing menopausal symptoms, like hot flushes and mood swings, is not clear (Aloysi et al., 2006).

Further, the Women's Health Initiative Memory study (WHIMS), an ancillary study to the WHI, which enrolled 7510 postmenopausal women over the age of 65 and investigated the benefits of either EPT or ET on cognition and the risk of developing AD, found slightly more cases of dementia in the treatment groups than in the corresponding placebo groups (Shumaker et al., 1998). Hence, the WHIMS findings do not support the use of HRT to reduce the risk of AD in women. The results of the study were generally disappointing with regard to the effect of HRT on cognitive ageing and dementia. It had little effect on memory performance and it improved neither memory nor cognitive abilities. On the contrary the WHIMS observed an increased risk for dementia with HRT.

The other published studies, which indicated a reduction of the risk to develop AD, often involved younger patients, who already have a lower risk compared to the ones that took part in the WHIMS (>65 years) (Craig et al., 2005).

The overall benefits of HRT are the amelioration of bothersome menopausal symptoms such as hot flushes, and the preventative effects against fractures and heart disease for younger women before entering the menopausal transition, if the HRT is properly timed.

1.3.2. Phytoestrogens

Phytoestrogens are plant-derived compounds, which act like endogenous estrogens (Fig. 1.6). Mixtures of isolated phytoestrogens or complex HMPs made from phytoestrogen containing plants are widely used to treat menopausal symptoms (Carroll, 2006;
Reinhard-Hennch et al., 2006). They gained great popularity when patients and doctors sought alternative treatment options to HRT after the results of WHI study were published in 2002 (see section 1.4 Risk of the treatments of menopausal symptoms). Another trend is the rising desire of women for a more “natural” treatment, which is often assumed to be milder or have less adverse effects (Ososki and Kennelly, 2003). Most of the phytoestrogens share a few structural ‘key features’ with E2, which enable the compound to bind to the estrogen receptor (ER). Distinctive structural features of ER ligands are an aromatic ring and a hydroxyl group, as well as a planar hydrophobic scaffold, plus a second in-plane hydroxyl group within a specific distance (Pike et al., 2000). Other characteristics influence the binding affinity of a compound for the ER (Brzozowski et al., 1997; Seo et al., 2006). Although some estrogenic compounds do not have any of the described features, they are very common features within the group of the phytoestrogens.

Phytoestrogens have been divided into several classes. The phenolic phytoestrogens, like isoflavones, coumestans and lignans, are the most abundant and best-studied compounds. Other classes include the anthraquinones, chalcones, flavones, prenylflavonoids and saponins (Ososki and Kennelly, 2003). Most intensively studied are among the isoflavones, the aglycones, daidzein and genistein and their glycosides, daidzin and genistin. Biochanin A and formononetin are 4'-methyl ethers of daidzein and genistein. They are soy-derived, like the isoflavone glycine (Fig. 1.6). In plants they usually occur as glycosides, but they easily degrade to their aglyconic form, via enzymatic reaction.

![Chemical structures of endocrine hormones and isoflavones](image)

**Figure 1.6:** Chemical structures of endocrine hormones: 1 17-β-estradiol, 2 estriol and 3 estrone and isoflavones: 4 Genistein (R=H), 4 Biochanin A (R=CH₃), 5 Daidzein (R=H), 5 Formononetin (R=CH₃) and 6 Glycitein.
A good source of isoflavones is the Fabaceae family, with soya beans (*Glycine max* L.), peanut (*Arachis hypogaea* L.) and red clover (*Trifolium pratense* L.), but they can also be found in seeds and nuts from sunflower (*Helianthus annuus* L., Asteraceae), walnut (*Juglans nigra* L., Juglandaceae). Isoflavones are also common in the Iridaceae and the Euphorbiaceae families.

Phenolic compounds can also be found among the coumestans and their most prominent compound is coumestrol (Fig. 1.7), which is present in ladino clover (*Trifolium repens* L., Fabaceae), strawberry clover (*Trifolium fragiferum* L., Fabaceae) and alfalfa/lucerne (*Medicago sativa* L., Fabaceae). Within the class of the lignans the most well known compounds are secoisolariciresinol and matairesinol. The parent compounds do not seem to match the chemical characteristics of other phytoestrogens, but they are converted and bioactivated by human intestinal microflora into enterodiol and enterolactone, which exhibit estrogenic activity (Fig. 1.7).

Flaxseed (*Linum usitatissimum* L., Linaceae) is rich in secoisolariciresinol. Other sources of lignans are rye (*Secale cereale* L., Poaceae), oilseeds, or brewed green or black tea and coffee. They can be found generally in cereals, fruits and vegetables. However they have not been studied as well as the isoflavones or coumestans.

![Chemical structures of phytoestrogens from the classes of the coumestans and lignans.](image-url)

Figure 1.7: Chemical structures of phytoestrogens from the classes of the coumestans and lignans. 1 coumestrol, 2 secoisolariciresinol, 3 matairesinol and their bioactivated forms 4 enterodiol and 5 enterolactone.
One of the most potent phytoestrogens known today is the flavonoid 8-prenylnaringenin (Fig. 1.8). The phytoestrogenic activity found in hops (Humulus lupulus L., Cannabaceae) has been attributed to the 8-prenylnaringenin and its related compounds, xanthumol and iso-xanthumol. Resveratrol (Fig. 1.8), a stilbene, has also been found to bind to the ER. It usually functions as a phytoalexin and is found in a variety of plants, e.g. in the skin of grapes (Vitis vinifera L., Vitaceae) (Ososki and Kennelly, 2003).

![Chemical structures of the phytoestrogens 1 8-prenyl-naringenin and 2 resveratrol.](image)

One question arises immediately when phytoestrogens are discussed as an alternative treatment to HRT. If phytoestrogens act like endogenous estrogens, why should they be different, or better?

Phytoestrogens have the ability to bind to two kinds of ER, the ERα and the ERβ. The ERs differ in their distribution and their affinity to ligands (Pearce and Jordan, 2004). Where E2 shows similar affinity to both subtypes, many phytoestrogens have a higher binding affinity to ERβ than to ERα (Harris et al., 2005). The different tissue distribution of the two ER subtypes results in a tissue dependent effect of the phytoestrogens.

Another striking difference to the endogenous estrogens is the ability to exhibit antagonistic activity. Where endogenous estrogens act only agonistic on the ER, the phytoestrogens can exhibit both agonistic and antagonistic effects. The opposing actions of the ERs on gene transcription are related to the cell context and depend on tissue specific cofactors (Koehler et al., 2005). Phytoestrogens can trigger an estrogenic response e.g. on the uterus, and, at the same time, an anti-estrogenic response e.g. on the breast tissue. This diverse activity has been attributed to a class of non-steroidal compounds which still share structural similarities with E2, the selective estrogen
receptor modulators (SERMs). Hence, phytoestrogens are often classified as SERMs (Beck et al., 2005).

The anti-estrogenic effects are not necessarily ER related, e.g. competing with endogenous estrogens in the binding to the ER. Other effects which lower the availability of endogenous estrogens, such as an increased synthesis of estrogen-binding globulin, inhibition of the 17-β-hydroxysteroid dehydrogenase or an increase in the elimination of estrogens also result in a reduction of estrogenic effects (Shu et al., 2009).

The reported physiological effects of the phytoestrogens are manifold. Some of them also exhibit agonistic activity on the progesterone and androgen receptors.

Phytoestrogens especially attracted attention in recent years, when it was assumed that they may have protective effects against breast cancer and other ageing related symptoms like cardiovascular disease and osteoporosis (Ososki and Kenneley, 2003). This assumption is based on relatively low breast cancer incidence rates in women from East Asia, where soy, a source for phytoestrogens, is part of the daily diet. Many studies tried to determine the influence of lifestyle and diet on breast cancer development in Western and Asian women. After rats, which were treated with genistein during prepubertal or pubertal development, had a reduced incidence and/or multiplicity of chemically induced mammary tumors (Brown et al., 1998) it was suggested that the observed effects may be related to alterations in gene expression and morphological changes in the mammary glands. The possible influences of early-, pre-menopausal or post-menopausal exposure to phytoestrogens have been described and reviewed in several publications (for review see (Hooper et al., 2010; Nagata, 2010)).

However, no clear evidence for a breast cancer preventive effect of a phytoestrogen-rich diet has been found and some laboratory findings even indicate stimulatory effects of phytoestrogens on breast cancer development (Kortenkamp, 2007a). An increase in the bone mineral density, and therefore a reduced risk of developing osteoporosis and cardioprotective activity, are other reported beneficial effects of the phytoestrogens, which were found in menopausal women (Beck et al., 2005).

Although several studies have been conducted in order to show the ability of phytoestrogens to reduce the frequency and severity of hot flushes, the clinical evidence is controversial. Nelson et al. (2006) compared the results of 17 studies, conducted with red clover and soy extracts. They found that the frequency of hot flushes was not reduced with red clover extract and findings for soy extracts were contradictory.
Purified, soy-based isoflavones were also investigated for their effects on cognition. A follow-up analysis of the WHIMS resulted in contradictory results and more recent studies also show discrepancies. Some found a positive effect on cognition for postmenopausal women, whereas some failed to observe any beneficial effect. Unfortunately, the composition of the phytoestrogens used during the RCT were not standardised and therefore varying compositions of phytoestrogens may have influenced the results. No study with sufficient numbers of participants and a standardised composition has been conducted yet, thus consensus about the efficacy of phytoestrogen on cognition or protective effects against AD cannot be reached (Zhao and Brinton, 2007).

Phytoestrogens are still the focus of many in vitro and in vivo studies today. Hopefully these studies will shed more light on the question as to whether phytoestrogens are a safe and efficient, hence better, alternatives to the HRT.

1.3.3. Selective serotonin re-uptake inhibitors

Treatment of menopausal women with selective serotonin re-uptake inhibitors (SSRI) has become a recognised non-hormonal option against hot flushes. First clinical trials to assess the anti-hot flush effect were performed with breast cancer survivors (Carroll and Kelley, 2009), where treatment with estrogens is contraindicated (Loprinzi et al., 2002). Since then, many studies, including those with healthy menopausal women, have been conducted with promising outcomes.

The SSRIs belong to a relatively modern class of antidepressants and are grouped solely according to their mode of action and not by structural similarity. Paroxetine is the most studied SSRI for the relief of hot flushes. The other well-studied antidepressant is venlafaxin, a selective serotonin and noradrenalin re-uptake inhibitor (SSNRI). It inhibits the re-uptake of serotonin independently of the dose, whereas the noradrenalin re-uptake inhibition is directly related to the dose. Desvenlafaxine (SSNRI) as well as sertraline, fluoxetine and citalopram (all SSRIs) are second and third options, when neither paroxetine nor venlafaxine are tolerated. Other antidepressants were also used for anti-hot flush treatment, but the SSRI/SSNRIs were superior in clinical trials (Carroll and Kelley, 2009).

The endogenous neurotransmitter 5-HT is released in the central and peripheral nervous system. In order to prevent over-stimulation of synaptic receptors, it is cleared from the synaptic cleft by re-uptake via transporters, the 5-HTTs, which are centrally
located in the pre-synaptic neural membranes and in the periphery in plasma membranes of various tissues (e.g. pulmonary). The SSRIs bind directly to the 5-HTT and prevent the 5-HT re-uptake into the synapses, hence they increase the time the 5-HT remains in the synaptic cleft (Ramamoorthy et al., 1993). The theories as to why influencing the serotonin system may affect the frequency and severity of the hot flushes is described in paragraph 1.2.1.1: Physiology of hot flushes.

The anti-hot flush effect of the SSRIs is already observable in the first week of treatment, unlike the antidepressant effect, which is noticeable after 6-8 weeks. It suggests that the modes of actions are independent of each other.

The clinical trials assessing the effectiveness of SSRI/SSNRIs struggle with the same issues as the other studies assessing hot flushes - the perception of the hot flush, the duration of the study, and the prevalence of Caucasian women in the patient groups. Furthermore, the initial studies with SSRIs/SSNRIs only assessed their effects in breast cancer survivors and often tamoxifen, a SERM, was co-administered, which most likely affected the outcome of the studies.

Venlafaxin reduced the average number of daily hot flushes by 61% compared to placebo (27% reduction) after 4 weeks of treatment in women with a history of breast cancer (Loprinzi et al., 2000). In a randomized controlled trial of 6 weeks in comparison to medroxyprogesterone acetate (synthetic progestin) however, venlafaxine was not superior (reduction of 79% compared to 55% respectively) (Loprinzi et al., 2006). Another study assessed the reduction of hot flushes in a randomised controlled trial with 80 healthy menopausal women using either venlafaxine or placebo over 12 weeks. The reported reduction of hot flushes was 51% for the venlafaxine group and 15% in the group treated with placebo. In addition to the advantage that healthy women were monitored over 12 weeks, only 42% of the women were Caucasian in this study (Evans et al., 2005).

Studies with paroxetine were also initially conducted with breast cancer survivors, before studies like the one conducted by Stearns et al. (2003) showed a substantial reduction in the number of experienced hot flushes with paroxetine (~62%), compared to placebo (~37%) in women experiencing natural or surgical menopause. Another study showed a significant reduction of the number and severity of hot flushes with paroxetine relative to placebo. The participating 50 women had used HRT over 5 years, but discontinued it one year before entering the study (Soares et al., 2008). Generally, the design of the studies has to be taken into account, as various circumstances and events
such as the history of cancer or treatment, could have influenced the response. However, the SSRI have been shown to alleviate hot flushes and especially the SSNRI venlafaxine can be considered as a treatment option for breast cancer survivors and healthy menopausal women for alleviation of hot flushes (Carroll and Kelley, 2009).

The association of AD and 5-HT recently caught the attention of researchers. Serotonergic receptors densely populate the hippocampus and the link between 5-HT levels and the AD pathology is therefore being investigated. The extracellular 5-HT levels have also been correlated with memory performances, and beneficial effects of SSRIs on hippocampal plasticity and neurogenesis have been shown. SSRIs may be able to modify the disease through effects on amyloid plaque formation and hippocampal neurogenesis (Chow et al., 2007). Recently, Rozzini et al. (2010) examined whether a combined AD treatment with acetylcholinesterase (AChE) inhibitors e.g. the natural product galanthamine (see section 4.2 Plant derived inhibitors of the AChE), plus SSRIs, may have beneficial effects for depressive AD patients. They observed a slightly better outcome for the combined therapy, which may result from protection against the negative effects that depression has on cognition. In conclusion, the anti-hot flush effect of SSRIs have been demonstrated in many studies and SSRIs are therefore an alternative to HRT for hot flushes. Additional desirable effects on other menopause related risks and symptoms, however, have not been shown.

1.3.4. Other treatment options for amelioration of hot flushes

Women who turn to the 'other options', for treatment of menopausal symptoms are usually reluctant to take HRT or SSRIs because of potential side effects. The main aim is usually a reduction of the hot flush frequency and severity. The largest group of alternative treatment options are products based on plants.

The other treatments play a minor role and have limited supporting evidence. Examples of alternative anti-hot flush treatments are a belladonna/ergotamine tartrate/phenobarbital combination, gabapentin, mirtazapine, clonidine and Vitamin E. The belladonna/ergotamine tartrate/phenobarbital combination (Bellergal), containing low-dose phenobarbital, ergotamine tartrate, and levorotatory alkaloids of belladonna, decreased the number of hot flushes by 75%, versus a 68% reduction in the placebo group in a clinical trial (Bergmans et al., 1987). This small improvement was not a significant reduction of the hot flush frequency and suggested a limited efficacy for this agent. Often women withdrew before studies ended owing to a lack of response and to toxicity, which included dry mouth, dizziness, skin rash, and sleepiness. Given the
limited efficacy and the availability of safer therapies, the drug Bellerghal, which was a popular agent in the 1970s and 1980s, is not available on the market anymore (Loprinzi et al., 2005; Kimmick et al., 2010).

Gabapentin is structurally related to the neurotransmitter the γ-aminobutyric acid, and is usually used for neurological disorders, including epilepsy. After it was first recognised as a possible alternative treatment for hot flushes in 2000, when a reduced hot flush frequency was observed in 6 cases (Guttuso, 2000), many promising studies followed. The observed effects are promising for a dose of 900 mg gabapentin daily. However, the known side effects of gabapentin, e.g. dizziness, nausea and tremor, are problematic (Carroll, 2006; Pachman et al., 2010).

The well-known anti-hypertensive drug, clonidine, is a centrally acting α₂-adrenergic agonist. One proposed mechanism of action of clonidine is that it raises the sweating threshold by reducing norepinephrine release. However, the results from 10 clinical trials for the treatment of hot flushes were inconsistent (Nelson et al., 2006). Although half of the trials showed that clonidine was effective in treating hot flushes, the use of clonidine for hot flush treatment is not recommended because of the significant side effects, including mouth dryness, constipation, itchiness, and drowsiness, which many women experienced (Pachman et al., 2010).

Vitamin E (Tocopherol) was first used as anti-hot flush treatment in clinical trials in the 1940s (Finkler, 1949; Mc, 1949). In 1998, 120 women took part in a randomized, crossover, clinical trial receiving vitamin E (800 IU daily) for 4 weeks followed by 4 weeks of placebo or vice versa. Vitamin E showed a reduction of hot flush frequency of about 1 hot flush per day, but was not preferred over the placebo by the patients (Barton et al., 1998). Even though the concerns about the carcinogenic potential of Vitamin E are not supported by a recent meta-analysis (Bardia et al., 2008), it is currently not recommended because of its low efficacy (Pachman et al., 2010).

Probably the most commonly used alternatives amongst the HMPs include extracts from black cohosh {Cimicifuga racemosa L.) rhizomes/roots. It has been traditionally used for 'female complaints', which include menstrual problems and childbirth, by American Indians (Geller and Studee, 2005). Clinical trials reported efficacy in alleviating hot flushes and other vasomotor symptoms, but other studies failed to confirm the superiority of black cohosh over placebo (Huntley and Ernst, 2003). A recent randomised, double-blind clinical trial comparing the reduction of vasomotor symptoms by different treatments showed 34% reduction in the black cohosh group, 57% in the
red clover group, 63% in the placebo group, and 94% in the HRT group, after 12 months. Hence, the efficacy of black cohosh could not be shown (Geller et al., 2009). Initially it was thought to act like a phytoestrogen, but the estrogenic activity was not confirmed in further studies. Recently, serotonergic activity of black cohosh rhizomes/roots extracts has been shown in vitro and the activity was attributed to the N-methyl-serotonin, which was isolated from the ethanolic and methanolic rhizomes/roots extracts. Further investigations on the mechanism of action are needed to clarify the mode of action (Powell et al., 2008).

A standardised rhubarb (Rheum rhamnictum L.) root extract (ERr 731), containing hydroxystilbenes, raponticin and desoxyrhaponticin, as well as their aglycones rapontigenin and desoxyrhaponticin, is used in Germany for women with menopausal symptoms. It reduced menopausal symptoms, particularly hot flushes, in a long term RCT (96 weeks). It further activated ERβ in vitro, but did not exhibit estrogenic activity via ERα. ERβ activation may be the mode of action of rhubarb HMP, but other mechanism of actions, including the inhibition of aromatase CYP 19, are hypothesised and need further clarification (Hasper et al., 2009).

A clinical trial investigating the efficacy of a combination product containing 120 mg sage extract (Salvia officinalis L.) and 60 mg Alfalfa extract (Medicago sativa L.) in reducing the hot flush frequency, showed very promising results. Most of the women reported that hot flushes and night sweats completely disappeared and all women reported a reduction in the hot flush frequency (De Leo et al., 1998).

Another clinical trial assessing the efficacy against hot flushes of a sage tablet containing 51 mg of a 68% aqueous-ethanolic extract, included 71 patients (mean age of 57 years), which were menopausal for at least 12 month and experienced at least 5 hot flushes daily. The frequency of hot flushes was reduced by 50% within 4 weeks and by 64% after 8 weeks. Furthermore reduced the treatment with the sage tablet the intensity of the experienced hot flushes (Bommer et al., 2011).

The Chinese medicine Dong quai (Angelica sinensis L.) roots are described as a ‘female tonic’ and preparations have been used for health concerns such as menstrual pain and for regulating the menstrual cycle. Ethanolic extracts showed estrogentic activity in vitro, but despite this effect, products based on dong quai were not found to have beneficial effects superior to placebo in clinical trials. Hence, taken alone, dong quai does not appear to be beneficial for menopausal hot flushes (Geller and Studee, 2005; Reinhard-Hennch et al., 2006).
Topically applied products, based on wild yam (*Dioscorea villosa* L.), have also been tested for relief of menopausal symptoms, but efficacy was not proven (Reinhard-Hennch *et al.*, 2006). Evening primrose (*Oenothera biennis*) oil, extracted from the seeds of the evening primrose plant, showed no benefits in the relief of hot flushes in a randomised, double-blind, placebo-controlled study (Chenoy *et al.*, 1994). HMPs containing *Hypericum perforatum* L. or *Humulus lupulus* L. are used in addition because of their anti-depressive or mood stabilisation activities (Reinhard-Hennch *et al.*, 2006).

Behavioural intervention is a non-pharmacological option, which is often tried either to support the pharmacological treatment or by women who dislike the idea of treatment with drugs and fear the adverse effects. Relaxation and respiration training can be beneficial for women suffering from hot flushes. The relaxation may decrease the adrenergic tone in the central nervous system and might therefore present a non-pharmacological alternative. Most trials on the efficacy of relaxation techniques on hot flush frequency had positive results, where designed with small sample sizes and included a variety of interventions and outcome measures. Interventions such as hypnosis and acupuncture may be used to support other treatments, but in themselves did not reduce hot flush frequency (Pachman *et al.*, 2010).

Additional efforts to maintain a low body temperature with low room temperatures, loose-fitting clothes, cold drinks, avoidance of alcohol and spicy food (increase in peripheral vasodilatation) have been reported as beneficial. However, these effects have not been assessed in clinical trials.

Overall, the treatment options have to be considered for each women individually, but a holistic approach, pharmacological therapy and behavioural modifications, appears to be a sensible approach to ameliorate hot flushes, stress symptoms and general well-being (Shanafelt *et al.*, 2002).

### 1.4. Risk of the treatments of menopausal symptoms

Although the advantages of HRT, which is the "gold-standard"-therapy for hot flushes and other life-quality affecting menopausal symptoms, are numerous, it has gained a negative reputation over the last few years. A gold standard therapy is 'the best or most successful diagnostic or therapeutic modality for a condition' and new tests or results are compared against it. Since recent research pointed at some severe disadvantages of HRT, it is questionable whether HRT should remain gold standard.

The benefits and harms of HRT were the focus of many studies following the WHI study from 2002. The WHI study was a two-armed 5 years follow-up study assessing
risks and benefits of EPT compared to placebo in healthy menopausal women. The effect on CHD was assessed as the beneficial outcome of the study. The assessed risk, or adverse outcome, was the breast cancer incidence. The study had to be stopped after EPT led to an increased risk of breast cancer among the participating women and the observed risk outweighed the benefits. Overall, no beneficial effects on CHD were found, along with an increase in breast cancer incidence. However, a reduction in osteoporotic bone fractures and a reduced risk to develop colon cancer were observed as beneficial effects (Rossouw et al., 2002). The WHI also observed the effects of ET only treatment and found a reduced risk for breast cancer, which was difficult to explain (Stefanick et al., 2006).

The completion of the WHI overlapped with one of the largest observational studies looking at women who received mammography screening. The results of this Million Women Study, conducted in the UK, showed that ET and EPT increase the breast cancer risk (Beral, 2003). The results obtained from both studies had, and still have, a great impact on further decision-making in terms of the treatment options for menopausal women. Eight years and many more studies later, still no agreement about the risk-benefit ratio of HRT has been achieved.

The experts of the International Menopause Society (IMS) deplored the rushed release of the WHI results in 2002. In their view the results were over-interpreted and it failed to highlight the importance of the timing, the duration and the start of HRT. They highlight that HRT treatment in the early postmenopause (~50-57 years) does not increase CHD risk in healthy women and may even decrease the risk in this age group. Further to that, no significantly increased risk of breast cancer was found in women without prior use of HRT and the risk increased only by eight extra breast cancer cases per 10,000 women per year after 5 years of EPT. However, EPT might dose-dependently increase breast density, a risk factor for breast cancer development. ET or low dose regimen were not found to change density. They conclude a decreased risk of AD, when women initiated ET early in the menopausal transition, but find that clinical evidence for a cognitive benefit among women initiating HRT late in the postmenopausal period is absent (Pines et al., 2008).

The North American Menopause Society (NAMS) emphasises in their most recent position statement on HRT for postmenopausal women, the increased risk for venous thromboembolism and the five-fold increased risk of endometrial cancer, a risk that increases up to ten-fold when treatment is continued up to ten years. Concerning the
breast cancer risk, they agree with the IMS, that EPT-naive patients (no previous treatment with EPT) have no increased risk to develop breast cancer and that time and duration of treatment play an important role in the risk. They also speculate that EPT might promote pre-existing cancers, which are too small to be diagnosed, because of the ability to promote breast cell proliferation and mammographic density (NAMS, 2010).

The unexpectedly reduced breast cancer incidence found with ET in the WHI trial was evaluated in a meta-analysis of a large number of trials (Greiser et al., 2005). Re-evaluation confirmed the effect of ET on breast cancer development found in the Million Women study and dismisses the findings of the WHI as due to chance (Hulley and Grady, 2004).

An extensive summary of the understanding of the risks and benefits of HRT today, however, is beyond the scope of this introduction and would not serve the aim and focus of this thesis.

HRT should generally be used responsibly, which includes considering that a woman's menopause-related symptoms, and therefore her risk-benefit ratio, continually changes with her age. Hence, whether the impact of menopausal symptoms on the quality of life justifies HRT treatment needs always to be addressed.

With the alternative treatments, there are concerns that phytoestrogens may contribute to breast cancer development, by promoting latent breast cancers through their ability to activate ER. An in vivo study showed that the isoflavone genistein could stimulate the growth of estrogen-responsive mammary tumours in a mouse model (Allred et al., 2001). This may be indicative of the contributory effects of phytoestrogens on the development of estrogen-dependent breast cancer.

A recently conducted meta-analysis, considering 92 RCT, showed that the incidence of breast cancer was not significantly different between the groups taking either phytoestrogens or placebo. The authors addressed the problem of defining "the phytoestrogens" by only including substances with a defined amount of isoflavones, lignans, or coumestans (Tempfer et al., 2009). A meta-analysis of RCT observing the effect of isoflavones on breast density, showed no increased breast density in postmenopausal women after treatment with isoflavones. The effect on premenopausal women was inconclusive and larger trials are needed to verify the effects (Hooper et al., 2010).
Comparisons between the studies are generally complicated because of varying outcome measures and varying measures of exposure to phytoestrogens. Overall, published studies introduced a great deal of uncertainty and generalised clinical recommendations for phytoestrogens are not yet available (Kortenkamp, 2007a).

The cardiovascular risk during long term SSRI treatment for the alleviation of hot flushes was assessed in the WHI. An increased stroke risk, especially haemorrhagic and fatal strokes, and all-caused mortality was associated with SSRI treatment, but the absolute observed risk was low (Smoller et al., 2009). Adjuvant therapy with SSRI during breast cancer treatment with tamoxifen is problematic, as SSRIs inhibit cytochrome P450 enzyme (CYP) 2D6, the enzyme which converts tamoxifen into its most active form, endoxifen (Pinkerton et al., 2009). The SSRIs are generally considered as a safe treatment option. The occurrence of adverse effects like nausea, dizziness, undesirable appetite increase, fatigue, mouth dryness, vaginal spotting, nervousness, negative mood changes, libido, or vaginal dryness and their influence on the benefit from SSRI treatment needs to be assessed individually.

The alternative products, especially those classified as food supplements, are often advertised as safe and harmless alternatives to HRT. For many of the alternative treatment options, data about safety and efficacy are limited or missing, and active components are often unknown. Additionally, variation between the compositions of the HMP is high and the food supplements especially do not comply with any standardisation or quality control and hence the benefits and the risk are often up to chance (Carroll, 2006; Reinhard-Hennch et al., 2006).
1.5. The plant of interest: *Salvia officinalis*

*Salvia officinalis* is one of the most prominent species of the genus *Salvia*. The name *Salvia* is derived from *salvere* (Latin), which means to save (Grieve, 1980), indicating the use as a medicinal plant and existing knowledge about its curative properties. The genus *Salvia* is widespread over many countries throughout the world. *Salvia officinalis* itself, and preparations made from the species, are acknowledged in many pharmacopoeias, for example Sage leaf, Sage oil and Sage Tincture can be found in the British Pharmacopoeia (BP, 2011) and the European Pharmacopoeia (PhEur, 2010). The use of the plant in traditional medicine is still a reason for wide interest in pharmaceutical research. *Salvia officinalis* was found to have anti-hydrotic, anti-oxidant, anti-microbial and anti-viral activities, as well as anti-inflammatory, anti-spasmolytic, anti-hypertensive and hypoglycaemic activities (Hagers, 1994; Baricevic et al., 2001). Beneficial effects on the CNS (Baricevic and Bartol, 2000; Wichl, 2004) and estrogenic properties have also been reported for *Salvia officinalis* (Paris and Moyse, 1971; Bartram, 1995; Duke, 2002).

The second part of this introduction will deal with the major chemical constituents of the species *Salvia officinalis*, and very briefly some other *Salvia* species, and review the traditional use of the plant and current research.

1.5.1. The genus *Salvia*

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Lamiidae
Order: Lamiales
Family: Lamiaceae
Genus: *Salvia* L.

The genus *Salvia* is one of the largest in the Labiatae family. It consists of around 900 species. *Salvia* plants are common in subtopic and temperate regions, especially the Mediterranean area, occurring from sea level to elevations of 11,000 ft and more. Its distribution has reached central Europe today (Hagers, 1994). The *Salvia* species are annual, biennial or perennial herbs or subshrubs. They have simple pairs of leaves that
are usually velvety or hairy and the characteristic feature of the Labiatae family the square stems. Leaves are often rugose, entire, toothed and lobed (Hagers, 1994). The corollas can have a variety of shapes, but must have two lips of unequal length, of which the lower lip is usually spreading and the upper lip of variable shape. The calyx is also two-lipped and the upper lip may be three-toothed, two-toothed or undivided, where the lower lip is typically two-toothed. *Salvia* flowers always have two fertile stamens and sometimes two additional infertile stamens. The flowers are clustered in racemes; spike-like racemes, spikes and panicles are usually large. The typical colors are blue, red and sometimes white and yellow (Hagers, 1994; Clebsch, 2003).

The range of traditional medicinal applications of *Salvia* is exceptionally wide; among others, it has been used against fever, as a carminative, spasmolytic, an anti-septic, astringent, a gargle or mouthwash against inflammation of the mouth or throat, a wound-healing agent, and against rheumatism or mental and nervous conditions. *Salvia* plants are also used in commercial industries for flavouring food, in cosmetic formulations, aromatherapy and insecticides (Dweck, 2000).

Pharmaceutical research mainly focuses on the species *Salvia miltiorrhiza* (BUNGE), *Salvia lavandulifolia* (VAHL), *Salvia triloba* (L.fil), *Salvia divinorum* (EPLING et JàTIVA) and *Salvia officinalis* (L.) (Hagers, 1994). Large numbers of pharmacologically interesting secondary metabolites from various chemical groups have been isolated. These include phenolic acids (caffeic-, chlorogenic-, ellagic-, ferulic-, gallic- and rosmarinic acid), as well as flavonoids and various terpenoids, such as mono-, sesqui-, di- and triterpenoids. The main components of *Salvia* species are flavonoids and terpenoids. The flavonoids,
triterpenoids and the monoterpenoids of the volatile oil can be found in the aerial parts, where diterpenoids are the main compounds founding the roots (Ulubelen, 2000). The main monoterpenoids in the volatile oil are cineol, thujone, campher, linalool and linalyl acetate. Known diterpenoids are from the abietane-, quinone or clerodane-type and phenanthrene-quinone diterpenoids, with royleanone- or tanshinone-structure are taxonomically specific for *Salvia* roots. The most common triterpenoids are usually pentacyclic and from the oleanane- or ursane-type (Hagers, 1994).

### 1.5.2. The species *Salvia officinalis*

*Salvia officinalis* is a polymorphic species. Initially it was differentiated between the three subspecies (ssp.), *lavandulifolia*, *maior* and *minor*, but the differentiation became obsolete and the subspecies are now recognised as three independent species. The ssp. *minor* as *Salvia officinalis* L., the ssp. *major* as *Salvia grandifolia* (HTL.) and the ssp. *lavandulifolia* as *Salvia lavandulifolia* (VAHL.) (Wichtl, 2004). Common names in English are Common sage, Dalmatian sage, Garden sage and sage. *Salvia officinalis* is distributed in the Mediterranean area, especially around the Adriatic Sea, but also in north and Central Spain, the south of France and west of the Balkans. The area from Dalmatian to Southeast Serbia and Macedonia is the origin of distribution. Plant material for commerce is cultured mainly in southeastern Europe e.g. Albania or Bosnia (Wichtl, 2004).

*Salvia officinalis* is a branching herb or subshrub that reaches around 60 cm in height; the stems are typically squared, angled and covered with trichomes. The leaves of *Salvia officinalis* (*Salvia officinalis fohum/Sage leaves*) are set in pairs, velvety, pale green (upper surface) to greyish-green (lower surface), are oblong-lanceolate to ovate and up to 10 cm

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Figure 1.10: *Salvia officinalis* leaves 1 (Moran, 2008) and an eight-cell head Labiatae trichome 2 (Venkatachalam *et al.*, 1984).
long and 3 cm wide (Fig. 1.9), with a finely crenulated margin, apex acute and a rounded or tapering base, which is frequently lobed. The leaves are also covered with glandular trichomes on both sides. Some of these have a unicellular stalk and an eight-celled head, a typical type in the Labiatae family (Fig. 1.10). The leaves have a strong, aromatic odour and taste astringent and bitter (BHP, 1996). The flower are in whorls and purple with lipped corollas (Grieve, 1980). The root of \textit{Salvia officinalis} are rarely investigated or described.

1.6. Chemistry of \textit{Salvia officinalis}

The phytochemistry of \textit{Salvia officinalis} is often considered in relation to the other \textit{Salvia} species or other members of the Labiatae family, as their phytochemistry is similar. Flavonoids and terpenoids are the main components found in the \textit{Salvia} species and they are dominant in the aerial parts. It is furthermore very rich in essential oils and the chemistry of the volatile compounds, which are found in many species of the Labiatae family (Ulubelen, 2000), are well-studied (Giannouli and Kintzios, 2000a; Ben Farhat \textit{et al.}, 2009). The essential oil consists mainly of terpenoids. The characteristic components of the oil are monoterpenoids that are synthesised in the leaf trichomes (Giannouli and Kintzios, 2000a). On the other hand, much attention has been directed to the biologically active, water-soluble components found in the aerial parts of \textit{Salvia officinalis}. The polar polyphenols include flavonoid-glycosides and phenolic acids. The majority of the phenolic acids found in \textit{Salvia} species derive from hydroxycinnamic acid, the caffeic acid derivatives, and the rosmarinic acid, the dimer of the caffeic acid (Lu and Yeap Foo, 2002). This chapter will briefly review the phytochemistry of \textit{Salvia officinalis} and its best-known components.

**Hydroxycinnamic acid derivatives**: Rosmarinic acid, a caffeic acid dimer, is the most dominant hydroxycinnamic acid derivative found in \textit{Salvia officinalis}, but free caffeic acid can also be found. Trimers like salvacic acid K and sage coumarin (Fig. 1.11) and tetramers like sagerinic acid have also been isolated (Lu and Yeap Foo, 2002).
Sometimes these compounds are referred to as ‘Labiatae tannins’, because they comply in their behaviour with the genuine tannins like proanthocyanidines or gallotannins. Other, less dominant hydroxycinnamic acid derivatives of *Salvia officinalis* include derivatives of 6-feruloyl-glucose and hydroxycinnamic esters of disaccharides, such as 1-caffeoyl-(6’-apiosyl)-glucoside (PhEur, 2010).

**Flavonoids:** Flavonoids are common in leaves and flowers, where they function as UV protective agents. *Salvia officinalis* leaves contain about 1-3% flavonoids (PhEur, 2010). The majority of the flavonoids of *Salvia officinalis* are flavones, flavonols, their 6-hydroxy-derivatives and glycosides. The most prominent flavones are the derivatives of luteolin (5,7,3',4'-tetrahydroxyflavone) and apigenin (5,7,4'-tri hydroxyflavone), whereas the most prominent flavonols are the kaempferol (5,7,4' trihydr oxyflavonol), and quercetin (5,7,3',4' tetrahydroxyflavonol) derivatives. Both groups are common in their glycosidic form, the flavones are common as 7-glucosides or 7-glucuronides, whereas flavonols are exclusively those of the 3-glycoside derivatives (Lu and Yeap Foo, 2002). Common luteolin derivatives are the luteolin-7-O-glucoside (cinaroside), luteolin-7-O-glucuronide, and also the luteolin-3'-O-glucuronide. Other derivatives are the luteolin-7-methoxy, 3'-methoxy, 6-methoxy (nepetin) the luteolin-6,7 dimethyl ether- (cirsiliol) and the luteolin-6-hydroxy-7-glucoside (Fig. 1.12). Known apigenin derivatives are apigenin-7-O-glucoside (cosmoisin), apigenin-7-methyl ether (genkwanin), apigenin-7,4'-dimethyl ether, 6-hydroxy-apigenin (scutellarein), 6-methoxy-apigenin (hispidulin),...
apigenin-6,7-dimethyl ether (cirsimaritin) and 6-methoxy-apigenin-7-O-glucoside (homoplantagenin) (Fig. 1.13). The 8-hydroxyapigenin (isoscutellarin) is the only 8-hydroxyflavone that has been reported for *Salvia officinalis* so far.

![Chemical structures of flavones in *Salvia officinalis*.](image)

Figure 1.12: Common flavones found in *Salvia officinalis*. Luteolin derivatives. (Glc = Glucose, GluA = Glucuronic acid).

The 7-glucosides are very common, but also 7-glucuronides and several apigenin derivatives, glycosylated with two or more sugars (*e.g.* apigenin-7, 4' diglucoside), exist. Apigenin-6,8-di-C-glucoside (vincenin-2) is the only C-glycosidic derivative found in *Salvia officinalis* (Lu and Yeap Foo, 2002).

![Chemical structures of flavones in *Salvia officinalis*.](image)

Figure 1.13: Common flavones found in *Salvia officinalis*. Apigenin derivatives. (Glc = Glucose)
Terpenoid components of the volatile oil: The essential oils of *Salvia* are chemically very complex mixtures and *Salvia officinalis* is the species with the highest amount of essential oil within the genus. The flowering parts have the highest essential oils content, followed by the leaves, and only a small amount can be isolated from the stems (Perry *et al.*, 1999). The essential oil of the leaves of *Salvia officinalis* mainly consists of monoterpenoids and is exceptionally rich in α and β-thujone (35-50%, more α-thujone), where the essential oil of the flowers is rich in β-pinene.

Other major monoterpenes found in the essential oils are camphor, 1,8-cineol and borneol (Fig. 1.14.A), as well as α-pinene, β-pinene and bornyl acetate. They are important compounds for food flavouring, general health care and act as antioxidant agents (Giannouli and Kintzios, 2000b). The essential oil components α-, β-thujone and camphor are used to differentiate between *Salvia officinalis* and *Salvia fruticosa*, which can be commercially substituted. The essential oil composition of *Salvia officinalis* contains between 45-68% of these components compared to 4.8-15.9% in *Salvia fruticosa*. Further differentiation between the species is possible by the amount of 1.8-cineol, which varies between 2.8-23% and 55-75% respectively.

Other essential oil constituents are sesquiterpenes like the monocyclic α-humulene, the bicyclic β-caryophyllene and viridiflorol (Fig. 1.14.B) and diterpenes such as manool (Länger *et al.*, 1996; Perry *et al.*, 1999).

Figure 1.14: Major terpenoids and phenolic diterpenes of *Salvia officinalis*. A monoterpenoids: 1 α and 2 β-thujone, 3 camphor, 4 1,8-cineol and 5 borneol. B Sesquiterpenes: 1 α-humulene, 2 β-caryophyllene and 3 viridiflorol.
Diterpenes: Carnosic acid is the main phenolic, abietane-type diterpene isolated from the leaves of *Salvia officinalis*. It is fairly unstable and auto-oxidises to form lactones. The major product is the bitter-tasting carnosol, which itself further degrades to other phenolic diterpenes with lactone structures, for example rosmanol (Fig. 1.15) (Brieskorn, 1991; Schwarz and Ternes, 1992). Other phenolic diterpene derivatives are known e.g. 7-methyl carnosolate and rosmanol 7-methylether (Mathe et al., 2007).

![Carnosic acid, carnosol, and rosmanol](image)

Figure 1.15: Phenolic, abietane-type diterpenes: 1 carnosic acid, 2 carnosol, 3 rosmanol.

The abietane-type diterpenes are also the most common diterpenes found in the roots of *Salvia* (Fig. 1.16). The quinones royleanone, 7α-oxyroyleanone, 7α-acetoxyroyleanone horminone and 7-O-acetylhorminone, can be, amongst other derivatives, found in the roots of *Salvia officinalis* (Masterova et al., 1996; Spiridonov et al., 2003)

![Royleanone and horminone](image)

Figure 1.16: Abietane-type diterpenes found in the roots of *Salvia officinalis*: 1 royleanone and 2 horminone.
**Triterpenes:** The main triterpene occurring in *Salvia officinalis* is ursolic acid. Oleanolic acid (Fig. 1.17) and triterpene alcohols namely α- and β-amyrin are found to a lesser extent (Brieskorn and Kapadia, 1980).

![Figure 1.17: Common triterpenes found in *Salvia officinalis*: 1 ursolic acid and 2 oleanolic acid.](image)

**Miscellaneous natural products:** *Salvia officinalis* extracts contain various polysaccharides, e.g. arabinogalactans, high-molecular weight pectin and glucuronoxylan-related polysaccharides, which have been isolated from *Salvia officinalis* leaves (Capek et al., 2003). Benzoic acid derivatives, such as p-hydroxybenzoic, gentisic, syringic acids, all, and other acids are only present in small amounts (Schulz and Herrmann, 1980; Wang et al., 2000). The same is true for phytosterols like β-sitosterol and stigmasterol (Brieskorn and Kapadia, 1980; Masterova et al., 1989).

1.6.1. **The chemistry of other *Salvia* species**

Among the hundreds of *Salvia* species, some have been studied more extensively than others, and their active constituents were isolated. These species include *Salvia lavandulifolia* (Vahl), *Salvia miltiorrhiza* (Bunge) and *Salvia divinorum* (Epling et Jätiva). Their chemistry overlap in certain areas with the ones found in *Salvia officinalis*, but characteristic differences can be found. *Salvia lavandulifolia* (Spanish sage) is endemic to Spain, but also grows in South France (Hagers, 1994). Mostly studied is the essential oil of *Salvia lavandulifolia*. It is especially rich in 1,8-cineol and camphor (Fig. 1.18), but contains only small amounts of thujone, which distinguishes it well from the essential oil of *Salvia officinalis* (Lawrence et al., 1970). Unlike *Salvia officinalis*, the leaves contain no carnosic acid or carnosol (Hagers, 1994), but as in *Salvia officinalis* royleanone diterpenes are present in the roots (Patudin et al., 1974).
**Salvia miltiorrhiza** (Tanshen) is distributed in Japan and China (Hagers, 1994). The well-known chemical constituents of this species belong to the class of the tricyclic abietane diterpenoids, which can be found in the roots. Characteristic for *Salvia miltiorrhiza* are the compounds tanshinone I and II (Fig. 1.19), that contain a furane ring attached to the phenanthrene core (Kakisawa *et al.*, 1968). Several different phenanthrofuranochinone-type diterpenes, such as cryptotanshinone, isocryptotanshinone, isotanshinone I and II as well as ferruginol and hydroxytanshinone, are present in the roots (Tang and Eisenbrad, 1992).

The tricyclic abietane diterpenoids are the main diterpenes in most *Salvia* species. The American *Salvia* species are an exception. The bicyclic neoclerodane diterpene salvinorin A (also divinorin A) is the active ingredient of *Salvia divinorum*, which is native to Mexico (Hagers, 1994). Salvinorin A was isolated from the leaves of *Salvia divinorum* together with salvinorin B (also divinorin B) (Fig 1.19) (Ortega *et al.*, 1982; Valdes *et al.*, 1984).
1.7. Bioactivity of *Salvia officinalis*

*Salvia officinalis* has a long history as a traditional medicinal plant and is still used for several medicinal indications in current times. The bioactivity of *Salvia officinalis* preparations has been of interest in many previous studies conducted. This chapter will briefly review the traditional use and current use of *Salvia officinalis*. The reported bioactivities found in more recent studies will be the focus of this review as well as the characteristic biological activities of some other *Salvia* species.

**Traditional use as a medicinal plant:** *Salvia officinalis* has been known for its healing properties and considered as a cure-all since medieval times. Additionally, it has always been used as a culinary herb. Documents from one of the earliest medicinal schools, the medical school at Salerno, mention sage in an adage: salvia salvatrix, natura conciliatrix (sage, the saviour; nature, the conciliator) (Chevallier, 2001). Traditionally it has been used to treat flatulent dyspepsia, pharyngitis, uvulitis, stomatitis, gingivitis, glossitis, hyperhidrosis and galactorrhoea (Barnes *et al.*, 2007). The Précis de Matière Médicale from 1971 recognises infusions and extracts of *Salvia officinalis* for internal use as a tonic, with choleretic, anti-sweat and hypoglycaemic properties. Estrogenic properties are also suggested. Externally astringent and healing properties are reported (Paris and Moyse, 1971).

**Current use as medicinal plant:** Martindale recognises the carminative and astringent properties of *Salvia officinalis* and its use for respiratory-track disorders, as a mouthwash and gargle for disorders of the mouth and throat, as well as its use in homeopathic preparations (Reynolds, 1996). The therapeutic indication in the ESCOP monograph for tinctures, infusions or dried extracts of *Salviae officinalis folium*, are similar to those reported in the Martindale and include inflammations and infections of the mouth and throat such as stomatitis, gingivitis and pharyngitis (ESCOP, 2003). Current books on medicinal herbs or herbal medicines often list many other indications for *Salvia officinalis*. Amongst those are, for example, acne, alopecia, Alzheimer's disease, depression (Duke, 2002), menopausal hot flushes, reduction of high sugar levels in diabetes, and anxiety (Bartram, 1995). The following paragraphs will briefly review the available scientific data of some of the described biological properties.

**Anti-oxidative activity:** *Salvia officinalis* has strong anti-oxidative potential. Initially an aqueous-methanolic extract has been found to inhibit lipid peroxidation dose-dependently (Hohmann *et al.*, 1999). In the following years, the anti-oxidant activity of
Salvia officinalis has been related to its phenolic compounds and compounds such as caffeic acid, rosmarinic acid, carnosic acid and carnosol (Ben Farhat et al., 2009). The phenolic acids, salvianolic acids I, K and L, and some phenolic glycosides such as 6-O-caffeoyl-β-D-fructofuranosyl-α-D-glucopyranoside (Wang et al., 1999) have also been identified as anti-oxidative compounds (Lu and Yeap Foo, 2001a; Lu and Yeap Foo, 2001b).

**Anti-inflammatory activity:** Initially, ursolic acid and its isomer, oleanolic acid, were found to show anti-inflammatory properties and inhibit tumorigenesis in mouse skin (Huang et al., 1994). Later anti-inflammatory properties have been found in n-hexane and chloroform extracts of Salvia officinalis leaves. Both extracts demonstrated dose-dependent, topical anti-inflammatory properties by inhibiting the croton oil-induced ear oedema in mice. In this study the ursolic acid was identified as the main compound involved in the anti-inflammatory activity and it showed a two-fold higher activity than the reference drug indomethacin (Baricevic et al., 2001).

**Anti-viral and anti-microbial activity:** Aqueous extracts of Salvia officinalis were tested for their anti-viral potential and were shown to be effective against Herpes simplex virus (Nolkemper et al., 2006; Geuenich et al., 2008; Schnitzler et al., 2008). Anti-microbial activity against vancomycin-resistant enterococci was found for aqueous-acetone Salvia officinalis extract. The oleanolic acid and ursolic acid, which were isolated as active constituents, showed anti-microbial activity also against Streptococcus pneumoniae and methicillin-resistant Staphylococcus aureus (Horiuchi et al., 2007a). The diterpene carnosol showed a weak anti-microbial activity against the vancomycin-resistant enterococci, but reduced the minimal inhibitory concentration of several aminoglycosides and exhibited synergistic effects with the gentamicin (Horiuchi et al., 2007b). The anti-bacterial activity of the essential oil of Salvia officinalis was assessed on bacterial strains derived from 100 urine samples obtained from patients with urinary tract infection. The essential oils showed very good activity against Klebsiella, Enterobacter species, Escherichia coli, Proteus mirabilis and Morganella morgani (Pereira et al., 2004). The royleanone diterpenes in the roots of Salvia officinalis exhibit antimicrobial activity against gram-positive bacteria (Staphylococcus aureus) (Masterova et al., 1996). It has been suggested that biological membranes are the main target of the royleanones. Their hydrophobicity allows penetration through the phospholipid membranes. This, and the presence of chemical groups capable of donating and accepting protons, is the reason for their protonophoric properties (Spiridonov et al., 2003).
Effects on cognitive performance: *Salvia officinalis* has a reputation for being beneficial effects on cognitive performance. Although the anti-inflammatory, anti-oxidant and estrogenic properties may contribute to the beneficial effects, the influence on the cholinergic system is considered to be most influential (Howes *et al.*, 2003). Several clinical studies and *in vitro* investigations have been conducted to verify this presumption.

An amelioration of the clinical ratings and the cognitive symptomatology of AD has been observed in a RCT with AD patients receiving 45% ethanolic *Salvia officinalis* extract over 16 weeks (Akhondzadeh *et al.*, 2003). Dried leaves of *Salvia officinalis*, which were encapsulated for the study, were found to improve mood in terms of alertness and anxiety in young, healthy participants (mean age: 24.4 years) following the administration of a single oral dose (Kennedy *et al.*, 2006). Furthermore, healthy participants, with a mean age of 72.9 years, showed a dose dependent, better performance of the secondary memory, especially in the task of word recognition and recall, after they were given a single dose of dried 70% ethanolic *Salvia officinalis* extract instead of placebo (Scholey *et al.*, 2008).

This extract, and an 80% ethanolic extract obtained from the dried leaves used in the study by Kennedy *et al.* (2006), were also found to inhibit the AChE *in vitro* (Kennedy *et al.*, 2006; Scholey *et al.*, 2008) and the same effect was found for the essential oil of *Salvia officinalis* (Perry *et al.*, 1996).

So far only the essential oil constituents of the species *Salvia lavandulifolia*, which were also found to inhibit the AChE enzyme, have been tested individually for their AChE inhibitory activity *in vitro*. The AChE inhibitory potency found for the single, major monoterpenes was considerably less than the potency of the known plant derived inhibitors like physostigmine and the activity of the essential oil was not readily explained by the activity of the single compounds (Perry *et al.*, 2000c).

Anticonvulsant activity: The flavonoid hispidulin, isolated from *Salvia officinalis* by using activity-guided fractionation with a benzodiazepine-binding assay, was found to have modulating activity on recombinant GABA₆ receptors and to possess anticonvulsant activity as well. On the other side, the major essential oil component of *Salvia officinalis*, α-thujone, is a known GABA₆ receptor antagonist and it is thought to influence the GABA-enhancing effects of hispidulin. Hence, *Salvia officinalis* compounds, especially flavonoids, may well be useful as GABA₆ receptor agonists or modulators for anxiety and epilepsy management (Johnston and Beart, 2004).
**Estrogenic activity:** It is claimed in several books on herbal medicines that *Salvia officinalis* has estrogenic properties (Paris and Moyse, 1971; Bartram, 1995; Duke, 2002). Only one reference reported the estrogenic activity based on experimental data. The uterus of ovariectomised mice was increased in size after injection of a purified ethyl acetate and petrol ether extracts of *Salvia officinalis*. Further, the histology of the uterus was similar to those after estrogen injections. The authors concluded that *Salvia officinalis* extracts must contain estrogenic compounds (Kroszcynski and Bychowska, 1939). So far there is limited experimental data available using modern techniques, such as cell-based reporter gene assays, to assess estrogenic potency which could confirm and further investigate the estrogenic activity. Only experiments with an ethanolic extract of *Salvia lavandulifolia* and its essential oils were undertaken, which showed very weak estrogenic activity *in vitro* (Perry et al., 2001).

**Anti-hydrotic activity:** Anti-hydrotic effects of *Salvia officinalis* and its use as adjuvant therapy against night-sweats during menopausal transition have been acknowledged by sources from the 17th and 18th century (Th. Syndenham, 1624-1689 and G. van Swieten, 1700-1772) (Brieskorn, 1991). Nowadays *Salvia officinalis* products are still recognised by dermatologist as an adjuvant treatment option for patients suffering from hyperhidrosis (Togel et al., 2002). Studies with healthy participants or hyperhidrosis patients, which were undertaken between 1923 and 1940, confirmed the anti-sweat effect of *Salvia officinalis* aqueous preparations (Hagers, 1994) However, recent good clinical data is not available to confirm the reported activity.

The reputation led to the assumption that *Salvia officinalis* may be effective in menopausal women suffering from hot flushes. More than 50% of a group of 30 menopausal women (age 46-52), who took part in a clinical study either experienced a natural or surgical menopause, reported a complete disappearance of hot flushes after 3 months of therapy with tablets containing 120 mg of a dried *Salvia officinalis* and 60 mg of a dried *Medicago sativa* (Alfalfa) extract. Basically, all women reported a reduction in the hot flush frequency (De Leo et al., 1998). Interestingly, the number of women with surgical menopause was equal to the number of women who still experienced hot flushes to some extent during the study. That may indicate that the benefits of the treatment were influenced by the kind (natural or surgical) of menopause women experienced. However this study highlighted that the anti-hot flush activity of *Salvia officinalis* should be further investigated.
Other biological activities: Components of *Salvia officinalis* such as phenolics caffeic acid, salvianolic acids K and L, and the methyl ester of salvianolic acid I, showed pronounced anti-leishmanial activities against intracellular amastigote stages within RAW cells in an *in vitro* model for leishmaniasis. In addition the phenolics activated *Leishmania*-infected RAW cells for release of TNF without showing any cytotoxicity against the host cells (Radtke et al., 2003).

For a study investigating the hypoglycaemic effects of *Salvia officinalis*, streptozotocin-induced diabetic rats were treated with essential oils and a MeOH extract. Determination of the serum glucose levels revealed a hypoglycaemic effect of the extract, but not the essential oil (Eidi et al., 2005).

Furthermore, a MeOH extract was tested active against pancreatic lipase, an enzyme involved in lipid digestion. The *in vivo* study, with olive oil-loaded mice, assigned the inhibitory pancreatic lipase activity to the diterpenes carnosic acid and carnosol. Carnosic acid also significantly inhibited triglyceride elevation and reduced the gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice after 14 days (Ninomiya et al., 2004).

Water-soluble polysaccharides of *Salvia officinalis* were examined for their immunomodulatory activities using *in vitro* mitogenic and comitogenic rat thymocyte tests. The polysaccharides showed evidence of immunomodulatory properties with a particularly high adjuvant activity (Ebringerova et al., 2003). The polysaccharide complex composed of galactose (17.9%), 3-O-methyl-galactose (3.0%), glucose (15.5%), mannose (8.3%), arabinose (30.4%), xylose (7.6%), fucose (2.6%), rhamnose (6.7%), and uronic acids (8.0%) showed a broad molecular-mass distribution pattern (Mw approximately 2,000 — 93,000). After subjecting the complex to ion-exchange chromatography, three fractions showed potential as adjuvant properties mitogens and comitogens (Capek and Hribalova, 2004)

1.7.1. Bioactivities of other *Salvia* species

The main ‘traditional’ use of *Salvia divinorum* is as a vision-inducing mint used by the Mazatec people of Oaxaca in Mexico. Different consumption methods are practised. Either the fresh leaves are chewed as a quid and kept in the mouth, eaten, consumed as an aqueous infusion or even smoked (Valdes, 1994). The clerodane diterpene, salvinorin A, was found to be the main active ingredient of *Salvia divinorum* in *in vivo* studies with animals and humans. It is the first highly selective non-nitrogenous κ opioid receptor agonist and thought to be responsible for the hallucinogenic properties of *Salvia divinorum* (for review see (Grundmann et al., 2007)).
Besides the water-soluble phenolic acids identified in *Salvia miltiorrhiza*, which include caffeic acid monomers and oligomers, the diterpenes are of great pharmacological interest. The most abundant and major constituents among the tanshinones are the tanshinone I, tanshinone IIA and the cryptotanshinone. Most pharmacological studies have focused on these compounds and the activities found include antibacterial, antioxidant, anti-inflammatory, and antineoplastic effects. The phenolic acids in Tanshen, on the other hand, are known to exhibit antioxidant, anti-blood coagulation and cell protective effects. The phenolic acids responsible for the anti-oxidative effects were salvianolic acid A and salvianolic acid B and therefore different to the ones responsible for the anti-oxidative effect of *Salvia officinalis* (for review see (Wang et al., 2007)).

The essential oil of *Salvia lavandulifolia* has traditionally been used to treat memory-related disorders. It was shown that the essential oil exhibits an effect on AChE in vivo and proposed that the monoterpenoids are responsible for the effect (Perry et al., 2002). Furthermore, the essential oil of *Salvia lavandulifolia* exhibited antioxidant activity and estrogenic activity. The estrogenic activity was also detected in the ethanolic extract and the aqueous layer of the ethanolic extracts of *Salvia lavandulifolia* (Perry et al., 2001).

1.8. Evaluation of safety aspects considering interactions with the cytochrome P450 enzyme 3A4

Possible health and safety risks of HMP can have various causes. They can arise because of a contamination of the HMP, active compounds of the plant itself, or the cause can be consumer-dependent (e.g. patients may show hypersensitivity to certain plant derived compounds). In the case of the active constituents of the plant itself, toxic effects may be observed or the compound may affect the bioavailability, pharmacokinetic or pharmacodynamic of other co-administered drugs, causing herb-drug interactions (Halkes et al., 2009).

These interactions between HMP and conventional drugs are a matter of concern and the body of reliable information to assess the risk and the likelihood of interactions is not yet available (Williamson et al., 2009). In addition to that, food-drug interactions are being recognised as a possibility affecting the safety of an HMP.

In a recent study around 70% of the women who use alternative therapies for menopausal symptoms answered in an internet-based questionnaire that they did not consult their doctor and were not given advice when buying alternative therapies. Only 31% were aware of the possibility of drug interactions (Cumming et al., 2007). This behaviour may be a concern in itself, but it also results in under-reporting of
interactions. Hence, available information on the safety of HMPs are generally poor and there is a great need to investigate the potential for herb-drug or herb-food interactions of HMPs.

The cytochrome (CYP) 3A4 enzyme is the predominant CYP isoform expressed in liver cells and one of the key enzymes responsible for human drug oxidation of the majority of commercially available drugs. Overall it is thought to be responsible for about 50% of the human drug metabolism and may therefore well be the most important CYP enzyme for drug-drug interaction in general (Benet, 1996; Guengerich, 1997).

Thus, research about the effects of HMP on the CYP3A4 enzyme is of importance in order to make the effects of complex HMP more predictable and the products safer.
1.9. The aim of the current study

The aim of this thesis was to investigate the mode of action of a crude *Salvia officinalis* tincture for the amelioration of hot flushes and the determination of its active principle(s). *In vitro* assays for the identification of estrogenic potential (Wilson *et al*., 2004), acetylcholinesterase inhibition (AChEI) (Rhee *et al*., 2001) and selective serotonin re-uptake inhibition (SSRI) (Powell *et al*., 2008) were employed to gain insights into *Salvia officinalis*’ pharmacological potential.

A clinical study conducted on the *Salvia officinalis* tincture confirmed its potential to reduce the occurrence of hot flushes in menopausal women (Bioforce, 2005). *Salvia officinalis* is generally known to help alleviate excessive sweating (Togel *et al*., 2002), and its ability to assist with menopause-related symptoms has been acknowledged for many years (Bartram, 1995; Duke, 2002). Nevertheless, the mode of action of the anti-hot flush activity of *Salvia officinalis* has not previously been assessed and the multi-target approach was chosen to identify pharmacologically promising anti-hot flush activity.

A complete understanding of the physiology of hot flushes has not yet been achieved. A clear connection between hormones, neurotransmitters and the hot flush occurrence is still missing and an ideal treatment has not been found (Kronenberg, 2010). However, it is certain that the decrease in estrogen levels correlates with hot flushes (Weiss *et al*., 2004), and *in vivo* studies also identified correlations of estradiol and serotonin with the regulation of the core body temperature (Rybaczyk *et al*., 2005). Accordingly, treatments such as hormone replacement therapy (HRT) and SSRI anti-depressants are common. In order to identify the anti-hot flush mode of action of the *Salvia officinalis* tincture (Menosan®, Bioforce AG, Switzerland) its potential to exhibit estrogenic activity and serotonin re-uptake inhibition was investigated.

Additionally, women seem to have a higher risk of developing Alzheimer's disease (AD) (Launer *et al*., 1999; Aloysi *et al*., 2006). AD results from a deficiency in cholinergic activity in brain and the effects of estrogens have been shown to be beneficial to the nervous system and prevent atrophy of cholinergic neurons (Castellani *et al*., 2010). The symptoms of the early stages of AD can be treated by acetylcholinesterase (AChE) inhibitors. *Salvia officinalis* has previously been shown to be beneficial in AD treatment in clinical trials and an ethanolic extract and essential oils of *Salvia officinalis* were also found to inhibit the acetylcholinesterase (AChE) *in vitro* (Perry *et al*., 1996; Kennedy *et al*., 2006; Scholey *et al*., 2008).
Tests for the inhibitory potential against AChE of the *Salvia officinalis* tincture were included in this thesis to obtain more mechanistic insights and to elucidate whether *Salvia officinalis* may be beneficial for multiple menopause-related issues.

HMPs are commonly used in self-medication and they should therefore meet exceptionally high standards of safety and risk assessment. As a key enzyme in human drug metabolism, the CYP3A4 isoform is also responsible for most drug-interactions known today and a risk assessment should therefore include their CYP3A4 enzyme inhibitory activity (Guengerich, 1997). An *in vitro* assay using recombinant CYP3A4 enzymes was employed (Modarai et al., 2007) to identify inhibition of CYP3A4 activity by the *Salvia officinalis* tincture. The investigation gives insights into a potential of the tincture to cause herb-drug and herb-food interactions.

After the initial mechanistic studies the significant biological activity was followed by activity-guided fractionation and structure elucidation of the active constituent(s) of the crude *Salvia officinalis* tincture.

In summary, this study was designed to give answers about the anti-hot flush mode(s) of action and the responsible bioactive constituent(s) of a *Salvia officinalis* tincture, which ameliorates hot flushes in menopausal women, as well as its safety assessment regarding the inhibition of CYP3A4.
2. Material and Methods

2.1. The *Salvia officinalis* tincture

The HMP Menosan® (batch 026 141) was provided by Bioforce, Switzerland. It was obtained by maceration of the fresh cut *Salvia officinalis* folium in 60% EtOH over a time period of >10 days, with intermittent stirring throughout. The drug extracting agent ratio was 1:17. The tincture was decanted, pressed off and filtered at the end of the maceration period. Relative density and ethanol content were found to be 0.9 and 66%, respectively. Dry residue was 1.7% (m/m), essential oil fraction yielded 90 mg/100g. Homoplantaginin content was 51 mg/100g and thujone content was 18 mg/100g of α-thujone and 3.2 mg/100g of β-thujone (Bioforce, 2008). The *Salvia officinalis* tincture was stored at 4 °C in the dark and it is referred to as crude *Salvia officinalis* extract throughout this thesis.

2.1.1. Liquid-liquid partitioning of the crude extract

First, a very simple and crude liquid-liquid partitioning was carried out, a step often used as preliminary fractionation. The crude ethanolic extract was partitioned against more lipophilic solvents (n-hexane and CHCl₃) and the partition model assumes that the two phases are mutually immiscible (n-hexane and 66% ethanolic solution or CHCl₃ and ethanolic solution in the presence of adequate volumes of water). Hydrophilic compounds remain in the aqueous-ethanolic layer and lipophilic compounds partition according their polarity into the organic layers (Heinrich *et al.*, 2004).

**Protocol for liquid-liquid partitioning**

The crude *Salvia officinalis* extract was partitioned against the same amount of n-hexane three times, by continuous shaking for 5 min (Fig. 2.1). The resulting n-hexane layer (n-hexane subextract) was separated from the aqueous-ethanolic layer (here referred to as aq-EtOH phase) in a separation funnel and evaporated to dryness under vacuum. The remaining aq-EtOH phase was partitioned three times against equal volumes of CHCl₃ in the same manner and both layers (chloroform and aq-EtOH subextract) were separated and evaporated to dryness. The aq-EtOH subextract was re-dissolved in water and freeze-dried subsequently to ensure removal of any solvent residues. Tert-butyl alcohol was used to ensure complete solubility of the extract in water. Extracts were stored at -20 °C in the dark.
2.2. Activity-guided fractionation

The aim of activity-guided fractionation is to isolate biologically active compounds from a natural source. After demonstration of a biological activity, extracts are fractionated by multistep chromatography. After each step, the biological activity of choice guides the fractionation process and those fractions that retain the activity are carried through further fractionations until the isolation of an active constituent is achieved (Hostettmann, 1999).

The extract, which was subjected to activity-guided fractionation and biological activity used are described in chapter 7 and the basic methods used are described here.

For the fractionation and isolation, vacuum liquid chromatography (VLC) and size exclusion column chromatography were used. The techniques were chosen depending on polarity and complexity of the fractions and judged by preliminary thin layer chromatography (TLC) and proton nuclear magnetic resonance spectroscopy (¹H-NMR) studies. The methods used for structure elucidation and compound identification include NMR spectroscopy, mass spectroscopy (MS), high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectroscopy (LC-MS) and Capillary (cap) NMR.

2.2.1. Chromatographic methods

Chromatography includes a number of techniques, which rely on the distribution of a compound between two phases, a mobile and an immobile (stationary) phase. The principle of the separation is based on the chemical differences of the compounds and
their different adsorption and/or partitioning between the phases. Compounds can be separated by their polarity, charge, size and specific affinity (Cannell, 1998b).

Column chromatography is a widely used method. It can be used for preparative or analytical purposes. Preparative chromatography tends to use larger quantities of a sample, with the aim of separating and isolating components for purposes such as structure elucidation. Analytical chromatography uses smaller quantities of a sample, allowing qualitative and quantitative analysis of various components in the sample. It is also used as pilot method for semi-preparative or preparative HPLC and for the determination of purity of the isolated components.

The quality of separation of compounds depends mainly on how long the compound remains in the stationary phase. The longer a component remains in the stationary phase, the longer it remains in the column. Other components, which partition better in the mobile phase than in the stationary phase spend a longer time moving down along the column. Therefore the partitioning of the component between the mobile and the stationary phase determines how long a component remains on the column or at what speed the component travels through the stationary phase (retention time).

The partitioning of the component between mobile phase and stationary phase, i.e. its chemical properties, determines the retention time specific for the component. This allows comparison between components. However, the retention time also depends on the nature of the chromatographic material used, its particle size and the packing (Cannell, 1998b).

In this thesis reversed phase silica gel (RP-18) was used as stationary phase for VLC and Sephadex LH-20 was used for size exclusion chromatography.

Silica gel is a three-dimensional polymer of silicon dioxide tetrahydral units. Its surface is covered with silanol groups (SiOH), which interact with polar compounds via dipol-dipol interactions, van der Waals dispersion forces or by forming hydrogen bonds. Hence the affinity of polar compounds to normal phase silica is high and compounds retain longer in the silica gel (stationary phase). RP-18 is a modified silica gel. Alkyl chains (octadecyl-: C-18 chains) are bonded covalently to the SiOH groups on the surface of the gel, which results in a higher affinity for non-polar compounds (Gibbons & Gray, 1998).

Sephadex is a hydrophilic beaded dextran that is cross-linked by glycerin-ether bonds. It is hydroxypropylated and therefore holds both hydrophilic and lipophilic characteristics and allows separation of both non-polar and polar compounds. When suitable solvents are added (e.g. chloroform (CHCl₃), MeOH/water) the sugar polymer swells and forms a gel matrix. The gel is loaded into a column and the ability of the gel matrix (a three
dimensional polymere) to form pores, is used to separate molecules of different size. The chromatography follows the principle of size exclusion (molecular range of 100-4000 Da for LH-20) and small molecules remain longer in the three-dimensional polymer than larger molecules. This is because small molecules can easily enter the pores and remain on the column where larger molecules, which are excluded by their size, travel faster through the column. The solvents can influence the wet particle size, swelling ability and the exclusion limit of the pores. Due to the hydroxyl-groups of the dextran, which may interact with some of the compounds, a small part of the separation with the modified gels is according to polarity (via adsorption) (Salituro and Dufresne, 1998; Heinrich et al., 2004).

TLC, the classical planar chromatography, was used to choose suitable mobile phases for the column chromatography and to monitor the separation processes.

2.2.1.1. Thin layer chromatography

TLC is the most widely used form of liquid chromatography. Aluminium TLC plates coated with unmodified silica gel or glass plates coated with modified silica gel (reverse phase silica, RP-18) are common. The thickness of the adsorbent may vary, as 1-2 mm sorbent layers are used for preparative purposes, whereas for analytical purposes usually plates with 0.2 - 0.5 mm layers of adsorbent are used. The mobile phase moves vertically up through an open layer, the stationary phase (TLC plate), by capillary action. Separation of compounds in mixtures is based on differences in their migration speed through the plate. Migration distance per time depends on the adsorption of the specific molecule to the stationary phase. The stronger substances are adsorbed to the stationary phase, the slower they move. Such substances stay longer in the stationary phase and are therefore less frequent in the mobile phase, which moves upwards. The distance a substance migrates therefore depends on its dispensation between the two phases.

TLC plates often incorporate a fluorescent indicator (F254). Compounds absorbing short-wavelength ultra-violet (UV) light (UV254) appear as black spots on a green background due to their ability to quench the fluorescence of the indicator (F254). Under long UV light (UV366) several compounds may fluoresce, which can be indicative of the nature of several types of natural products. Non UV-active compounds can be visualized by using different staining reagents, which are applied to the plate after development (Heinrich et al., 2004). The compounds appear as coloured spots. The colours obtained with certain visualising reagents may be specific for certain classes of natural products. Vanillin/sulphuric acid is a universal spray reagent and not specific for
a certain class of compounds. More specific spray reagents are Dragendorff reagent, which allows detection of alkaloids, ninhydrin for detection of amino acids or Naturstoffreagent®—polyethylene glycol (NST-PEG) for flavonoids and other phenolic compounds.

TLC is a useful method, which gives information about the complexity of a mixture, the polarity of its constituents and may help classifying them by applying visualizing reagents. It is further a useful tool for the identification of the best solvent system for column chromatography.

**Protocol for analytical TLC**
All samples were dissolved in an appropriate solvent and loaded in similar concentrations as a small dot or slim band onto aluminium TLC plates coated with unmodified silica gel 60 F$_{254}$ (Merck, Germany) or onto glass plates coated with modified silica gel 60 RP-18 F$_{254}$ (Merck, Germany) 1 cm from the bottom edge of the plate.

Loaded TLC plates were placed into a glass TLC chamber. The chamber was saturated with an appropriate solvent mixture. The composition of the solvent mixture (mobile phase) depended on the nature and polarity of the extracts.

As soon as the mobile phase had travelled up to 1 cm from the upper edge of the TLC plate, the plate was removed from the chamber, dried and viewed under UV at short (254 nm) and long wavelengths (366 nm) (UV Cabinet, Camag). Spots that showed UV quenching activity under UV$_{254}$ were marked carefully with pencil on the left of the spot (\(\square\)). Spot that showed fluorescence at UV$_{366}$ were marked on the right (\(\square\)). The plate was sprayed with 4% vanillin (Sigma Aldrich, UK) in sulphuric acid (Sigma Aldrich, UK) and heated shortly (1 min) at 170°C.

Alternatively, for compounds exhibiting characteristic fluorescence, the NST-PEG was used instead. The plates were first sprayed with 1% (w/v) methanolic diphenylborinic acid 2-aminoethyl ester (Acros Organics, UK) followed by 5% (w/v) ethanolic polyethylene glycol 4000 grade (Fisher Scientific, UK), heated shortly and observed under UV$_{366}$ light. The developed TLC plates were scanned or photographed immediately after the application of the detection reagent.

**2.2.2. Principle of Vacuum-liquid chromatography**
VLC is a form of column chromatography where vacuum is applied to accelerate the flow-rate of the eluent. It is a quick and simple way for a coarse separation of complex mixtures and allows separation of fairly large amounts of sample within reasonable
time. In principle, various different stationary phases can be employed, such as normal phase silica, RP-18 silica and Al₂O₃ (Sticher, 2008), which are packed in columns with a sintered glass frit at the bottom. In this study, modified silica gel RP-18 was used for fractionation of the subextract.

**Protocol for VLC**

Reverse phase silica gel LiChroprep RP-18 (40-63 μm, Merck, Germany) was used for VLC. The column size, dimensions and the amount of RP-18 material used depended on the amount of extract/fraction to be separated. Columns were wet packed under vacuum with stationary phase in MeOH into a column with a sintered glass frit at the bottom (pore size 3) on the day of the chromatography. To ensure good separation the column was tightly and evenly packed without cracks or pockets of air. The packed column was conditioned in 25% steps to 100% water (25% MeOH/75% water, 50% MeOH/50% water, etc.). The fractions chosen for chromatography were dissolved in a minimal amount of water and carefully applied onto the column. Varying amounts of solvent and gradients from 100% water to 100% MeOH (Fisher Scientific, HPLC grade) were employed as eluents. A weak vacuum was applied from the bottom of the column and forced the mobile phase through the column. In order to ensure good separation, the column was allowed to run dry prior to addition of next solvent system.

Fractions obtained with the different solvents were analysed by TLC and ¹H-NMR analyses and combined when they seemed to consist of very similar compounds. They were dried under nitrogen and solvent residues were finally removed under vacuum at 30°C with a rotary evaporator. All fractions were stored at -20°C.

### 2.2.3. Principle of size exclusion chromatography

The basic size exclusion chromatography, also known as gel chromatography, utilises a cross-linked dextran. It is an excellent non-destructive method for separation of compounds of similar polarity and with a high recovery rate as compounds rarely adsorb (Heinrich et al., 2004). It is a chromatographic method driven only by gravity, as the gel has very poor mechanical stability (Hostettmann et al., 1998).

**Protocol for size-exclusion chromatography**

Sephadex LH-20 (GE Healthcare, UK) was soaked in MeOH and allowed to swell for at least 6 h before packing the column. Cotton wool was used to create a stopper at the
bottom of the column. In order to ensure that the column was evenly packed and air bubbles were prevented, the Sephadex LH-20 material was poured in one continuous motion into the column and allowed to settle.

The extracts chosen for size-exclusion chromatography were added on top of the column. They were dissolved in the solvent, which was used for the packing of the Sephadex LH-20 column. A flow rate of max. 1 ml/min was maintained throughout and fractions were collected in small amounts (5-20 ml), or if possible according to coloured bands. The similar fractions were pooled on the basis of TLC analysis. They were dried under nitrogen and solvent residues were finally removed under vacuum at 30°C with a rotary evaporator. All fractions were stored at -20 °C.

2.2.4. High pressure liquid chromatography

HPLC is a form of liquid chromatography, which operates at a high pressure to facilitate the flow of the mobile phase through a highly packed and thus relatively impermeable stationary phase (column). Depending on the amounts of sample to be separated it can be distinguished between analytical (separation and detection of microgram quantities), semi-preparative and preparative HPLC (separation of multi-milligram quantities). The high pressure speeds up the process and improves separation. The main difference between a preparative HPLC and a low-pressure column chromatography is the particle size in the column. Particles used in HPLC columns are generally more consistent and usually smaller (~5 μm) than those used for low-pressure chromatography. This smaller tightly packed sorbent requires the high pressure to force the solvent through the columns. The advantage of the small particle size and the consistency is a higher resolution, high selectivity, robustness, high stability and efficiency, which allows better separation of complex mixtures (Marston and Hostettmann, 2009).

HPLC is commonly coupled to UV or photodiode array detector systems. UV detection systems rely on chromophore groups present in the target compounds (e.g. double bonds, aromatic rings). Reverse phase (RP-18) columns have been successfully employed for the separation of polyphenolic and flavonoids, generally employing water/acetonitril or water/methanol mobile phases often containing acids such as formic acids and run either in gradient or isocratic elution modes.

Protocol for HPLC analysis

The purity of the samples was measured on a 1200 series quaternary liquid chromatography system (Agilent) consisting of a degasser (G1322A), quaternary pump (G1311A), autosampler (G1329A), fraction collector/autosampler thermostat
(G1330B), thermostatted column compartment (G1316A), diode array detector (G1315D), refractive index detector (G1362A) and analytical fraction collector (G1364C). The experiments were run and analysed using the software ChemStation (Agilent).

The sample to be analysed by HPLC was dissolved at a concentration of 1 mg/ml in an appropriate solvent, which nicely dissolved the sample (usually the eluent at t=0 min). The sample solution was filtered (pore size, Ø 0.45 µm) before it was loaded onto the RP-18 column (LUNA 5 µm C18 (2), 100 Å, 250 x 4.6 mm, Phenomenex, 00G-4252-E0). The injection volume was 10 µl and the flow rate was 1 ml/min. The eluent system used was a mixture of H₂O and acetonitrile (ACN) (Fisher Scientific, HPLC grade). Solvents were acidified with 1% formic acid, in order to facilitate separation, reduce peak tailing and prevent the deprotonation of the phenolic hydroxyl groups. Solvents were degassed by sonification for 15 min prior to their use.

HPLC analysis was run with a gradient, 0-5 min (96% H₂O), 5-15 min (85% H₂O), 15-25 min (50% H₂O) and 25-27 min (96% H₂O). Compounds were detected with UV at 254 nm and 360 nm.

2.3. Methods for structure elucidation and compound quantification

The process of the structure elucidation of the isolated natural product involves various spectroscopic methods, which can give valuable structural information about the chemical structure of the compound. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the classical methods, which were used in this study. In addition ultra-high-pressure liquid chromatography at elevated temperature coupled to time-of-flight mass spectrometry (UHPLC-TOF-MS) and capillary (cap) NMR analyses were used for identification and quantification of the chemical constituents in the minor fractions and parent extracts.

2.3.1. Nuclear magnetic resonance spectroscopy

NMR spectroscopy is still the most powerful method for the structure elucidation of organic molecules. Its great advantage lies in the possibility not only to determine the number of different nuclei in the structure, but also their chemical environment and their interconnection. NMR spectroscopy is based on the absorption of energy by a nucleus in a magnetic field. The absorption of the energy changes the energetic state of the nucleus and can be detected, amplified and recorded as resonance signal. Nuclei with a magnetic moment other than zero (e.g. ¹H, ¹³C, ¹⁵N) can be recorded and therefore used for structure elucidation (Günther, 1995). Further information about the
interconnection of the nuclei can be obtained in two-dimensional (2D) spectra by applying two separate radiofrequency pulses with different increments between the pulses. Several 2D correlation experiments, such as correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) are now widely employed in structure elucidation by NMR (Marston and Hostettmann, 2009). Ideally, the yield of the isolated compound should be more than 1 mg to allow structure elucidation using conventional NMR analysis (Hu et al., 2005) and have a purity of 95-100% (Cannell, 1998b). The capNMR has an increased sensitivity compared to the conventional NMR spectroscopy and $^1$H-spectra can be obtained with 5-10 μg of sample (Hu et al., 2005).

2.3.1.1. Proton NMR ($^1$H-NMR) spectroscopy

$^1$H-NMR spectroscopy records resonance signals for the protons in the molecule. The signals depend on the chemical environment of the proton and are expressed in parts per million (ppm), the chemical shift. Protons in a high electron environment are shielded and appear upfield at low ppm values, as a higher field strength is required to record the resonance of the nucleus. Deshielded protons, in low electron environments, appear respectively downfield at high ppm values where the strength of the field is lower.

The chemical shift of a proton can be characteristic for a certain functional group of molecule structure (methyl protons 0.7-1.5 ppm, aromatic protons 6-9 ppm, aldehyde protons 9-11 ppm). The area under the resonance signal corresponds with the number of protons that give rise to the signal and can be determined by integration of the signal ( Günther, 1995).

The multiplicity of the resonance signal gives further information about the adjacent protons and therefore the chemical environment of the proton. Signals appear as singlet, duplet, triplet, quartet or multiplet. The signals arise from spin-spin coupling, an interaction between the protons transmitted by bonding electrons through which they are connected. The split pattern is determined by $2nI+1$, where $n$ represents the number of adjacent equally coupling protons and $I$ the spin number. Spin-spin interactions between nuclei, and hence their position to each other, can be determined from the peak distance. $J$ values allow to distinguish between a axial or equatorial position of a proton in respect to a neighbouring proton or $\alpha, \beta$ configuration of the sugar moiety within a glycoside ( Harborne, 1993). The coupling-constant $J$ is independent from the magnetic field and is expressed as a frequency in Hertz (Hz) ( Günther, 1995).
2.3.1.2. Carbon NMR (\(^{13}\)C-NMR) spectroscopy

The \(^{13}\)C-NMR spectrum gives further information about the environment of chemical groups and the number of carbon nuclei, which form the framework of the molecule. The method is less sensitive than \(^1\)H-NMR spectroscopy. The abundant isotope \(^{12}\)C does not have a magnetic moment. The NMR spectroscopy is therefore limited to investigating the less abundant (~1.1%) \(^{13}\)C nucleus and its magnetic moment is four times lower than that of \(^1\)H protons. Fourier transform spectroscopy made a significant contribution to improve the signal to noise ratio of \(^{13}\)C-NMR by allowing to record a large number of spectra in a relatively short time and with smaller amounts of samples. \(^{13}\)C-NMR spectroscopy has since then developed into a standard method for structure elucidation. Furthermore, the signals of \(^{13}\)C-NMR spectra are easier to interpret than \(^1\)H-NMR spectra, because in the routine decoupled spectra \(^{13}\)C atoms do not show homonuclear coupling or heteronuclear coupling. The assignment of quaternary carbons that are part of functional groups (C=O, C=NR) often provides crucial information for structure elucidation, which can be obtained from \(^{13}\)C-NMR spectroscopy (Günther, 1995).

The Distortionless Enhancement by Polarisation Transfer (DEPT) sequences offer the advantage of detecting the carbon multiplicities. DEPT spectra are based on \(^{13}\)C signals, but by tilting the \(^1\)H pulse, the carbons can be assigned to methyl- (CH\(_3\)), methylene- (CH\(_2\)) or methine- (CH) groups. Quaternary carbons can not be observed. The DEPT90 spectrum only exhibits methine resonances, whereas the DEPT135 shows signals for protonated carbon atoms such as CH and CH\(_3\) as positives signals and CH\(_2\) as negative signal (Neri and Tringali, 2001).

2.3.1.3. Two-dimensional (2D) NMR methods

A 2D experiment is based on two axes displaying 1D NMR experiments. Cross-peaks between the signals can indicate which nuclei are spin-spin coupled. These correlations show as ‘spots’ on the 2D spectrum and are used to obtain structural information by correlating the chemicals shifts between \(^1\)H-\(^1\)H or \(^1\)H-\(^{13}\)C.

The proton correlation spectroscopy (COSY) is based on homonuclear coupling and correlates protons (\(^1\)H-\(^1\)H). The \(^1\)H-\(^1\)H correlations and coupling can occur through two (geminal coupling, J) or three bonds (vicinal coupling, J). COSY is a useful tool to elucidate partial structures of the molecule.
Heteronuclear 2D experiments (\(^1H\)-\(^13C\)) give information about the correlations between protons that are directly attached to a carbon. Assignment of the carbon signals observed in the \(^13C\)-NMR spectrum to the proton is based on their chemical shift correlations. These are linked by the coordinates of each cross-peak between the chemical shift scale of the proton and the chemical shift scale of the carbon. Most of these shift correlations are based on correlations over one bond, hence direct neighbours in the molecule and are recorded as heteronuclear multiple quantum coherence (HMQC) spectrum.

Long-range correlations can further give crucial information about \(^1H\)-\(^13C\) coupling over multiple bonds (two \(^2J\) and three-bonds \(^3J\) and therefore give final details about the structure. This heteronuclear multiple bond coherence (HMBC) analysis is a powerful tool for clear assignment of a chemical structure, as carbon-carbon correlations and correlations of quaternary carbons with nearby protons can be confirmed indirectly. It is used to elucidate the gross structure of a compound (Günther, 1995; Neri and Tringali, 2001).

2.3.1.4. Capillary NMR

CapNMR spectroscopy is a relatively new method with many advantages, which are especially useful for natural product research. The isolation of active constituent from a natural source is challenging and often the amounts of isolated biologically active compounds are very small. Conventional NMR spectroscopy often needs milligram amounts of the compound for a complete structure elucidation. CapNMR spectroscopy allows to record spectra with much small amounts such as 5-10 µg for \(^1H\) and COSY experiments and 30 µg or 70 µg for HMQC and HMBC respectively. The samples are injected into a capillary tube and the size of the flow cell is reduced so that the total injection volume is 5 µl (only 1.5 µl of active volume). This allows acquiring all necessary experiments for structure elucidation using only 50 µl of deuterated solvent altogether and without using NMR tubes. Another advantage is the reduced recording time. \(^1H\)-experiments can be recorded within 5 min, COSY in 1.5 h, HMQC in 5 h and HMBC experiments within 10-15 h (Hu et al., 2005). The disadvantage of capNMR methodology is that the machines are still very expensive and thus only few laboratories are able to afford one.
Protocol for NMR spectroscopy

$^1$H-NMR experiments on all extracts and fractions were run on an AVANCE 400 MHz spectrometer (Bruker) with 128 scans. The sample was dissolved in 600 µl chloroform-$d$ (CDCl$_3$) (Cambridge Isotopes Laboratories) or in 600 µl methanol-$d_4$ (MeOD) (Cambridge Isotopes Laboratories) and transferred into a NMR tube with 5 mm diameter and 17.8 cm length (Aldrich, Z276278). The Bruker software Topspin 1.3 was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak (MeOD: $\delta$ 3.31, $\delta$ 4.80 (residual water peak), CDCl$_3$: $\delta$ 7.27).

For the structure elucidation of an isolated compound its NMR spectra were run in (CD$_3$)$_2$SO (DMSO-$d_6$) (Cambridge Isotopes Laboratories). 1D experiments ($^1$H-NMR, $^{13}$C-NMR, DEPT90°, DEPT135° and 2D experiments (COSY, HMQC, HMBC) were performed on an AVANCE 500 MHz spectrometer (Bruker). Analysis of pure compounds by $^1$H-NMR was performed with 128 scans, for $^{13}$C NMR with 14,000 scans with a resonance frequency of 125 MHz and for DEPT90 with 7,000 scans. The COSY experiment was performed with 16 scans, HMQC with 32 scans and HMBC with 64 scans. The Mnova® (Mestrelab research, version 6, 2010) was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak of (CD$_3$)$_2$SO: $^1$H= $\delta$ 2.5 and $^{13}$C = $\delta$ 39.5.

CapNMR experiments were run on a Varian Unity Inova 500 MHz NMR instrument. It was equipped with a 5µl microflow capNMR probe from Protasis/MRM and an active volume of 2.5 µl. CapNMR experiments were performed in collaboration with Professor Jean-Luc Wolfender and his PhD student Laurence Marcourt from the University of Geneva, Switzerland.

2.3.2. Mass spectrometry

MS is a spectroscopic method, which can be used to determine the molecular weight of a compound and its fragments and to ascertain the exact number of atoms. The principle of MS depends on the production of charged ions in a gas-phase (vapourisation), which are then accelerated, deflected and detected. The ions are deflected by a magnetic field and separated according to their mass-charge ratio ($m/\zeta$); the lighter the ion or the more charge it carries, the more it is deflected. The ions are collected and the abundance of each ion species is detected (Hoffmann and Stroobant, 2007). Electrospray ionization (ESI) is a soft ionisation technique that creates singly charged ions and avoids fragmentation of the molecule. The samples are dispersed into small droplets from the electrically charged tip of the capillary, which is situated within the ionisation source. The following process of solvent evaporation, droplet contraction
and Coulombic explosions results in single charged molecule-ions. The charged ions are directed towards the analyser of the MS. Positive ionisation generates protonated ions \([\text{M+H}]^+\) or sodium adduct ions \([\text{M+Na}]^+\) and negative mode ionisation generates deprotonated ions \([\text{M-H}]^-\). The ionisation mode depends on the ability of the compound to readily accept or loose a proton (Hoffmann and Stroobant, 2007).

Tandem MS (MS/MS or MS2) uses collision-induced dissociation (CID) to achieve fragmentation of the molecule. The electrically charged molecules are accelerated and collision with neutral molecules (helium, nitrogen) results in bond breakage and the fragmentation of the molecular ion into fragments. Tandem MS can give useful information about the masses of sugar moieties and the corresponding aglycone as a fragment (Fabre et al., 2001).

**Protocol for MS**

Mass spectrometry measurements were performed by the analytical services of the School of Pharmacy.

Mass spectra were acquired using a LCQ mass spectrometer (Finnigan MAT) equipped with an electrospray ionization (ESI) source. The operating parameters were as follows: the spray needle voltage was set at 4.5 kV and the spray was stabilised with a nitrogen sheath gas (700 lb/in.²). ES capillary voltage was +30 V, helium was used as auxiliary gas (15 lb/in.²), and capillary temperature was 200 °C, collision energy 45% of 5 V for MS/MS. A syringe pump delivering 3 μL/min was used for the direct loop injections of pure standards dissolved in MeOH (about 0.3 μg/l). MS experiments were performed in negative mode. The negative MS² spectra were obtained with a CE-nano-ESI-(MS)² with 35 collision-induced dissociation (CID). The -ESI (low resolution) and accurate mass (high resolution) experiments were applied to isolated compounds.

High resolution accurate mass spectra were acquired using a Micromass Q-TOF Ultima Global Tandem Mass Spectrometer (Micromass). Samples were measured in positive mode under Electrospray ionization mode using 50% ACN in water and 0.1% formic acid as solvent.

**2.3.3. Liquid chromatography-mass spectrometry**

Coupling a chromatographic method with another analytical method has become a commonly applied powerful tool in characterisation of natural products. HPLC is the most commonly coupled with MS. The LC-MS is a very sensitive method, which can yield structural information about a molecule and about its molecular weight. LC/MS
interphases have to achieve vaporisation of the solvent and ionisation of the probe in order to allow analysis in the mass analyser (Hostettmann and Wolfender, 2001).

The packing material of the used columns affects the separation of the samples. The particle size of the packing material is a variable in the van Deemter equation, which describes the relationship between the flow rate (linear velocity) and the plate height (for column efficiency). The smaller the particle size the more efficient the separation. A newer development is the Ultra Performance Liquid Chromatography (UPLC), which describes LC utilising columns packed with chromatographic materials with a particle size < 2 μM. Higher flow rates are possible and results with the small particle size in an increased analysis speed and a better resolution and sensitivity (Swartz, 2005). With the sub-2 μM columns the analysis time can be shortened up to 9 times compared to systems with columns packed with a 5 μM particle size. The back-pressure also increases 9 times and UPLC analysis are therefore very high pressure analysis (up to 100 MPa) (Novakova et al., 2006).

Protocol UPLC-MS

LC–MS analyses were performed on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, Milford, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Waters, Milford, MA, USA). The detection was performed in positive ion mode in the \( m/z \) range 100–1000. The separations were carried out on Waters Acquity UPLC BEH C18 column (2.1mm × 150mm, 1.7 μm) at 40°C. The solvent system was A = 0.1% (v/v) formic acid–water, B = 0.1% (v/v) formic acid–ACN and gradient elution was performed at a flow rate of 0.4 ml /min using 2% B – 95% B in 30 min.

For quantification experiments a gradient elution on the UHPLC-TOF-MS system using a Waters Acquity BEH RP 18 shield 100 mm × 1.0 mm, 1.7 μm column or a Waters Acquity BEH RP 18 shield 50 mm × 2.1 mm, 1.7 μm column was carried out. The detection was performed in positive ion mode in the \( m/z \) range 100–1000. The solvent system was A = 0.1% (v/v) formic acid–water, B = 0.1% (v/v) formic acid–ACN and the gradient elution was performed at a flow rate of 0.74 ml /min using 10% B – 50% B in 10.0 min, at 40°C. During all experiments a UV trace 210-460 nm was recorded. UHPLC-TOF-MS analyses were performed in collaboration with Professor Jean-Luc Wolfender and his PhD student Philippe Eugster from the University of Geneva, Switzerland.
2.4. Biological methods

2.4.1. *In vitro* assays for the identification of the mechanism of action

The different methods to investigate the mechanism of action of the crude *Salvia officinalis* extract were chosen. The applied *in vitro* methods are briefly introduced in this chapter. Detailed information of each method is given in the respective chapters.

The *in vitro* assays had to meet a number of requirements. Assays should be suitable for herbal extracts, which includes that solubility and detection levels are in an acceptable range. Ideally the set-up of the assay should allow testing of dilution series with an appropriate effort in time and costs. The assay finally chosen for the activity-guided fractionation should meet all these requirements to a higher extent as number of fractions could increase necessary cost and time enormously.

The *Salvia officinalis* tincture was tested for inhibitory potential against CYP3A4 to provide important information to ensure the safety of the HMP. CYP3A4 is the most abundantly expressed CYP enzyme in the liver and is thought to be responsible for the metabolism of about 50% of the commonly used drugs (Benet, 1996; Li and Jurim-Romet, 1997). The inhibition of the purified CYP3A4 enzyme was assessed using cDNA expressed cytochrome P450 enzyme 3A4 in a fluorometric microtiter plate assay (Crespi et al., 1997; Crespi and Stresser, 2000). Details can be found in 4th CHAPTER: Evaluation of the Cytochrome P450 enzyme 3A4 inhibitory effects of *Salvia officinalis*.

The acetylcholinesterase (AChE) is currently the key enzyme in the treatment of AD. Several studies connected plants of the genus *Salvia* with a potential in maintaining cognitive functions (Perry et al., 2001; Akhondzadeh et al., 2003; Houghton et al., 2006). AD is an issue of importance especially for menopausal women. HRT has been found to influence cognitive functioning of menopausal women, but the underlying mechanism is not clear (Aloysi et al., 2006). In order to elucidate whether the beneficial activities of the crude *Salvia officinalis* extract may be linked by relying on the same active compound it was tested for its potential to inhibit AChE in a colorimetric assay. The assay is based on the well-known method described by Ellman (Ellman et al., 1961) and is carried out in a 96 well microtiter plate format. Details can be found in 5th CHAPTER: Inhibitory potential of *Salvia officinalis* against acetylcholinesterase (AChE) enzyme.

The selective serotonin re-uptake inhibition assay (SSRI assay) utilised human embryonic kidney cells (HEK-293 cells), which harbour the human serotonin
transporter (5-HTT). The assay was conducted in 24 well plates, where cells were treated with the extract. Re-uptake of tritiated 5-HT was monitored by scintillation counting (Rodriguez et al., 2003). The group of the SSRI antidepressants are commonly used to treat hot flushes (Soares et al., 2008; Freeman et al., 2011) and this assay was used to assess whether the *Salvia officinalis* extract exhibits SSRI-like effects, which might influence the serotonergic system. Details can be found in 6th CHAPTER: Evaluation of the serotonin re-uptake inhibitory potential of the crude *Salvia officinalis* extract.

Two breast cancer cell-based assays were used to test whether the *Salvia officinalis* tincture possesses estrogenic potency. Classical hormone replacement therapy acts through activation of ERs and is still the most effective treatment for hot flushes (NAMS, 2010). Two breast cancer cell lines (T47D-KBlux and MCF-7) were utilised in 96 well plate assays. The assays differ in outcome measure. The assay using the T47D-KBlux cells allowed quantification of estrogenic responses as a result of binding and activation of the estrogen receptor by a luminescence reaction product (ERLUX assay) (Wilson et al., 2004). MCF7 cells were utilised to measure estrogenic potency by cell proliferation (ESCREEEN assay) (Soto et al., 1995). Details can be found in 7th CHAPTER: Defining the estrogenic potency- Estrogen receptor α activation by the crude *Salvia officinalis* extract.

Toxic effects of the extract against the cells used in the cell-based assays can produce false-positive or false-negative results. A toxic effect against the cells utilised in the ERLUX assay would result in a false-negative results by showing no estrogenic response where cell death masks the actual effect. Cytotoxic effects against the cells utilised in the SSRI assay, which measure a re-uptake of 5-HT, would result in a reduced re-uptake due to a reduced cell number and hence give a false-positive result. In order to evaluate possible cytotoxic effects of the extract against the cells a well-known cytotoxicity assay based on the determination of the mitochondrial activity of the viable cells was utilised (Mosmann, 1983). Cells were incubated with a thiazolyl blue tetrazolium bromide (MTT) and metabolically intact cells cleave the MTT and resulting purple formazan allows determination of the percentage of viable cells. Details about the Cytotoxicity assay can be found in the 6th and 7th CHAPTERs.
3. CHAPTER: Preliminary chemical profiling of the crude $Salvia$ officinalis extract

3.1. Liquid-liquid partitioning of the $Salvia$ officinalis extract

The liquid-liquid partition of the $Salvia$ officinalis extract (4.75 l) was performed as described in Figure 3.1. It yielded $n$-hexane subextract (SE) (5.9 g), chloroform ($CHCl_3$) SE (18.1 g) and aqueous-ethanolic (aq- EtOH) SE (46.7 g).

![Diagram of liquid-liquid partitioning scheme for the $Salvia$ officinalis extract.](image)

Figure 3.1: Liquid-liquid partitioning scheme for the $Salvia$ officinalis extract.

![Partitioning: a) crude $Salvia$ officinalis extract, b) $n$-hexane layer (top), c) $CHCl_3$ layer (bottom) and aq-EtOH layer (top)](image)

Figure 3.2: Partitioning: a) crude $Salvia$ officinalis extract, b) $n$-hexane layer (top), c) $CHCl_3$ layer (bottom) and aq-EtOH layer (top)
3.2. Chemical profiling of the *Salvia officinalis* extract and subextracts

For chemical profiling the crude *Salvia officinalis* extract as well as the *n*-hexane-, CHCl₃- and aq-EtOH SE were analysed by TLC and by ¹H-NMR spectroscopy.

3.2.1. TLC analyses of the crude *Salvia officinalis* extract and the subextracts

The TLC analysis (stationary phase: silica gel 60 F₂₅₄) of the crude *Salvia officinalis* extract, the *n*-hexane-, the CHCl₃- and the aq-EtOH SE is presented in Figure 3.3. After the plates were developed in appropriate solvent systems, they were sprayed with an universal staining agent, 4% vanillin/sulphuric acid, which aided to visualise secondary metabolites (e.g. blue, purple, pink, yellow, orange spots). The spots marked by "[" absorbed the UV light at UV₂₅₄nm and spots marked with "[]" showed fluorescence at UV₃₆₆nm.

The TLC analysis of the crude *Salvia officinalis* extract showed blue, purple and yellow spots after being sprayed with 4% vanillin/sulphuric acid spray reagent. The TLC analysis of the *n*-hexane SE showed mainly blue and purple spots, which may be indicative of terpenoids, steroids or fatty acids. Essential oil compounds such as 1,8-cineol appear as bright blue spots on TLC and terpene alcohols generally as blue-violet spots, when sprayed with 4% vanillin/sulphuric acid spray reagent. The *n*-hexane SE...
further showed various different compounds, which quenched the absorbed light at $\text{UV}_{254\text{nm}}$ and/or showed fluorescence at $\text{UV}_{366\text{nm}}$.

The $\text{CHCl}_3$ SE was rich in compounds, which appeared as brown, green or yellow spots on the TLC. The green spot at the front most likely belonged to chlorophyll, which is ordinary in aerial parts of plants. Some compounds, which absorbed light at $\text{UV}_{254\text{nm}}$ were detected in the $\text{CHCl}_3$ SE and the aq-ETOH SE and a few compounds showed fluorescence at $\text{UV}_{366\text{nm}}$.

The TLC analysis of the aq-EtOH SE was dominated by red and yellow spots, which might derive from phenolic compounds like flavonoids or other hydrophilic compounds, which accumulate in the aqueous layer after liquid-liquid partitioning.

3.2.2. $^1$H-NMR analyses of the crude *Salvia officinalis* extract and subextracts

The $^1$H-NMR analysis (Fig. 3.4) of the crude *Salvia officinalis* extract (red spectrum) in MeOD was dominated by primary metabolites such as lipids and fatty acids ($\delta$ 0.2 ppm, 5.2 ppm) and sugars ($\delta$ 3-4 ppm).

![Figure 3.4: $^1$H-NMR spectra (400 MHz) of crude *Salvia officinalis* extract (red, in MeOD) and $\alpha$-hexane SE (blue, in CDCl$_3$), the CHCl$_3$ SE (green, in MeOD) and the aq-EtOH SE (dark yellow, in MeOD).]
The $^1$H-NMR spectrum of the $n$-hexane SE in CDCl$_3$ (blue spectrum) was dominated by signals which are typical for fatty acids ($\delta$ 0-2 ppm, 5.2 ppm) and which have been transferred by the liquid-liquid partitioning into the $n$-hexane SEs. The CHCl$_3$ SE was very similar to the $n$-hexane SE and also showed typical signals for fatty acids ($\delta$ 0-2 ppm, 5.2 ppm). Differences between the two SE are in the aromatic signals ($\delta$ 6-8 ppm), which were observed in the CHCl$_3$ SE, but not in the $n$-hexane SE. The most polar compounds such as sugars ($\delta$ 3-4 ppm) have been transferred mainly to the aq-EtOH SE (orange spectrum). The signals in the aromatic region ($\delta$ 6-8 ppm) are similar to the once observed in the CHCl$_3$ SE.

The results obtained by TLC and $^1$H-NMR analyses showed that a coarse separation of the compounds found in the crude Salvia officinalis extract according to the polarity was achieved by the liquid-liquid partitioning. The TLC and $^1$H-NMR analyses of the SEs emphasised that the crude Salvia officinalis extract and was rich in phytochemically interesting secondary metabolites.
4. CHAPTER: Evaluation of the Cytochrome P450 enzyme 3A4 inhibitory effects of *Salvia officinalis*

Drug-interactions occur when the behaviour or the effects of one drug is changed by the presence of another drug, a herbal medicine, food or other substances. An interaction can for example lead to an increase of the toxicity or a reduction of the efficacy of the drug.

Food components can alter drug absorption through alterations in drug solubility or by affecting the bioavailability of certain drugs by presystemic intestinal metabolism. For example, the effect of concomitant intake of grapefruit juice has been studied and it is thought that the flavonoid narigenin is involved in the inhibition of CYP3A enzymes (Ameer and Weintraub, 1997; Evans, 2000). Many HMPs are based on spices, herbs or berries, which are widely used for flavouring food or consumed as juices. Beside grapefruit juice, cinnamon, black or white pepper and ginger, are examples of food which were shown to have the potential to exhibit interactions with CYP enzymes (Kimura *et al.*, 2010).

One issue for the safety assessment of HMP is the natural variations in the herb itself, hence the variations in the products made from it. HMPs are usually complex mixtures of many bioactive or inactive compounds. The complex nature of the product makes it difficult to assign a desired effect to a specific compound and the same difficulty exists for the undesired effects and the potential to interact with other drugs or enzymes. Furthermore, once isolated, the effect of a single compound might not be the same as when the compound is present in a mixture (Williamson *et al.*, 2009).

The main herb-drug interactions of concern are pharmacokinetic and pharmacodynamic interactions and modulations of drug-transporter proteins. Pharmacokinetic interactions are highly enzyme dependent and are known to affect the absorption, distribution, metabolism and excretion of a drug. Possible interactions are manifold, but most importantly include the cytochrome P450 (CYP) enzymes.

Drug-transporter protein interactions can result in similar effects, as transporter protein, may reduce drug absorption and distribution or increase its elimination. The P-glycoprotein transporters are, amongst the carrier-mediated processes, the best-studied ones. They are efflux pumps located in certain cells, which enable drugs to cross membranes through a carrier-mediated process. Pharmacodynamic interactions occur when one drug has a direct influence on the effect of another drug. This influence can be through synergistic or additive effects, which may result in toxic or antagonistic effects, which often cancel out the therapeutic benefits (Williamson *et al.*, 2009). Rarely
one mechanism is responsible for the interactive potential of a product and as many drugs interact through multiple mechanisms final conclusions should always consider multiple factors.

The isoform CYP3A4 is the most abundantly expressed CYP enzyme in the liver and accounts for the majority of human drug metabolism ((Shimada et al., 1994) for review see (Guengerich, 1997; Wrighton et al., 2000)).

The potential of *Salvia officinalis* to cause herb-food and herb-drug interactions with conventional drugs has not yet been thoroughly studied. Investigating the effect of *Salvia officinalis* on the CYP3A4 enzyme gives information about possible adverse reactions. In this chapter the inhibitory potential of the *Salvia officinalis* tincture and its three subextracts against CYP3A4 are presented. A fluorescence-based, sub-cellular (cell-free) *in vitro* assay was chosen, which measures the metabolite formation of a substrate for the enzyme, in the presence of another drug or test substance (Donato and Castell, 2003; Modarai et al., 2007). A possible involvement of thujone, one of the major volatile oil components of *Salvia officinalis*, has additionally been assessed and is described in the following sections.

### 4.1. The cytochrome P450 enzyme system

The majority of drugs are modified, hence metabolised, before the body can eliminate them. Lipophilic drugs are modified to more hydrophilic compounds by a series of different reactions, which are subdivided into phase I and phase II reactions. Phase I reactions are mainly oxidisation reactions and result in more polar compounds. Phase II reactions mainly involve conjugation reactions, with glucuronic acid, glutathione and sulfate.

CYP enzymes are major players in the oxidative metabolism of the phase I reactions. The majority of the CYP enzymes are located in the liver, in the endoplasmatic reticulum of hepatocytes (Donato and Castell, 2003). Their catalytic cycle is briefly reviewed in the following sections. The cytochrome P450 system is a heme-containing, membrane-bound, multi-enzyme system, named after “pigment 450” (Guengerich, 2001). That is because of its unusual spectral properties of displaying a typical absorption maximum of a reduced CO-bound complex at 450 nm.

Cytochrome indicates the hemoprotein, P stands for pigment and the 450 reflects the absorbance maximum ($A_{max}$) of the CO complex at 450 nm. Quantification of cytochrome P450 enzymes is often based on the ability of the reduced P450 to produce an absorption peak at 450 nm, which is caused by the shift of the heme's $A_{max}$ (the Soret peak) upon CO binding to 450 nm (for review see (Bernhardt, 2006)). Cytochromes
P450 enzymes are external monooxygenases, mixed function oxidases, and they split bimolecular oxygen and introduce one of the atoms onto their substrate; the remaining oxygen atom is converted to water (Bernhardt, 2006). Its catalytic function depends on a coenzyme, the cytochrome-P450 oxidoreductase and to some extent cytochrome b5 (Guengerich, 1997).

The general reaction catalysed by the cytochrome P450 system is:

\[
\text{NADPH} + \text{H}^+ + \text{O}_2 + \text{R} \rightarrow \text{NADP}^+ + \text{H}_2\text{O} + \text{RO}
\] (Guengerich, 2001)

NADPH: nicotinamide adenine dinucleotide phosphate, NADP+: nicotinamide adenine dinucleotide phosphate oxidised form, H\text{\textsubscript{2}}O: water, R: substrate and O\text{\textsubscript{2}}: molecular oxygen.

The general catalytic cycle of the cytochrome P450 enzymes starts with the binding of substrate to the P450 iron in the ferric state near the heme region (Fig 4.1).

The next step is the introduction of a first electron from NADPH-P450 reductase NADPH via an electron transfer chain onto the Fe\textsuperscript{3+} atom on the heme, reducing it to Fe\textsuperscript{2+} (ferrous iron). Binding of a molecule of oxygen leads to an unstable complex (Fe\textsuperscript{2+}O\textsubscript{2}), which is capable of accepting a second electron to produce a ferric peroxy anion (Fe\textsuperscript{3+}O\textsubscript{2}) the second electron is provided by either the NADP-P450 reductase directly or via involvement of cytochrome b5. Upon protonation of the ferric peroxy anion a ferric hydroperoxy complex is formed (Fe\textsuperscript{3+}O-H\textsubscript{2}O), which undergoes subsequent heterolytic cleavage of the O-O bond, followed by the formation of FeO\textsuperscript{3+} and H\textsubscript{2}O.

![General catalytic cycle of P450 enzymes](image)

Figure 4.1: General catalytic cycle of P450 enzymes (Berg \textit{et al.}, 2002) adapted from (Guengerich, 2001)

The FeO\textsuperscript{3+} complex (formally equivalent to a Fe\textsuperscript{5+}=O species named compound 1) then reacts with the substrate (RH), yielding the hydroxylated product (ROH), effectively

---

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adding an oxygen atom to the substrate, which dissociates and regenerates the ferric ion to let the catalytic cycle start again (for reviews see (Guengerich, 2001; Bernhardt, 2006)).

4.1.1. The Cytochrome P450 enzymes

The majority of herb-drug and drug-drug interactions, especially the oxidative metabolism of drugs, involve cytochrome P450 enzymes (CYP). The CYP enzymes are membrane-bound proteins. They belong to a very large family of enzymes and around 30 different isoenzymes have been found in the liver tissues to date and some, at smaller amounts, were isolated from extra-hepatic tissues. CYP enzymes are grouped upon structural similarities into families (e.g. CYP3), subfamilies (e.g. CYP3A) and then subdivided in isoforms (e.g. CYP3A4). Only a few subfamilies are responsible for the metabolism of the majority of commonly used drugs, with CYP3A being one of them.

The induction of CYP enzymes simply involves an increase in the expression of the particular isoform, followed by an increase in drug oxidation and its elimination. CYP enzyme induction, unlike inhibition, has no immediate effect and may develop over days or weeks and continue for a similar time, even after the application of the inducer is discontinued. Gingko, Echinacea and St. John's Wort are examples of herbal inducers of CYP3A4 in vitro (Guengerich, 1997; Williamson et al., 2009).

Inhibition of CYP enzymes, on the other hand, is a phenomenon, which happens rather immediately and directly results in a decrease in enzyme activity. This direct effect on the availability of functioning enzyme can be considered as more severe compared to the delayed induction of enzymes. Furthermore, most drug-drug interactions that are reported can be attributed to inhibition of CYP enzymes (Guengerich, 1997).

The cytochrome P450 system appears to be particularly vulnerable for interactions with herbal preparations, as these multiple component mixtures can modulate many different isoenzymes at the same time. Finally, the drug, whose metabolising enzyme is inhibited, may accumulate in the body and increase the risk of toxic effects. Potential to inhibit CYP3A4 in vitro was found for e.g. Grapefruit, turmeric and again Gingko and Echinacea. However, many of the effects are not clinically supported, or the observed effects were low, and suggested only modest effects in vivo. Although there are known difficulties with extrapolation from the in vitro to in vivo scenario, the screening of isolated isoenzymes allows identification of HMP, which may have potential to cause significant herb-drug interactions in vivo (Foster et al., 2003; Williamson et al., 2009).
4.1.2. Importance of investigating the inhibition of the CYP isoform 3A4

Inhibitors or inducers of CYP3A4 are a major cause for drug-drug interactions. The influence of a compound against CYP3A4 is therefore assessed in early stages of drug discovery (Ekins et al., 2003) and information about the behaviour against CYP3A4 is more often required for regulatory purposes. It has also been found that CYP3A4 is the main drug-metabolising enzyme which activity is altered by food and it therefore appears to be the major enzyme involved in food-drug interactions (for review see (Fujita, 2004)).

Only a handful of different CYP isoforms are responsible for the metabolism of the majority of the commonly used drugs (e.g. 1A2, 2C9, 2C19, 2D6 and 3A4) (Guengerich, 2001). CYP3A4 accounts for on average 29% of the CYP isoforms found in the liver (Shimada et al., 1994).

Furthermore, the inhibition of the CYP enzymes has an immediate influence on the human drug metabolism and is more common than the induction of CYP enzymes (see 4.1.1 and (Guengerich, 1997)).

In order to obtain a clear picture about the direct effects of the crude <i>Salvia officinalis</i> extract on the most abundantly expressed CYP isoform, we focussed on the inhibitory potential of the crude <i>Salvia officinalis</i> extract and its subextracts on the CYP isoform 3A4 by using complementary deoxyribonucleic acid (cDNA) expressed recombinant enzymes.

4.2. Methodological consideration - <i>in vitro</i> investigation of the potency to inhibit the CYP3A4 enzyme

<i>In vitro</i> drug metabolism studies are useful tools, which aid characterisation of metabolites and metabolism pathways, and therefore give very valuable information for further <i>in vivo</i> studies. The <i>in vitro</i> models used today include metabolising enzymes (e.g. CYP and UDP-glucuronosyltransferase), subcellular fractions (e.g. microsomes, cytosols) and, the ones most suitable to mimic the physiological conditions, the cellular organells (e.g. hepatocytes and liver slices) (Jia and Liu, 2007).

Naturally, each and every model has its advantages and disadvantages. Hepatocytes and liver slices mimic closely the physiological conditions with respect to enzyme and cofactor composition. They allow observation of phase I and phase II metabolism, as well as interactions with transporter proteins. Compared with subcellular models, studies with hepatocytes and liver slices allow more accurate predictions of <i>in vivo</i> metabolism.
However, they show a natural variation in their enzyme composition and the enzyme activity is fairly unstable, which reduces the reproducibility of the experiments. Microsomes are organelles obtained by subcellular fractionation of tissues. They contain a physiological mixture of CYP isoforms. Subcellular systems are easily available, but they do not contain phase II enzymes. On the other hand, metabolic processes or uptake via transporters do not affect the obtained results, and the reproducibility of the results is high.

The cDNA expressed recombinant human CYP enzymes have been expressed in a variety of cells (e.g. yeast, bacteria, mammalian and insect cells). Compared with microsomes, they have similar advantages and disadvantages, but allow the assessment of the effects on a single enzyme. This is a desirable feature, as the presence of many CYP isoforms, e.g. at different concentrations in the microsome model, makes the picture of the observed interactions more complicated (Jia and Liu, 2007). Keeping the picture simple, by investigating the effects on one particular enzyme isoform has certainly its advantages, even though the physiological composition of enzymes is not retained.

Knowing which enzyme isoform is responsible for the metabolism of a drug is important in order to predict possible drug-drug interaction with co-administrated drugs (Li and Jurima-Romet, 1997).

HMPs are complex mixtures and any constituent of the mixture might contribute to a change in pharmacokinetic behaviour (Williamson et al., 2009). Determining the effects of a complex mixture on a mixture of various enzyme isoforms makes it very difficult to determine the enzyme isoform, which is affected by the HMP and therefore responsible for the observed effect. Although this can be determined by monitoring the metabolising rate of selective substrates, compounds also may be metabolised by more than one isoform and different kinetic behaviour can lead to a complicated, unclear picture (Harper and Brassil, 2008).

Generally, it is most likely that many different isoforms will contribute to the overall metabolism of the product (Williamson, 2005). In order to ensure the safety of an HMP it is useful to investigate the influence on the metabolism of other co-administrated drugs, whose metabolising enzyme isoform is known. For that reason the influence of the crude Salvia officinalis extract was assessed against a single isolated isoform. Because of the relatively simple experimental set-up, the high reproducibility of the results and in order to clearly reveal, which enzyme isoform is inhibited, we decided to work with the cDNA expressed recombinant human CYP3A4 enzyme (Modarai, 2008). The basic principle of the CYP enzyme system, the model of the cDNA expressed recombinant
human enzymes and our reasoning why we decided to focus on enzyme inhibition is described in the following sections.

4.2.1. cDNA expressed recombinant enzymes, supersomes and fluorescence based determination of enzyme inhibition

Heterologous expression of drug-metabolising human CYP enzymes made them more easily available for in vitro studies. These cDNA expressed human cytochrome P450 enzymes are frequently used to assess the involvement of the CYP family in the metabolism of drug candidates. CYP-engineered cells, or in our case microsomes from engineered cells (supersomes), have facilitated the identification of the enzyme isoform which is primarily responsible for the metabolism of a drug. Additionally, they provide a useful tool for identifying drug metabolism related interactions (Donato and Castell, 2003). Working with supersomes has advantages and disadvantages, which will be briefly discussed in the following section.

CYP isoforms have been expressed using yeast (Saccharomyces cerevisiae, Schizosaccharomyces pombe), bacteria (Escherichia coli), mammalian and insect cells. Usually the CYP enzyme is expressed in conjunction with its cofactors in order to provide a system with optimal catalytic activity. BD Supersomes™ enzymes are recombinantly expressed drug metabolizing enzyme reagents. They consist of microsomes, prepared from baculovirus infected BT1-TN-5B1-4 insect cells from the eggs of Trichoplusia ni (Tiger moth), engineered to express a pure single CYP isoform. The CYP activity is supported by the presence of its human cytochrome c reductase and human cytochrome b5 (BDbioscience, 2010). Baculovirus/insect cells expressed enzymes have high catalytic activity and are therefore suitable for in vitro assessments (Crespi et al., 1997). The enzyme activity in the supersomes is higher than in human liver microsomes. High enzyme activity is beneficial, as it affects the turnover number of CYP enzyme. A greater substrate turnover allows identification of interaction with poorly metabolized compounds, which is a general limitation of microsome preparations, as incubation can only be performed for a short time and therefore poorly metabolised substrates are not assessed (Venkatakrishnan et al., 2000; Donato and Castell, 2003).

The performance of the enzyme depends on various accessory non-CYP factors, such as electron-transfer proteins NADPH cytochrome P450-oxidoreductase and cytochrome b5, membrane lipid composition, and ionic strength of the in vitro incubation matrix. These conditions should be kept very similar to those of the human liver microsomes in order to allow identification of potential inhibitors of the enzyme in vivo. The fact that with the supersomes a single CYP isoform interacts with the
accessory proteins, whereas in liver microsomes multiple CYPs compete for the accessory proteins, is a difference which is difficult to control and may lead to incorrect predictions of the contribution of the enzyme isoform on the metabolism of a drug in other systems. This highlights the fact that supersomes cannot represent the conditions in the human liver (Venkatakrishnan et al., 2000), but they are valuable tools to identify the specificity and potency of inhibitors of cytochrome P450 enzymes. The supersomes have shown comparable results with the traditional human liver microsome preparations for selective inhibitors of CYP enzymes (Crespi et al., 1997) and assays using supersomes are accurate, rapid, robust and reproducible (Crespi and Stresser, 2000; Modarai, 2008). They further allow to obtain a pool of useful information before the in vivo or clinical trials begin and further investigations can be planned more cost-effective and ethically accepted (Donato and Castell, 2003).

The classical in vitro approach for studying CYP enzyme inhibition involves the use of one drug as a substrate for the enzyme and measures its metabolite formation in the presence of another drug or test substance. The inhibition of the CYP enzyme is detected as a decrease in metabolite formation compared to the control (for review see (Donato and Castell, 2003)). Most of the in vitro methods are relatively expensive, time consuming and often require HPLC separation for metabolite quantification.

In this thesis a fluorometric assay in microtiter plate format was utilised to assess the inhibitory potential of the crude *Salvia officinalis* extract and its subextracts using cDNA expressed cytochrome P450 enzyme 3A4. This method was previously shown to be less time consuming, but efficient and cost-effective and obtains a high reproducibly and comparable results with other more complex methods (Crespi et al., 1997; Modarai et al., 2007).

4.3. Previous studies of the effect of *Salvia officinalis* on drug metabolism

*Salvia officinalis* preparations and extracts have been subjected to a couple of experiments investigating the effects on enzymes or transporter proteins, which play a major role in human drug metabolism. The inhibitory potential of a dried, aqueous *Salvia officinalis* extract preparation against cDNA baculovirus expressed CYP3A4 mediated metabolism of testosterone was assessed. The formation of the metabolite 6-β-OH-testosterone was measured by HPLC and the IC$_{50}$ value for the *Salvia officinalis* extracts was 483 μg/ml (Hellum and Nilsen, 2008). Foster et al. (2003) reported in the same in vitro model over 95% inhibition by an aqueous *Salvia officinalis* extract against the CYP isoforms 2C9, 2C19, 2D6, 3A4 at a concentration of 25 mg/ml. This inhibitory potential of the
aqueous *Salvia officinalis* extract against CYP2D6 was confirmed later and the reported IC\(_{50}\) value was 796 µg/ml (Hellum and Nilsen, 2007).

Studies investigating the effects of a dried aqueous *Salvia officinalis* preparation on CYP induction did not reveal a clinically significant inductive effect on the CYP enzymes 1A2, 2D6 and 3A4 (Hellum *et al.*, 2007). The same preparation however induced CYP2C19 activity by 70% at a concentration of 82 µg/ml in human hepatocytes (Hellum *et al.*, 2009).

Further, a small reduction of the digoxin transport across the monolayer of Caco-2 cells indicated a weak ability of the dried aqueous *Salvia officinalis* extract preparation to inhibit P-glycoprotein efflux transport activity (Hellum and Nilsen, 2008).

### 4.4.4. Experimental procedure — the CYP assay

#### 4.4.1. Chemicals, reagents, solutions

Human CYP3A4 with P450 reductase and cytochrome b5 (supersomes®), were purchased from Gentest, Woburn. Microsomes, prepared by baculovirus infected insect cells (BTI-TN-5B1-4), also contained cDNA expressed human P450 cytochrome c reductase and human cytochrome b5. 7-Benzylxy-4-(trifluoromethyl)-coumarin (7-BFC) was also purchased from Gentest, Woburn.

Ethanol (EtOH) (99-100%) and acetonitrile (ACN) were obtained from Fischer Scientific, Leicester, UK. Ketoconazole (98% pure), α-thujone (96% pure), α/β-thujone mixture (99% pure) and all other chemicals were purchased from Sigma-Aldrich Company Ltd., Dorset, UK, unless stated otherwise. Test samples were dissolved in 66% or 100% EtOH.

#### 4.4.2. The CYP assay

The CYP assay was performed according to Stresser and Crespi (*Crespi et al.*, 1997; Crespi and Stresser, 2000) and is described in detail in the manufacturers instructions to be found on www.gentest.com (Gentest, 2010). Minor modifications of the assay set-up were undertaken by Modarai (Modarai, 2008). For the assessment, cDNA expressed human CYP3A4 enzyme was used, which were expressed in a baculovirus/insect cell expression system by the manufacturer. 7-Benzylxy-4-(trifluoromethyl)-coumarin (7-BFC) was used as substrate for the enzyme reaction and its fluorescent metabolite (7-hydroxy-4-(trifluoromethyl-coumarin, 7-HFC) is formed via hydroxylation.

It has been shown that solvents can inhibit enzyme activity. Therefore, the concentration of EtOH was kept below the tolerable concentration of 3% EtOH/well (Busby *et al.*, 1999). To control for small solvent effects, EtOH test wells with ethanol
concentrations equivalent to those present in the extract test wells were included in each plate (EtOH test, eight replicates). Blank wells, containing the cofactor solution and the enzyme substrate mixture, were used to determine background fluorescence (blanks). In order to control for intrinsic fluorescence, introduced by the extract itself, extract blanks were implemented (eight replicates), which were identical with the blanks, but the enzyme substrate mixture was added after the reaction was terminated (extract blanks). Additional wells, which allowed evaluation of maximal enzyme activity on each plate, contained only cofactor solution and enzyme substrate mixture (positive controls) and were used to monitor the performance of the enzyme.

Briefly, a 0.5 M K$_2$HPO$_4$ buffer (pH7.4) was used for the preparation of a cofactor solution and was adjusted to pH7.4 with 0.5 M KH$_2$PO$_4$ solution. The cofactor solution in deionised water contained 10% of buffer, 0.0026 mol/l NADP$^+$, 0.0066 mol/l C$_8$H$_{11}$Na$_2$O$_4$P × xH$_2$O, 0.0065 mol/l MgCl$_2$ × 6 H$_2$O and 2% of a solution containing 40 units/ml of glucose-6-phosphate dehydrogenase (G-6-PDH) in 5 mM Na$_3$C$_6$H$_5$O$_7$ solution.

To ensure solubility of all assay components in the 96 well plates (Greiner Bio one, black polystyrene, UK), 4% ACN was added to an appropriate amount of the cofactor solution.

One hundred μl of this cofactor/ACN mixture was distributed to all test wells (extract test, extract blanks, EtOH test, positive control and blanks) except the wells for the highest extract test/blanks and EtOH test concentrations (first column). Test solutions (extract and EtOH) were mixed with cofactor solution in appropriate concentrations. Duplicate of 150 μl of extract/cofactor mix and EtOH/cofactor mix were added into the wells of the first, empty column (for extract test, extract blanks and EtOH test). Removing 50 μl from the wells of the first column and adding them to the next column performed a 1:3 serial dilution. This step was repeated along the plate up to column 8. Plates were warmed at 37°C for 10 min.

The enzyme and substrate mixture contained pre-warmed (37°C) 0.5 M potassium phosphate buffer (pH7.4), 0.1 mmol/l 7-benzyl-4-(trifluoromethyl)-coumarin (small aliquots were prepared beforehand in ACN) and 0.1 nM CYP 3A4 enzyme. One hundred μl of this mixture was added rapidly to the wells containing ‘extract test’ (T) and ‘EtOH test’ (E) solutions or positive control wells, resulting to 200 μl final assay volume per well. Plates were incubated for 30 min at 37°C and the enzymatic reaction was terminated by adding 75 μl of stop solution (20% Tris (0.5 M)/80% ACN). The remaining enzyme substrate mixture was added to the ‘extract blank’ (e) and ‘blank’ (b)
wells. Plates were placed on a plate shaker for 1 min before the fluorescence signals were read with a FLUOstar Optima (BMG, Labtech Ltd.) plate reader at excitation wavelength of 405-410 nm and 530 nm for emission. Extracts were tested three times in duplicates.

4.4.2.1. Quantification of inhibitory potential and controls

Although the concentration of EtOH was kept below the tolerable concentration of 3% EtOH/well (Busby et al., 1999), there may still be an unspecific inhibition due to the inevitable presence of EtOH in the test solution. Another concern is that natural products' intrinsic fluorescence might mask enzyme inhibition (Zou et al., 2002; Modarai, 2008). To distinguish between these effects and the inhibition caused by the extracts, the degree of inhibition by the extract was computed as the ratio between the fluorescence measured in the presence of the extract (extract test wells, T) and the fluorescence observed in absence of the extract (EtOH test wells, E). To correct the values, EtOH test (E) readings were corrected for background fluorescence introduced by the enzyme and the substrate (blanks, b). The fluorescence readings obtained in the presence of the extract (extract test, T) were corrected for fluorescence introduced by the extract itself (extract blanks, e). The results were expressed as % enzyme activity.

The values were calculated with the following formula:

\[
\% \text{ enzyme activity} = \frac{(T-e)}{(E-b)} \times 100
\]

The curve fitting and data analysis were performed using Graph Pad Prism® v4.03 (Inc, USA). Ligand concentrations were log transformed and curve fitting was performed using a sigmoidal dose-response model with variable slope.

\[
\text{IC}_{50} = \frac{\text{Min} + (\text{Max} - \text{Min})}{(1 + 10^{\log \text{IC}_{50} - X \cdot p})}
\]

Half maximal inhibitory concentrations (IC\textsubscript{50}) were evaluated by non-linear regression analysis using the four parameter Hill model. Min and Max are minimal and maximal observed effects, 'X' represents the concentration of sample and 'p' is the slope parameter. Data were corrected by setting the minimal observed enzyme activity to 0% and the maximal observed enzyme activity to 100%.
4.4.2.2. Quenching effects

Quenching of test compounds limits the applicability of the assay, as it may result into misinterpretation of the observed effects by mimicking inhibition of the enzyme (Zou et al., 2002). Quenching describes any process, which decreases the fluorescence intensity of a given substance. A variety of processes can result in quenching, such as excited state reactions, energy transfer, complex-formation and collisional quenching. When external influence (quenching substance) interferes with the excited state, for instance via energy or charge transfer, dynamic quenching occurs. When inhibition of the formation process (during ground state) occurs, for instance via complex formation, it is referred to as static quenching (Modarai, 2008). To determine if the test samples exhibit quenching, the assay was performed as described in the protocol using solvent controls throughout. The fluorescent signals were determined. Test samples were added to the wells directly afterwards and fluorescence was determined again. Fluorescent signals should not be affected (significant decrease of signal) by the samples. Quenching values are determined with following formula:

\[
\text{Quenching} = \left( \frac{\text{fluorescence of metabolite} - \text{fluorescence in the presence of sample}}{\text{fluorescence of metabolite}} \right) \times 100
\]

4.5. Results

4.5.1. Inhibition of CYP3A4 by ketoconazole

Ketoconazole, a well known CYP3A4 inhibitor (Li et al., 1995), was used as reference inhibitor (0.0006 μM to 1.25 μM), to assess the the accurate functioning of the CYP assay. Data were obtained in two independent experiments and normalised according to the details in section 3.4.2.1. Concentrations of half maximal inhibition (IC\text{50}) were estimated using non-linear regression (sigmoid, 4 parameter Hill model) and the IC\text{50} value of 0.12 μM was in agreement with published literature values of 0.13 μM (Yamamoto et al., 2002), 0.1 μM (Dierks et al., 2001; Hellum and Nilsen, 2008), 0.083 μM (Crespi et al., 1997) and 0.12 μM (Modarai et al., 2007), which confirmed that the assay was performing well in our hands (Fig. 4.2). The ketoconazole experiments gave an impression of the small data variation, which can be expected in the assay.
4.5.2. Quenching effects and intrinsic fluorescence

Quenching effects could produce false-positive results (i.e. simulate 100% enzyme inhibition) or result in a concentration-dependent decrease in the fluorescence signal, which might interfere with an accurate determination of the IC₅₀ value. The highest concentrations of the extracts were therefore tested for quenching effects (crude Salvia officinalis extract 191.25 µg/ml, n-hexane SE 75.00 µg/ml, CHCl₃ SE 60.00 µg/ml and aq-EtOH SE 625.00 µg/ml). Neither the crude Salvia officinalis extract, nor the n-hexane- or CHCl₃ SEs exhibited strong quenching effects (~10% for all extracts). Only the aq-EtOH SE showed moderate quenching effects (~20%), which may have contributed to any inhibition by the subextract. The observed quenching effects were considered as weak to moderate as Zou et al. (2002) observed quenching effects of 15% for quercetin at much lower concentrations (100 µM = 30 µg/ml).

Intrinsic fluorescence, on the other hand, could mask inhibition of the enzyme or shift an observed effect to higher concentrations and therefore produce false-negative results (i.e. simulate 0% enzyme inhibition). In order to control for intrinsic fluorescence by the extracts the raw data obtained for the extract test wells were corrected with data obtained from the extract blank wells (see section 4.4.2.1). Figure 4.3 shows the intrinsic fluorescence of the extracts (extract blanks) relative to background fluorescence (set to 100%) by the cofactor solution and the enzyme substrate mixture (blanks) in %.
Data for intrinsic fluorescence are presented to allow assessment of the magnitude of the effects. The n-hexane- and the CHCl₃ SEs did not exhibit any intrinsic fluorescence except the highest concentration of the CHCl₃ SE (60 µg/ml), which showed an effect about 20% higher than the overall background fluorescence observed in the assay. The same applies for the crude *Salvia officinalis* extract, where the two highest concentrations showed intrinsic fluorescence of ~10% or ~20% at 63 µg/ml or 192 µg/ml respectively. One single observation of intrinsic fluorescence ~50% higher than background fluorescence was seen at 0.08 µg/ml for the crude *Salvia officinalis* extract, but both repeats of the experiment did not confirm this effect. The aq-EtOH SE showed intrinsic fluorescence at the three highest concentrations. The observed effects were around 15% (69 µg/ml), 35% (208 µg/ml) and 90% (625 µg/ml) higher than the background fluorescence. Any inhibition of the CY3A4 enzyme may therefore have been masked or the effect may have occurred at higher concentrations by the intrinsic fluorescence of the aq-EtOH extract at these concentrations.
4.5.3. Inhibition of CYP3A4 by the crude *Salvia officinalis* extract and the subextracts

The inhibitory potential against CYP3A4 was assessed for the crude *Salvia officinalis* extract at concentrations ranging from 0.09 - 191.25 µg/ml. The dried CHCl₃- and aq-EtOH SE were dissolved in 66% EtOH solution and tested at 0.03 - 60.00 µg/ml and 0.28 - 625.00 µg/ml, respectively. The n-hexane SE was dissolved in 100% EtOH and the inhibitory effects were assessed between 0.03 - 75.00 µg/ml. All extracts were capable of inhibiting CYP3A4 (Fig. 4.4). The crude *Salvia officinalis* extract, the CHCl₃ and the n-hexane SEs showed a dose dependent inhibition of the CYP3A4 enzyme ranging from 100% enzyme activity to 0% enzyme activity or 0% inhibition and 100% inhibition, respectively. The inter-assay data variation for the individual extracts was very small for all the experiments.

![Graph A](image1)

![Graph B](image2)

Figure 4.4: CYP3A4 inhibition by the crude *Salvia officinalis* extract (red), n-hexane SE (blue), chloroform SE (green) and aq-EtOH SE (yellow). Data were obtained in three independent experiments, run in duplicate. Data were fitted using non-linear regression model (sigmoid, 4 Parameter). A represent the individual data from the three independent experiments and dashed lines in B represent 95% confidence intervals of the regression analysis of A.
Among the four tested extracts, the n-hexane SE was the most potent inhibitor of the enzyme (IC$_{50}$ value of 1.8 µg/ml). The CHCl$_3$ SE and the crude Salvia officinalis extract showed a similar inhibitory potency with IC$_{50}$ values of 3.1 µg/ml and 6.8 µg/ml. However, compared to the other three extracts the aq-EtOH SE was a moderate inhibitor of the CYP3A4 enzyme, with an IC$_{50}$ value (estimated by regression analysis) of 281.2 µg/ml, i.e. around a hundred times less potent than the other extracts (Tab. 4.1).

<table>
<thead>
<tr>
<th>Extract</th>
<th>crude Salvia officinalis extract</th>
<th>n-hexane SE</th>
<th>CHCl$_3$ SE</th>
<th>aq-EtOH SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ [µg/ml]</td>
<td>6.8</td>
<td>1.8</td>
<td>3.1</td>
<td>281.2</td>
</tr>
</tbody>
</table>

Table 4.1: Concentrations of half maximal inhibition (IC$_{50}$ values) of the crude Salvia officinalis extract, the n-hexane, the CHCl$_3$ and the aq-EtOH SE against purified cDNA expressed CYP3A4.

4.5.4. The influence of the thujone content in the crude Salvia officinalis on the inhibitory potential against CYP3A4

Of the four extracts, the n-hexane SE was the most potent inhibitor of the CYP3A4. It was the most non-polar SE. The terpenoid thujone is one of the major compounds in the volatile fraction of Salvia officinalis (Perry et al., 1999). Hence the n-hexane SE was of all the tested extracts the one highly likely to contain the highest concentration of thujone. The cytochrome P450 system has been described as the principal system for the metabolism of thujone diasteromers and CYP3A4 oxidises α- and β-thujone (Hold et al., 2001). It was therefore hypothesised that the thujone present in the n-hexane SE may be responsible for the inhibitory effect on the CYP3A4 enzyme.

In order to investigate the possible contribution of thujone to the inhibitory effects of the crude Salvia officinalis extract, and its SE (Fig. 4.5), commercially available, chemically pure α-thujone and a α/β-thujone mixture (90-95% α-thujone, 5-10% β-thujone) were tested for their inhibitory potential against CYP3A4. In order to be able to link the results obtained in the CYP assay, the thujone samples were tested at concentrations similar to those found in the highest concentration of crude Salvia officinalis extract tested in the CYP assay (191.25 µg/ml). The amounts of α/β-thujone were calculated based on the results on the quantitative analysis of the thujone content provided by Bioforce (see section 2.1: The Salvia officinalis tincture). The crude Salvia officinalis extract (batch no. 026141) contained α-thujone (162 µg/ml = 18 mg/100g) and β-thujone (28.8 µg/ml = 3.2 mg/100g) (see chapter 2, (Bioforce, 2008)). The amount of thujone in the
crude *Salvia officinalis* extract at 191.25 μg/ml (highest tested concentration) amounted to 2.36 μg/ml of α/β-thujone. The tested concentrations for the pure thujone samples ranged from 0.02 to 46.6 μg/ml for α-thujone and from 0.02 to 47.25 μg/ml for the α/β-thujone mixture (Fig. 4.5).

Figure 4.5: CYP3A4 inhibition α-thujone (light blue, dashed line) and α/β-thujone mix (dark purple, 90-95% α-thujone, 5-10% β-thujone) in μg/ml (A) and μM (B). Data shown in A are from two independent experiments, run in duplicate. Data were fitted using non-linear regression model (sigmoid, 4 Parameter Hill).

Figure 4.6: CYP3A4 inhibition by the crude *Salvia officinalis* extract (red), α-thujone (light blue, dashed line) and α/β-thujone mixture (dark purple, 90-95% α-thujone, 5-10% β-thujone). Data are from at least two independent experiments, run in duplicate. Data were fitted using non-linear regression model (sigmoid, 4 parameter Hill). Second x-axis (bottom) represents the concentration of the thujone diastereomers in the crude *Salvia officinalis* extract at tested concentrations displayed in the first x-axis (top).
The chemically pure α-thujone showed CYP3A4 inhibition of ~20% at 15 μg/ml and ~40% at 47 μg/ml. Inhibition of CYP3A4 was observed for the α/β-thujone mixture at 15 μg/ml and 47 μg/ml, where the activity of the enzymes was inhibited by ~30% and ~50% respectively. Figure 4.6 displays the results from Figure 4.5 in comparison with the dose-response inhibition curve of the crude *Salvia officinalis* extract (reproduced from Fig. 4.4). The fraction of the thujone in the crude *Salvia officinalis* extract at 191.25 μg/ml is indicated by dotted line (2.3 μg/ml of α/β-thujone). The pure α-thujone or the α/β-thujone mixture showed no inhibitory potential against CYP3A4 (100% enzyme activity) at the concentration of thujone present in the crude *Salvia officinalis* extract (2.3 μg/ml). It was therefore concluded that inhibition of the CYP3A4 enzyme by the crude *Salvia officinalis* extract cannot be due to the thujone alone, as the concentrations of thujone in the extract were not high enough and hence other compounds in the crude *Salvia officinalis* extract must have contributed to the inhibitory effect of the extract.
4.6. Discussion

The crude *Salvia officinalis* extract inhibited the CYP3A4-mediated metabolism of the substrate 7-BFC in a dose dependent manner. Quenching of the fluorescent signal, which could mimic inhibitory activity, was very minor (~10%) for the crude *Salvia officinalis* extract, the *n*-hexane- or the CHCl₃ SE. The same was observed for the intrinsic fluorescence, which could have masked inhibition of the CYP3A4. It was only observable for the highest concentration of those three extracts and in moderate ranges (~10-20%). Both effects did not affect the assessment of the inhibitory potential of the crude *Salvia officinalis* extract, the *n*-hexane or the CHCl₃ SE. The most potent inhibitor of CYP3A4 was the *n*-hexane SE (IC₅₀ 1.8 µg/ml), followed by the CHCl₃ SE (IC₅₀ 3.1 µg/ml) and the crude *Salvia officinalis* extract (IC₅₀ 6.8 µg/ml).

Only the two highest tested concentrations of the aq-EtOH extract (625 µg/ml and 208 µg/ml) showed inhibition of CYP3A4, but complete inhibition (0% CYP3A4 activity) of CYP3A4 was not observed at the tested concentrations. The highest concentration of the aq-EtOH subextract, however, showed quenching effects (mimicking inhibition), and the concentrations 625 µg/ml and 208 µg/ml showed strong (up to 50%) intrinsic fluorescence. Although the raw data were corrected for intrinsic fluorescence, a strong effect around 50% could have an influence on the results. Further, the quenching effect of the aq-EtOH SE may have mimicked inhibition observed and that could have interfered with the accurate determination of the IC₅₀ value (Zou et al., 2002; Modarai, 2008). Hence, it is not certain, whether the aq-EtOH SE shows weak inhibitory potential against CYP3A4. Accordingly, it is difficult to say whether the observed effect can be solely explained by quenching of the fluorescent signal or whether an effect might have been masked by the intrinsic fluorescence of the aq-EtOH SE.

The crude *Salvia officinalis* extract, the *n*-hexane- and the CHCl₃ SE showed complete inhibition of the CYP3A4 enzyme (0% CYP3A4 activity) in a dose-dependent manner *in vitro* and as the observed EC₅₀ values are relatively low, they may potentially cause herb-drug interactions when taken along with conventional medicines that are metabolised by CYP3A4.

The hypothesis that thujone contributed substantially to the observed effects was not confirmed, as the inhibitory potencies of either a purified α-thujone or a α/β-thujone mixture were generally very weak (no inhibition below ~100 µM observed) or absent at the concentration present in the crude *Salvia officinalis* extract.

The IC₅₀ values estimated by regression analysis for α-thujone and the α/β-thujone mix were 117 µM (18 µg/ml) and 146 µM (22 µg/ml) respectively (Fig. 4.5). Abass *et al.*
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(2010) recently reported IC$_{50}$ values of 15.4 μM for inhibition of CYP2A6 and 17.5 μM for inhibition of CYP2B6 by α-thujone. Other studies investigating the inhibitory potential of the thujone diastereomers have to our knowledge not been published. Hence, it can be concluded that thujone diastereomers are metabolised by CYP3A4 (Hold et al., 2001), but their inhibitory potential against the CYP3A4 enzyme is generally weak and 10 times lower than the inhibitory potential against CYP2A6 and 2B6 (Abass et al., 2010).

Utilising cDNA expressed CYP enzymes for inhibition studies is straightforward, relatively simple, shows good reproducibility and is therefore a commonly used in vitro method. However, as the different enzymes, which are involved in human drug metabolism, are not expressed to the same level in vivo, extrapolation to physiological conditions requires normalisation to expression levels (Harper and Brassil, 2008). The other in vitro method, the human liver microsomes, mimic the physiological conditions by providing a physiological mixture of the different enzymes at their expression levels. That however has other previously named drawbacks and further introduces variations to the test system, as significant variability in CYP enzyme levels has been observed in human liver microsomal preparations from different donors (Yeo et al., 2004; Harper and Brassil, 2008).

Ideally further experiments that characterise the effect of the crude *Salvia officinalis* extract on CYP3A4 should include in vitro methods utilising hepatocytes, because the intact cells provide a physiological level of cofactors and enzymes, metabolic pathways and active gene expression which makes them the model closest to in vivo (Donato and GasteU, 2003).

The results obtained for the crude *Salvia officinalis* extract indicate a relatively strong inhibitory potential. Hellum et al. (2008) tested an aqueous *Salvia officinalis* extract in the same supersome enzyme preparation of CYP3A4 and reported only moderate inhibitory effects (IC$_{50}$ 483 μg/ml). This result is similar to our findings for the aq-EtOH SE (EC$_{50}$ 281.2 μg/ml). In their study a dried HMP was used and re-dissolved in water, which was used for the initial extraction process by the producer, and the concentrations tested in the assay ranged from 0 to 3 mg/ml.

Beside the possibility that differences in the plant materials could cause variations in the obtained extracts, the different extraction solvents may be a reason for the different outcomes in the in vitro experiments. The findings in this thesis highlight that the relatively non-polar SE (n-hexane- and CHCl$_3$ SEs) show higher inhibitory potential against the CYP3A4 enzyme than the polar SE (aq-EtOH SE). The polar, aqueous
extract tested by Hellum et al. (2008) may be low in the non-polar constituents, which seem to be responsible for the inhibitory effect of *Salvia officinalis* extracts.

Comparing our results with other published data on HMP, in order to make qualitative conclusions in respect to potency is rather difficult, as most of the studies conducted with HMP do not present dose-response curves, hence rarely report IC$_{50}$ values. Mostly the total CYP enzyme inhibition is given in percent and often the exact concentration of the extract present in the reaction volume is not clearly stated (Budzinski et al., 2000; Foster et al., 2001).

However, a few other studies testing HMP in the same *in vitro* model were published and allow classifying the observed inhibitory potency in relation to their results. Modarai et al. (2007) considered their reported IC$_{50}$ values for an *Echinacea purpurea* preparation of 22.18 µg/ml as weak inhibitory potential. Hellum and Nilsen (2008) on the other hand believe that a St. John's Wort preparation with an IC$_{50}$ value of 15.4 µg/ml may be an issue for herb-drug interactions in the small intestine. With an IC$_{50}$ value of 6.8 µg/ml, the potency of the crude *Salvia officinalis* extract was twice as high as the potency reported for the St. John's wort preparation.

The interactions of St. John's Wort however are a fairly complex matter and represent an excellent example for the fact that *in vitro* data cannot necessarily be transferred to the *in vivo* situation. *Hypericum perforatum* (St. John's Wort) extracts were found to activate CYP3A4 *in vivo* (Madabushi et al., 2006), but pure constituents of the extract, including hyperforin, were found to inhibit CYP3A4 *in vitro* (Unger, 2004).

Overall the results obtained in this thesis indicate that the inhibitory potential of the crude *Salvia officinalis* extract against CYP3A4 warrants further investigation. Inhibition and induction studies in hepatocytes may give useful information for further *in vivo* studies that are necessary to find out whether the inhibitory activity can be confirmed *in vivo*, or whether inhibitory active constituent of *Salvia officinalis* may behave similar to *Hypericum perforatum* constituents and the *in vitro* results may not be relevant *in vivo*.
5. Inhibitory potential of *Salvia officinalis* against acetylcholinesterase (AChE) enzyme

Women seem to suffer from a higher risk of developing Alzheimer's disease (AD) (Launer *et al*., 1999) and the withdrawal of endocrine hormones after the menopause is thought to be responsible for this tendency (Shumaker *et al*., 1998; Resnick *et al*., 2004). AD is a neurodegenerative disease, accompanied by a deficiency in cholinergic activity in the brain. The aetiology is still unknown, but various factors, e.g. oxidative and inflammatory processes and neurotransmitter disturbances, are thought to influence the risk of developing the disease or its progression. Treatments to achieve a slow down of the progression or prevent the occurrence of the disease, such as non-steroidal anti-inflammatory drugs, antioxidants like Vitamin E and C, neurotropins (nerve growth factors) and hormone replacement therapy (HRT), are being investigated (Castellani *et al*., 2010).

Currently drugs like donezepil hydrochloride (Aricept®), rivastigmine (Exelon®), galanthamine (Reminyl®) and memantine (Ebixa®) are the main agents used to treat the progression of AD. Memantine is a N-methyl-D-aspartate receptor antagonist and the others are all AChE inhibitors (Alzheimer's Society, 2011). A loss of cholinergic function has been found to be closely related to cognitive dysfunction and the class of the AChE inhibitors are the best developed therapeutics to slow down the progression of AD (Castellani *et al*., 2010). The natural product galanthamine is derived from the bulbs of the common snowdrop and several Amaryllidaceae species. It has been considered as a safe treatment and was shown to be preferred by patients over donezepil and rivastigmine (Van Puyvelde and Mets, 2011).

It also has been suggested that phytoestrogens improve cognitive function. However, the effects and the mode of action of phytoestrogens on AD are still unclear (Houghton and Howes, 2006).

Neuroprotective and AChE inhibitory activities of different *Salvia* sp. have been described (Houghton and Howes, 2005), and estrogenic (Perry *et al*., 2001), antioxidant (Ben Farhat *et al*., 2009) and anti-inflammatory (Baricevic *et al*., 2001) activities are also known. The reported benefits of *Salvia officinalis* for cognitive function (Akhondzadeh *et al*., 2003; Scholey *et al*., 2008) may be related to one or more of these properties. Many compounds which have been identified as AChE inhibitors are alkaloids, terpenoids or coumarins (for review see (Mukherjee *et al*., 2007)). The triterpenoid ursolic acid,
isolated from *Origanum majorana*, which is also present in *Salvia officinalis*, showed AChE inhibitory potential (Chung *et al.*, 2001). It has been suggested that there is a link between the antioxidant and the estrogenic activities of *Salvia* species (Perry *et al.*, 2000a) and it is conceivable that a link between AChE inhibitory potency and estrogenic potency exists. Thus, a triterpenoid compound similar to ursolic acid might inhibit the AChE and the same compound may possess estrogenic activity. In order to investigate a possible structural link, the potential of the crude *Salvia officinalis* extract to influence cognition by acting estrogenic was assessed in this thesis (chapter 7) and its potential to inhibit the AChE was assessed in the following sections. In order to elucidate whether the extract has the potential to act as an AChE inhibitor, the crude extract and its subextracts (SE) were tested in a microtiter plate assay based on the colourimetric method first described by Ellman (Ellman *et al.*, 1961).

### 5.1. Acetylcholinesterase

AChE is an enzyme which hydrolyses the ester bonds in acetylcholine (ACh). ACh is a neurotransmitter by which electrical impulses are transmitted between nerve cells or nerve cells and muscles. The major receptors that are involved in this transmission are the muscarinic and nicotinic receptors. Muscarinic receptors stimulate the parasympathetic nervous system, whereas the nicotinic receptors are found in the CNS and in the motor end plates, the connections between the nerves and skeletal muscle (for review see (Houghton *et al.*, 2006)).

Stimulation of the nicotinic receptors in the CNS is related to cognitive processes and memory. When ACh, which is stored in vesicles in the nerve endings, is released, it enters the synapse and binds to the nicotinic receptor (Fig. 5.1). In the synapse, the hydrolysing enzyme AChE is present in large amounts and the half-life of ACh in the synapse is therefore very short (Silman and Sussman, 2005).

![Figure 5.1: Release of ACh into the synaptic cleft. (adapted from (WPC, 2000; HUJ, 2006)).](image-url)
The cholinergic hypothesis, which associates AD symptoms with cholinergic deficiency, is supported by neurochemical and neuroanatomical studies. These suggest that the cholinergic neurons projecting to the neocortex and hippocampus are those predominantly affected by AD. One effective way to stabilise the cholinergic neurotransmission by increasing the ACh levels in the synaptic cleft, is to inhibit the AChE (Greenblatt et al., 2004).

Isolated AChE has been used in in vitro studies for many years. The electric organ of the electric eel (Torpedo californica, Tc) is a good source for stable AChE enzymes of high purity and with good activity (Rothenberg and Nachmansohn, 1947). The structure of the TcAChE is very similar to that of the mammalian AChE (Bourne et al., 1995) and has therefore been used for most of the published in vitro work. The widely used method by Ellman employs AChE isolated from bovine erythrocytes (Ellman et al., 1961), but homogenates of human brain tissue (Perry et al., 1996) and enzymes from human erythrocytes are also used to assess enzyme inhibition in vitro. Other isolated AChE enzymes are BfAChE derived from the snake Bungarus fasciatus (Bf) (Cousin et al., 1996) or the fruit fly Drosophila melanogaster (Harel et al., 2000). Neither, however, is used to identify potential new drugs for the treatment or prevention of AD.

Another enzyme, which has attracted some attention, is the human butyrylcholinesterase (BuChE). Its structure is very similar to that of the mammalian AChE and the TcAChE (Vellom et al., 1993). No clear physiological function has yet been assigned to BuChE (Masson et al., 2009), but BuChE-positive neurons may play a role in attention, executive function, emotional memory and behaviour (Lane et al., 2006). Furthermore, BuChE activity progressively increases as the severity of dementia advances, while AChE activity declines (Perry et al., 1978), and inhibition of BuChE may therefore provide additional benefits for AD patients (Lane et al., 2006).

5.2. Plant-derived inhibitors of the AChE

Studies investigating AChE inhibitors from natural sources have focused on alkaloids and most of the plant-derived inhibitors belong to this class of natural products. Amongst those are the well-studied compounds physostigmine, galanthamine and huperizine A.

Physostigmine, isolated from the Calabar beans (seeds of Physostigma venenosum, Fabaceae), is the prototype of the AChE inhibitor. In order to be suitable for treatment of AD, transport through the blood-brain barrier needed to be improved and several more lipophilic compounds related to physostigmine have been synthesised, one of which is rivastigmine (Exelon®) (for review see (Houghton et al., 2006)).
Galanthamine, a widely used drug (Reminyl®) in AD treatment today, was initially isolated from snowdrops (Galanthus spp., Amaryllidaceae). Today, other members of the same plant family are used as a natural source, which include daffodils (Narcissus spp.) and snowflakes (Leucojum spp., esp. Leucojum aestivum), but most of the drug is obtained synthetically. In 1951 a Russian pharmacologist showed the AChE inhibiting properties of galanthamine for the first time and soon after, in 1952, its structure was identified. Galanthamine was licensed for treatment of AD in 2000 (for review see (Heinrich, 2010)).

The club moss Huperzia serrata is one the natural sources of huperzine A and has been used in traditional Chinese medicines for the treatment of AD. It has been shown in clinical trials that huperzine A promotes the improvement of general cognitive function of AD patients. However, it is currently not used for treatment of AD and further clinical trials are needed (Li et al., 2008).

The in vitro activity of several other plant-derived alkaloids against AChE has been reported. Other classes of compounds with inhibitory activity include terpenoids (especially monoterpenoids for Salvia species), glycosides and coumarins. The diversity of plant families which are a source of AChE inhibitors is high and include Amaryllidaceae, Compositae, Euphorbiaceae, Lamiaceae, Rutaceae, Solanaceae and many more (reviewed by (Houghton et al., 2006; Mukherjee et al., 2007)).

5.3. The potential of Salvia species for treatment of Alzheimer’s disease

Several Salvia species have been investigated for their inhibitory potential against AChE in vitro or for their influence on cognition in clinical trials (Akhondzadeh et al., 2003). The essential oil of aerial parts of Salvia lerifolia showed inhibitory activity against AChE in vitro with an EC₅₀ value of 0.32 μl/ml (Loizzo et al., 2009). The essential oil of Salvia lavandulaefolia leaves was found to inhibit the activity of the human brain tissue derived AChE at 0.1 μl/ml by over 60% (Perry et al., 1996) and the human erythrocyte AChE with an EC₅₀ value of 0.03 μl/ml (Perry et al., 2000b). This activity was confirmed by in vivo experiments, which showed a reduced AChE activity in the striatum of rats after treatment with essential oil of Salvia lavandulaefolia (Perry et al., 2002). The roots of Salvia miltiorrhiza contain compounds with AChE inhibitory potential, of which the isolated dihydrotanshinone and cryptotanshinone were the most potent ones (IC₅₀ 1.0 μM and 7.0 μM respectively) (Ren et al., 2004). Further the essential oil of Salvia officinalis was found to inhibit the AChE enzyme over 50% at a concentration of 0.1 μl/ml and
inhibition was also found by the 80% EtOH extracts of the *Salvia officinalis* leaves (dried and fresh). The extract of dried *Salvia officinalis* leaves inhibited the enzyme activity by over 60% (2.5 mg/ml) and the extract obtained from fresh leaves inhibited the enzyme by over 45% at 2.0 mg/ml (Perry *et al.*, 1996). The inhibitory potential of the essential oils of *Salvia officinalis* leaves from different sources was later confirmed with IC_{50} values ranging between 0.05 - 0.07 mg/ml (Savelev *et al.*, 2004).

The potential of *Salvia officinalis* leaf extracts to influence cognition of AD patients was reported by Akhonzadeh *et al.* (2003), who observed a significantly better outcome in a placebo controlled clinical trial, where patients were treated with 60 drops of a *Salvia officinalis* extract (45% ethanolic). Scholey *et al.* (2008) reported an improved cognitive function in healthy older individuals after treatment with a standardized 70% ethanolic *Salvia officinalis* leaf extract, which also inhibited the AChE enzyme in *in vitro* studies.

### 5.4. Methodological considerations — *in vitro* identification of potential AChE inhibitors

Inhibition of AChE has been investigated *in vitro* for more than 50 years. In order to screen possible inhibitors of the enzyme, several assays have been developed over the years. Commonly used methods for the assessment are based on the method by Ellman (*Ellman et al.*, 1961). The basic principle of the method is the measurement of thiocholine, which is produced through enzymatic reaction with AChE. The substrate acetylthiocholine is hydrolysed and the obtained hydrolysis product undergoes a continuous reaction with the 5,5'-dithiobis-2-nitrobenzoate ion (Ellman's reagent, DTNB) to produce a yellow compound, 5-thio-2-nitro-benzoic acid, which is detected at 405 nm either after or throughout a pre-determined time period (Fig. 5.2).

![Figure 5.2: Acetylthiocholine (1) and DTNB (2) form after enzymatic hydrolysis the yellow-coloured 5-thio-2-nitro-benzoic acid (3).](image)

Initially, the standard visible spectroscopy procedures used for detection required cuvettes, which made the assessment of large numbers of possible new inhibitors of the AChE difficult, as several ml of reaction volume were needed (Houghton and Howes, 2006).
In order to use less material and test larger numbers of samples in a shorter period of time, the method has been adapted for screening in microtiter plate format (Rhee et al., 2001; Brühlmann et al., 2004). The smaller scale and the higher throughput also allows the possibility of on-plate positive and solvent controls and running several replicates for each determination, which improves statistical treatment of results (Brühlmann et al., 2004). Ellman's method has also been adapted for thin layer chromatography (TLC) (Kiely et al., 1991; Rhee et al., 2001), where samples are run on TLC plates in the usual way and sprayed with a mixture of acetylthiocholine and DTNB after application of a solution of AChE. Samples which inhibit the enzymatic hydrolysis show pale to white inhibitory zones against the yellow-orange background on the plate. Rhee et al. (2003b) have addressed the possibility of false-positives, as inhibition of the reaction between thiocholine and DTNB by naturally occurring compounds in an extract has been observed. Detection of false-positives is possible by running a control plate where the enzyme solution is omitted.

A similar TLC based method utilises the enzymatic hydrolysis of 1-naphthyl-acetate. The formation of the resulting naphthol is quantified by measuring the formation of a purple-coloured diazonium dye with the Fast Blue salt reagent (Marston et al., 2002).

A high throughput chemiluminescent assay for the detection of AChE inhibition, with lower detection limits than the colourimetric assays, has been developed recently (Guardigli et al., 2005). The detection of inhibition of AChE is based on a series of coupled enzyme reactions, involving AChE, choline oxidase and horseradish peroxidase, and is quantified by measurement of the resulting chemiluminescent emission.

HPLC-coupled on-line detection methods, such as a colourimetric flow assay based on Ellman’s method (Ingkaninan et al., 2000) and a fluorometric flow assay method using a direct fluorogenic substrate (Rhee et al., 2003a), have also been developed.

In the first HPLC method, reagents are mixed continuously and the absorbance of the yellow product, the 5-thio-2-nitrobenzoate, is detected at 405 nm. Injection of an AChE inhibitor reduces the formation of the yellow product and the change in the absorbance reading is used to quantify the inhibition of the enzyme. This method allows simultaneous separation and detection of bioactive compounds from complex mixtures (Ingkaninan et al., 2000).

In the second one, the fluorometric flow assay, the fluorogenic substrate 7-acetoxy-1-methyl quinolinium iodide is hydrolysed by AChE to give 7-hydroxy-1-methyl quinolinium iodide, a highly fluorescent compound, which allows measurement of the AChE inhibition fluorometrically at an excitation wavelength of 405 nm and an
emission wavelength of 505 nm. The assay proved to have higher sensitivity compared to the colourimetric flow assay, as the detection limit for galanthamine was about 20 times lower than the method based on UV detection. Finally, the fluorometric flow assay has a minimised risk for false-positives, as the product of the enzymatic reaction is measured as such and no further chemical reaction is needed for its quantification (Rhee et al., 2003a).

The high sensitivity and the simultaneous separation of inhibitors are advantages of the on-line methods. However, these systems are more suitable for the screening of larger numbers of samples, as the set-up of the system is time-consuming and costly. Furthermore, the presence of fluorescent compounds with similar excitation and emission wavelengths to the product of the enzyme reaction can mask the inhibitory activity and there is the possibility that those compounds are present in complex mixtures like crude plant extracts. The TLC based methods seem to be more suitable for an initial screening of crude plant extracts. Potentially interesting extracts can be identified in relatively short time and with small effort and initial information about the chemical nature of the active compound can be predicted. However, the TLC plate may introduce a subtle change of the 3D structure of the enzyme, as adsorption of AChE onto silica plates may result in a change of conformation of the enzyme and thereby alter accessibility of the enzyme for inhibitors. Additionally, there is a chance that the activity of possible AChE inhibitory compounds in the extract is lost as a result of the conditions used to develop the plate (Di Giovanni et al., 2008).

The microplate assays are water-based and only minimal amounts of solvents (if any) are needed to dissolve the test samples. The aqueous milieu, however, is a difficulty of the enzyme assays performed in solution, as it limits the choice of a suitable co-solvent to solubilise test compounds. However, the microtiter-plate format allows the assessment of IC_{50} values, whereas TLC based assays give minimal inhibitory concentrations. It further shows a good reproducibility and a higher throughput. Both features are desirable for an in vitro method and especially when it is to be used for activity-guided isolation. The microtiter plate assay, based on the method by Ellman, was therefore chosen for the assessment of the inhibitory potential of the crude Salvia officinalis extract and the subextracts (SEs) against AChE.
5.5. Experimental procedure – the AChE inhibition assay

5.5.1. Chemicals, reagents, solutions

AChE from *Electrophorus electricus* (electric eel) Type V-S, lyophilised powder, (≥1,000 U/mg protein), galanthamine hydrobromide (≥ 94% pure) and physostigmine (≥ 98% pure) and all other chemicals or reagents were purchased from Sigma-Aldrich Company Ltd., Dorset, UK unless stated otherwise.

Enzyme stock was prepared in Tris buffer (50 mM, pH 8) with 1.000 units/ml. All test solutions were prepared in Tris buffer (50 mM, pH 8) with 66% EtOH. Positive controls were prepared in 10% methanolic Tris buffer (50 mM, pH 8). Stocks were stored at 4°C.

5.5.2. The AChE inhibition assay

The AChE inhibition assay is based on the method first described by Ellman (Ellman *et al.*, 1961). Ellman’s cuvette assay has been modified in order to adjust it to the high throughput microtiter plate format and the method described by Rhee *et al.* (2001) was followed, with minor modifications. Briefly, for the microplate assay, 25 µl Tris buffer (50 mM, pH 8) was added to all test wells (duplicate columns of 6 wells/sample). 25 µl of test solution were added to the first wells of the columns and by removing 25 µl from those wells and adding them to the next column a 1:2 serial dilution was performed. Subsequently, 50 µl of Tris buffer, 25 µl acetylthiocholiniodide solution (15 mM, deionised water) and 125 µl Ellman’s reagent (5,5’-dithiobis-2-nitrobenzoic acid, DTNB, 3 mM) in Tris buffer containing 0.1 M sodium chloride (0.01 M) and magnesium chloride (0.02 M) were added to each well of the clear polystyrene 96 well plates (Falcon, BD Bioscience, UK). The plates were mixed on the plate shaker for 2 min in the dark, before the absorbance (background reading, spontaneous hydrolysis of substrate) was read at 405 nm on a plate reader (Labsystem Multiskan Multisoft). Subsequently, 25 µl of AChE solution (0.22 U/ml in 50 mM Tris buffer (pH8) containing 0.1% bovine serum albumin) were added to give a final enzyme concentration of 0.02 U/ml. Plates were again mixed in the dark for 2 minutes on the plate shaker and read at 405 nm to determine the development of the resulting, yellow anion 5,5’-thio-2-nitro-benzoic acid. Solutions of acetylthiocholiniodide, DTNB and AChE were freshly prepared. Solvent controls (3.6% EtOH/well) and on-plate positive controls (galanthamine, 2 fold dilution series, 0.7 µM to 100 µM) were included on each plate to control whether the assay was performing well.
5.5.3. Calculation of the inhibition of the AChE enzyme and statistical approach

Inhibition of AChE was quantified by subtraction of corresponding background absorbance from raw data, before resulting values were divided by the average of the on-plate solvent controls \((n = 6)\). The application of *in vitro* assays based on the quantification of a reaction product via UV absorbance is limited by the presence of other compounds with a similar absorbance maximum or by intensely colourful samples such as some herbal extracts. The Lambert-Beer Law, which states a linear relationship between concentration and absorbance, applies only to diluted solutions (Rucker *et al.*, 1988). In order to obey the Lambert-Beer Law, absorbance values \(>1\) were excluded from the data analysis. There was variation in the data from the AChE inhibition assay, and therefore the statistical significance of inhibition of the enzyme by test samples had to be determined by using statistical hypothesis testing methodologies. The one-sample *t*-test was used; this was justified because the inhibition assay read-outs were normally distributed. A significance level of 5% was regarded as being statistically significant.
5.6. Results

5.6.1. Evaluation of different solvent controls

Solubility problems with the standard solvent used for the samples in the AChE assay were observed with the dried crude *Salvia officinalis* extract and the SE. The solvent consisted of Tris buffer (50 mM, pH 8) containing 10% MeOH (equal to 0.9% MeOH/well) (Rhee et al., 2001). In order to find a solvent which dissolves the samples, but shows no inhibition of the enzyme, the influence of concentrations of various solvents was evaluated. The influence of DMSO (1.8% and 2.7%) or EtOH (3.6% and 6%) on the AChE activity was evaluated and compared to the effect of the standard solvent (0.9% MeOH) (Fig. 5.3). DMSO at concentrations of 1.8%/well and 2.7%/well inhibited the AChE. The same applied for EtOH at 6.0%/well. 3.6% EtOH/well did not exhibit any inhibitory effect on the enzyme and AChE showed even a slightly higher activity than with the standard solvent concentration of 0.9% MeOH/well. A maximal concentration of 3.6% EtOH/well was chosen for all AChE inhibition experiments presented in this thesis.

![Figure 5.3: Effects of solvents on the AChE activity](image.png)

Figure 5.3: Effects of solvents on the AChE activity. Different concentrations of DMSO and EtOH (in %/well) were compared to the commonly used solvent concentration of 0.9% MeOH/well. The dotted line represents activity of AChE enzyme which was considered as 100%. Data are from at least 8 replicates. Error bars represent 95% confidence intervals.

5.6.2. Galanthamine and physostigmine

Galanthamine and physostigmine (0.7 μM to 100 μM), both potent AChE inhibitors from natural sources, were tested to monitor the performance of the enzyme and the assay (Fig. 5.4). Both AChE inhibitors showed dose-dependent inhibition of the AChE enzyme and confirmed that the assay was performing well. Results were obtained in
four independent experiments. Data variation for galanthamine results was smaller than for physostigmine, hence it was chosen as the on-plate positive control to monitor the performance of further assays with test extracts. The dose-response curves for galanthamine and physostigmine did not reach inhibition values lower than \(~30\%\) (galanthamine) or \(~20\%\) (physostigmine), as the lowest concentrations tested were too high to show no inhibition of the enzyme. The IC_{50} values (3.18 \(\mu\text{M}\) for galanthamine and 8.7 \(\mu\text{M}\) for physostigmine) were in good agreement with those published in the literature for the inhibition of the AChE derived from the electric eel (Rhee et al., 2001; Rhee et al., 2003b; Kivrak et al., 2009; Pereira et al., 2010).

Figure 5.4: AChE inhibition by positive controls: A Galanthamine (cyan) and B physostigmine (blue). Data are from four independent experiments, tested in duplicate. Error bars represent 95\% confidence intervals.

### 5.6.3. The crude *Salvia officinalis* extract and the subextracts

The crude *Salvia officinalis* extract was tested for AChE inhibitory potential at concentrations ranging from 3.4 to 216 \(\mu\text{g}/\text{ml}\) (Fig. 5.5). The \(n\)-hexane- (2.3 to 37.5 \(\mu\text{g}/\text{ml}\)), \(\text{CHCl}_3\) - (1.9 to 120 \(\mu\text{g}/\text{ml}\)) and aq-EtOH SE (10.7 to 340.9 \(\mu\text{g}/\text{ml}\)) were also tested, but, because of the varying self-absorbing properties, different concentrations were chosen (Fig. 4.5). The colour of the crude *Salvia officinalis* extract, for example, is of very strong intensity and its deep, dark-brown appearance interfered with the measurement of the absorbance at 405 nm; hence test concentrations much higher than 200 \(\mu\text{g}/\text{ml}\) for the crude *Salvia officinalis* extract did not obey the Lambert-Beer Law. The same reasoning was applied to choose the highest test concentrations of the SE.

A very moderate inhibitory potential against the AChE was observed for the crude *Salvia officinalis* extract (\(~10\%\) at 108 \(\mu\text{g}/\text{ml}\) and 216 \(\mu\text{g}/\text{ml}\)) and the \(\text{CHCl}_3\) SE (\(~5\%\) at 120 \(\mu\text{g}/\text{ml}\)). The aq-EtOH SE did not show any observable inhibition of the AChE and the \(n\)-hexane SE led to very slight increases in AChE activity, equivalent to "negative
inhibition. The one-sample t-tests confirmed the absence of statistically significant inhibition by the extracts, except for the crude *Salvia officinalis* extract at 108 μg/ml.

![Graphs showing AChE inhibition by different extracts](image)

Figure 5.5: AChE inhibition by the crude *Salvia officinalis* extract (red) and the n-hexane, CHCl₃ (green) and aq-EtOH (yellow) SE. Data are from at least three independent experiments, tested in duplicate. The effect of the positive control galanthamine (cyan) at 3.18 μM (IC₅₀ concentration) is presented next to the y-axis. Error bars represent 95% confidence intervals. *P<0.05, significant inhibition (>0).
5.7. Discussion

Inhibitory potential against the AChE enzyme was only statistically significant for the crude *Salvia officinalis* extract at 108 μg/ml. All other test concentrations or extracts did not show significant inhibition of the AChE in the AChE assay. Although statistically significant, the observed inhibition at 108 μg/ml is very small (<10%) and it furthermore seems likely that the result is due to chance or experimental error, as the next higher concentration did not show an inhibitory effect. The inhibition by the positive controls (galanthamine and physostigmine) was in good agreement with literature data and solvent controls (3.6% EtOH) did not show any inhibition against the AChE enzyme. Thus, the controls confirmed that the microplate assay performed well.

Our results could not confirm the inhibitory activity previously reported by Perry *et al.* (1996), as we were not able to test equally high concentrations of extracts in our assay. Perry *et al.* (1996) found inhibition of AChE enzyme by an 80% ethanolic extract of fresh and dried leaves of *Salvia officinalis* with 47% inhibition at 2.0 mg/ml and 68.2% inhibition at 2.5 mg/ml respectively. The extracts tested by Perry *et al.* (1996) were obtained by maceration of the plant material in 80% EtOH at room temperature for 3-14 days. The crude *Salvia officinalis* extract tested in this work was obtained by a similar process by using 60% EtOH in a maceration process over more than 10 days.

Even after considering variations during the experimental set-up (e.g. Perry *et al.* (1996) used homogenates of normal human brain tissue in a cuvette assay and not purified AChE from the electric eel in a microtiter plate assay), a similar inhibitory potential by the crude *Salvia officinalis* extracts would be expected. Inhibition at the tested concentrations used in this work, however, was observed, but it was very weak. Only inhibition values >20% were considered as active by Perry *et al.* (1996) and all observed inhibition of the AChE in our work was below this benchmark. It was not possible to test the crude *Salvia officinalis* extract for AChE inhibition at concentrations much higher than 200 μg/ml and therefore the test concentration of 2.0 mg/ml or 2.5 mg/ml tested by Perry *et al.* (1996) was not reached.

Most of the previous work on the inhibitory potential of *Salvia* species reported inhibitory potential of the essential oils obtained from *Salvia officinalis* (Savelev *et al.*, 2004), *Salvia lavandulafolia* (Savelev *et al.*, 2004), *Salvia potentillifolia* (Kivrak *et al.*, 2009), *Salvia leriifolia* (Loizzo *et al.*, 2009) or *Salvia fruticosa* (Savelev *et al.*, 2004). Further investigation of the individual activity of the major monoterpenoid constituents of *Salvia lavandulafolia* essential oil (e.g. borneol, pinene, camphor, 1,8-cineol) failed to identify a
particularly potent compound and a synergistic relationship of the essential oil constituents was proposed (Perry et al., 2000b).

The most non-polar, n-hexane SE of the crude *Salvia officinalis* extract, used in this thesis, should contain the major fraction of the essential oil constituents of the extract and also terpenoid compounds such as ursolic acid. It was therefore expected to find an inhibitory potential in this SE. However, there was no AChE inhibition detectable for this SE. The fact that the assessment of the n-hexane SE was restricted to particularly low concentrations by the limitations of the assay (the highest concentration tested was 37.5 µg/ml) may explain the absence of any noticeable inhibitory potential. Testing the n-hexane SE in the aqueous milieu of the assay was especially challenging, as precipitations were noticeable at higher concentrations. In addition, the SE was of very dark colour, and in order to obey the Lambert-Beer Law, tested concentration had to be low to avoid intensively coloured or turbid test solutions. The observed negative inhibition values could result from enzyme induction or be caused by compounds which interfered with the measurement of the UV absorbance. The induction of the AChE enzyme by other compounds in *in vitro* assays has, to our knowledge, never been reported and seems highly unlikely. Herbal extracts are known to contain several UV-interfering compounds (Cannell, 1998b). These compounds, however, should not have influenced the results, as high absorbance values (>1) were excluded and the raw data were normalised for background absorbance. Thus, the observed negative “inhibition” values, which were very low, may have resulted from general experimental variation. Since previous studies on *Salvia officinalis* gave indications that extracts/compounds exhibit inhibitory potency against AChE (Perry et al., 1996), further tests applying a method based on fluorescence (Rhee et al., 2003a) or chemiluminescence (Guardigli et al., 2005) might be a suitable alternative for the intensely coloured crude extracts and subextracts, which interfered with the measurement of the coloured anion 5-thio-2-nitrobenzoic acid at 405 nm. Overall it was not possible to identify inhibitory activity against AChE in this work. This would suggest that the tested crude *Salvia officinalis* extract doesn’t have any beneficial effects for AD treatment and it was therefore not possible to establish a link between possible estrogenic effects and AChE inhibition.
6. Evaluation of the serotonin re-uptake inhibitory potential of the crude *Salvia officinalis* extract

Serotonin (5-HT) re-uptake inhibition is the mechanism of action of a class of antidepressants, the selective serotonin re-uptake inhibitors (SSRIs). When the Woman’s Health Initiative (WHI) study (Rossouw *et al.*, 2002) raised concerns about risks of the classical hormone replacement therapy (HRT) (Carroll and Kelley, 2009), SSRIs became the focus of many clinical studies, which assessed alternative treatment options against hot flushes.

Women can be less tolerant to temperature changes around the menopause and as a result they experience hot flushes. The tolerance to temperature changes is reduced, because of a narrowed thermoregulatory zone (Fitzpatrick, 2004). Influencing the serotonergic system via the 5-HT receptors 5-HT\textsubscript{\textalpha}, and 5-HT\textsubscript{\textbeta} and the 5-HT transporters (5-HTT), which are generally involved in thermoregulation, is thought to be beneficial for the alleviation of hot flushes (Hedlund and Sutcliffe, 2004).

Paroxetine, sertraline, fluoxetine and citalopram are SSRIs, which increase the available concentration of central 5-HT by inhibiting the re-uptake of the neurotransmitter into the presynaptic neurons. Over the last years SSRIs, along with other antidepressants, have been studied in clinical trials for the relief of hot flushes, with promising results (Carroll and Kelley, 2009). SSRIs have been shown to be effective in reducing the frequency and severity of hot flushes (Soares *et al.*, 2008; Freeman *et al.*, 2011). Because the effect is not dose-dependent, low doses ensure good tolerability (Albertazzi, 2006).

The *Salvia officinalis* extract, studied in this thesis, also showed beneficial effects by reducing the hot flush frequency in a clinical trial (Bioforce, 2005). Although the efficacy of the SSRIs on hot flushes seems to be independent of their mood enhancing effects (Albertazzi, 2006), the reported beneficial effects of *Salvia* on depressions may be indicative of 5-HT related activities (Bartram, 1995; Reynolds, 1996; Duke, 2002). The anti-depressant effects of *Salvia* have not yet been investigated pharmacologically (Perry *et al.*, 2000a).

Furthermore, it has not been assessed whether *Salvia officinalis* has the potential to inhibit 5-HT re-uptake, nor whether the anti-hot flush effect of *Salvia officinalis* arises from its influence on the thermoregulation via the serotonergic system. Therefore, in order to elucidate the mode of action responsible for the anti-hot flush effect of the crude *Salvia officinalis* extract and the subextracts (SEs) were tested for 5-HT re-uptake inhibition *in vitro*.
6.1. 5-HT and its transporter

The monoamine neurotransmitter 5-HT is synthesised in the serotonergic neurons of the CNS (central nervous system, central 5-HT) and the enterochromaffin cells of the gastrointestinal tract (peripheral 5-HT). Its receptors are almost ubiquitously expressed, which results in manifold physiological roles of 5-HT in the human body (Jonnakuty and Gragnoli, 2008).

The peripheral 5-HT plays a role in platelet aggregation and in regulation of smooth muscles in the cardiovascular and gastrointestinal system. The central 5-HT is related to a variety of behaviour disorders including depression and anxiety and is known to influence a range of behavioural, physiological, and cognitive functions such as memory, mood, emotions, wakefulness, sleep, appetite, and temperature regulation (Jacobs and Azmitia, 1992).

5-HT is synthesised in the human body from the naturally occurring essential amino acid L-tryptophan via a series of reactions. The blood–brain barrier is impermeable to peripheral 5-HT, but not to 5-hydroxytryptophan, an intermediate product of the 5-HT synthesis. Hence, the concentration of central 5-HT depends on the availability of peripheral 5-hydroxytryptophan. Once produced, central 5-HT is stored in secretory granules and released from serotonergic neurons into a synapse. The action of central 5-HT is terminated by uptake via the 5-HTT, which are located in the presynaptic membrane, and returned to the presynaptic serotonergic neurons, where it is recycled or metabolised (for review see (Kriegerbaum et al., 2010)).

Although several 5-HT receptor subtypes exist, only one kind of transporter is known to be responsible for the clearance of extracellular 5-HT, the 5-HTT, also referred to as human serotonin transporter (hSERT). The 5-HTT belongs to the family of the \( \text{Na}^+ \) and \( \text{Cl}^- \) coupled, plasma membrane transporters, closely related to the norepinephrine and dopamine transporters. By re-uptaking the released 5-HT from the synaptic cleft into the presynaptic neurons, the transporters terminates the synaptic activity of the released 5-HT. 5-HTT antagonists, like SSRIs, limit the clearance of 5-HT from the synaptic cleft by simple diffusion or lower affinity transport pathways and elevate the 5-HT concentrations available for the 5-HT receptors by prolonging their presence in the synaptic cleft (Blakely et al., 1997).
6.2. 5-HT re-uptake inhibitors – plant derived alternatives?
The SSRIs are a relatively modern class of antidepressants. They are solely classified by their pharmacological mode of action and not like the older AD according their chemical structure. Currently there is no SSRI on the market, which is a natural product or just structurally related to a natural product.

SSRIs act within the brain to increase the amount of 5-HT in the synaptic gap by inhibiting its re-uptake. Several SSRIs have been tested as a non-hormonal treatment option for the most common menopausal symptom, the hot flushes. Paroxetine, sertraline, fluoxetine and citalopram are SSRIs, which are used for the relief of hot flushes. The same applies for venlafaxin and desvenlafaxine, which are selective serotonin and noradrenalin re-uptake inhibitors (SSNRIs), and therefore closely related (Carroll and Kelley, 2009).

Even though all available SSRIs are synthetic, 5-HT re-uptake inhibitors from natural sources with reported antidepressant effects and possibly 5-HT re-uptake inhibitory or general serotonergic effects are being investigated. Commonly used natural products for mild to moderate depressions include Hypericum perforatum (St John’s wort) preparations (Mennini and Gobbi, 2004). The mechanism behind the antidepressant effect of Hypericum perforatum is still not clear. Previously, it was reported that it is based on inhibition of the 5-HT re-uptake (Muller et al., 1997) and that most of the effect was due to its constituent hyperforin (Gobbi et al., 1999). However, further work led to the conclusion that the mechanism of hyperforin is different from that of the classic SSRIs and that direct interaction with 5-HTT is not the mechanism by which hyperforin affects 5-HT neurotransmission in vivo (Gobbi et al., 1999; Hirano et al., 2004; Mennini and Gobbi, 2004).

A few other studies assessed the potential to inhibit the 5-HT re-uptake of herbal extracts or single constituents isolated from extracts. 5-HT re-uptake inhibition by liquorice extract constituents was tested and the isoflavan glabridin, isolated from the acetone extract of the roots of Glycyrrhiza glabra, was found to show good inhibitory potency and inhibited the 5-HT re-uptake in a dose-dependent manner (Ofir et al., 2003). The antidepressant-like effect of aq-EtOH extracts from stems of the Kielmeyera coriacea Mart. (Clusiaceae, syn. “pau-santo”), used in Brazilian folk medicine, inhibits the re-uptake of 5-HT in a concentration-dependent manner in vitro, which suggests a mechanism similar to the SSRIs (Goulart et al., 2007).

In order to investigate the mode of action responsible for the anti-hot flush effect of Cimicifuga racemosa (black cohosh), ethanolic and methanolic extracts of the roots/rhizomes were tested for 5-HT re-uptake inhibition. An inhibitory effect of the
extracts was not observed and hence results could not confirm this mode of action for Cimicifuga racemosa (Powell et al., 2008).

6.3. The genus Salvia – known to inhibit the 5-HT re-uptake?

Salvia officinalis, Salvia lavandulaefolia and other Salvia species have been recommended in folk medicines for the treatment of depressions and anxiety (Bartram, 1995; Reynolds, 1996; Duke, 2002). Even so, the antidepressant activity of these subspecies has not been investigated thoroughly and available results for other species are sparse. An antidepressant-like effect of the essential oil of Salvia sclarea was observed in rats using the forced swim test, but was found to arise from dopaminergic, rather than serotonergic effects (Seol et al., 2010). Salah and Jager (2005) investigated activity on the 5-HT re-uptake inhibition by an aqueous and an ethanolic (70%) extract of Salvia triloba, but no significant activity was detected.

6.4. Methodological considerations

The serotonergic system can be influenced either by direct receptor interaction, agonistic or antagonistic activity on the 5-HT receptors or by influencing the transmembrane target proteins, the 5-HT transporter (5-HTT). Common assays used to investigate influences on the 5-HT pathways are competitive receptor binding assays using 5-HT receptor subtypes (Roth et al., 1994; Powell et al., 2008), intracellular cAMP assays (Albert, 1990; Powell et al., 2008), exchange or superfusion assays and re-uptake assays (Sitte et al., 2000; Rodriguez et al., 2003; Powell et al., 2008).

The 5-HT re-uptake occurs through specific transporter proteins, which have been cloned from several species (e.g. rat, Drosophila) including human (Ramamoorthy et al., 1993). Various cell lines, stably transfected with the human 5-HTT, were used in several studies investigating the binding potency of antidepressants like paroxetine, fluoxetine, citalopram, all SSRIs, but also of some tricyclic antidepressants or corresponding metabolites (Ramamoorthy et al., 1993; Tatsumi et al., 1997). The basic methods used for 5-HT re-uptake inhibition only vary by slight modifications or the employed cell lines.

To investigate whether the crude Salvia officinalis extract might have effects similar to SSRIs, we chose to assess the activity in an SSRI assay. The assay was chosen, because SSRIs are known to act through the 5-HTT and not through binding to the 5-HT receptors. Major differences between human 5-HTT and the Drosophila 5-HTT for substrate recognition and translocation have been shown (Rodriguez et al., 2003). Therefore, a HEK293 cell line stably transfected with the human 5-HTT (or hSERT) was used (HEK293-hSERT) to allow a better prediction of effects in humans. Further advantages of the assay are its simplicity and rapidity. In order to exclude false-positive
results, which includes reduced re-uptake of 5-HT due to cell death, the cytotoxicity was assessed as described in chapter 7.5 by determination of the mitochondrial activity of the viable cells (Mosmann, 1983).

6.5. Experimental procedure - the SSRI assay

6.5.1. Chemicals, reagents, solutions

Cell culture reagents were purchased from Invitrogen Ltd., Paisley, UK. Hydroxytryptamine Creatinine Sulfate (tritiated serotonin, 5-[1,2-^H(N)], 1 mCi) was purchased from Perkin Elmer, UK. 5-Fluoxetine hydrochloride (≥98% pure) and all other chemicals were from Sigma-Aldrich Company Ltd., Dorset, UK unless stated otherwise. Sample stocks were prepared in DMSO and kept in the freezer.

6.5.2. Routine cell culture - HEK293-hSERT cells

The cell line HEK293-hSERT was a kind gift from Dr. Randy D. Blakely of the Vanderbilt School of Medicine (Nashville). Cells were maintained in poly-D-lysine (50 mg/ml) coated 75 cm² flasks cell culture flasks (Greiner, Gloucester, UK) in Dulbecco's Modified Eagle Medium (DMEM with Glutamax) supplemented with 10% dialysed FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and Geneticin (0.8 µM) in a humidified incubator at 37°C, 5% CO₂. Media was replaced every other day and cells were sub-cultured, when the cells reached about 80% confluence. For sub-culturing, the cells were passaged by washing with 5 ml HBSS, followed by disaggregation with 1 ml 0.05% trypsin solution (containing 0.02% EDTA) to achieve a single cells in the suspension. The cell suspension was split into 75 cm² flasks.

6.5.3. The SSRI assay

The 5-HT re-uptake inhibition was assessed as described by Rodriguez et al. (2003) with minor modifications. The cells (100,000 cells/well) were seeded into poly-D-lysine (50 mg/ml) coated 24 well plates (Nunc, Thermo scientific, UK) and allowed to attach for 48 hours at 37 °C (5% CO₂, humidified incubator). The medium was removed and wells were washed with 200 µl wash-Krebs-Ringer-Hepes-buffer (wash-KRH) containing sodium chloride (120 mM), potassium chloride (4.7 mM), calcium chloride (2.2 mM), HEPES (10 mM), potassium dihydroxy phosphate (1.2 mM) and magnesium sulfate (1.2 mM) in endotoxin-free water (cell culture grade). Test samples were diluted with assay Krebs-Ringer-Hepes-buffer (assay KRH) identical to wash-KRH except for the addition of D-glucose (10 mM), ascorbic acid (100 µM) and paraglyne (100 µM). The highest DMSO concentration in the well was 1%. The test sample in assay-KRH solution was
applied (500 µl/well) and cells were incubated for 10 min at 37°C, before 5-HT solution (20.5 pmoles 5-HT and 0.1 pmoles \(^{3}\text{H}\)-5-HT) was added to each well. Plates were incubated for further 10 min. Washing three times with ice-cold wash-KRH terminated the re-uptake of 5-HT, before the cells were solubilised in 0.1% sodium hydroxide solution. Uptake of \(^{3}\text{H}\)-5-HT was quantified using scintillation counting (Beckmann LS6000IC, UK). On each plate assay blanks (20.5 pmoles 5-HT), solvent controls (1% DMSO) and positive controls (fluoxetine 100 µM) were included.

6.5.3.1. Quantification of the 5-HT re-uptake inhibition and statistical approach

Quantification of the re-uptake of \(^{3}\text{H}\)-5-HT was assessed using scintillation counting and 5-HT re-uptake was calculated as pmoles per minute per \(10^5\) cells seeded. The inhibition was calculated as percent of the average of on-plate assay blanks, which were corrected for non-specific uptake (in the presence of 100 µM fluoxetine). Results were plotted using Sigma Plot 8.02 (2002).

Significance of differences between solvent control (DMSO) and test sample activities were determined using one-sample t-test. \(P < 0.05\) was regarded as being statistically significant.

6.5.4. Cytotoxicity in the SSRI assay

In order to assess the cytotoxicity of the test samples on the HEK293-hSERT cells, the cells were treated as described in routine cell culture for HEK-293-hSERT cells (see section 5.5.2). Cells were seeded, incubated and treated in the same manner as described for the SSRI assay (see section 5.5.3) except for a 20 min incubation period with test samples (no 5-HT solution was added). After 20 min the assay-KRH was replaced by 250 µl media and additional 50 µl of MTT solution (5 mg/ml in HBSS) per well. Cells were incubated for 2 h to allow viable cells to cleave the yellow MTT salt. The cells were lysed afterwards with a 250 µl of solubilisation solution (described in 6.5.3). The plates were read at 570 nm (Labsystem Multiscan, UK).

6.5.4.1. Quantification of toxic effects

The raw data of the cytotoxicity assay were divided by the average of on-plate solvent controls and multiplied by 100, to express the results as % of viable cells compared to solvent controls. Bar charts were prepared using Sigma Plot 8.02 (2002). The significance of toxic effects of test samples compared to solvent control (DMSO) was determined using one-sample t-test. \(P < 0.05\) was regarded as being statistically significant.
6.6. Results

6.6.1. Implementation of the SSRI assay
In implementing the SSRI assay it was important to confirm that the HEK293-hSERT cells, were stably transfected and that they expressed the hSERT (5-HTT). It was also important to ensure that the SSRI assay was performing well in our hands. Therefore saturation concentrations, which for transporters are equivalent to the transport capacity ($V_{\text{max}}$), and 5-HT affinity ($K_m$), were determined. One hundred thousand cells/well were treated with increasing 5-HT concentrations (0.1 - 400 nM) and additional 0.1 pmoles $^3$H-5HT/well. The re-uptake was determined after 10 min of incubation at 37°C. The Michaelis-Menten model, which was used for the data analysis was:

$$Y = \frac{V_{\text{max}} \times X}{(K_m + X)}$$

$V_{\text{max}} = \text{maximal velocity}$

$K_m = \text{Michaelis-Menten constant or half maximal velocity (}V_{\text{max}}/2).\text{)}$

The $V_{\text{max}}$ obtained in five independent experiments (20 individual data points) was 4.7 ± 1.7 pmol/min/10^5 cells and $K_m$ value was 0.267 ± 0.19 $\mu$M. Both values are in agreement with literature data (Qian et al., 1997) and confirmed the stable expression of the hSERT (5-HTT) and the performance of the SSRI assay (Qian et al., 1997).

6.6.2. The crude *Salvia officinalis* extract and the subextracts
The inhibitory potential of the crude extract and the aq-EtOH SE was assessed at four different concentrations (4.6 $\mu$g/ml to 125 $\mu$g/ml, 1:3 dilution series). The $n$-hexane and CHCl$_3$ SE were tested at 31.25 $\mu$g/ml and 125 $\mu$g/ml. All data are shown as percent of maximal 5-HT re-uptake (assay blanks) (Fig. 6.1). Values around 100% re-uptake do not represent inhibitory effects on the 5-HT transporter. On-plate solvent controls (1% DMSO) and positive controls (fluoxetine, 100 $\mu$M) were used to monitor the performance of the assay throughout. Inhibition of the 5-HT re-uptake was observed in one experiment (underlined data points). All extracts showed inhibition of the 5-HT re-uptake around 20-40% in this experiment. The results of the other two or three repeats show a re-uptake of 5-HT of 100% or higher and therefore no inhibition of the 5-HT re-uptake. When the averaged data were tested for statistical significance by using the one-sample $t$-test, any statistically significant inhibition by the extracts was absent.
The on-plate solvent controls with 1% DMSO showed a great variability (data range 70% to 150%), whereas the positive controls (fluoxetine, 100 μM) showed much less variability in the data.

The observed inhibition of the 5-HT re-uptake (underlined data points) is within or very close to the range of the solvent controls and might therefore be due to experimental variations. Overall, none of the tested extracts showed strong, reproducible activity in the SSRI assay. The crude extract and the aq-EtOH SE were tested using a wider concentration range compared than employed with the n-hexane- or the CHCl₃ SE. Further experiments with the n-hexane- and the CHCl₃ SE at lower concentrations were not pursued, after the initial results did not indicate promising activity in the SSRI assay.
6.6.3. Cytotoxicity against HEK-293-hSERT cells

The cytotoxicity was assessed in order to avoid false-positive results for selective 5-HT re-uptake inhibition (Fig. 6.2). In case the extract exhibits toxic effects on the cells the measured 5-HT re-uptake would be lower than the 5-HT re-uptake in the presence of solvent controls.

![Cytotoxicity Results](image)

Figure 6.2: Cytotoxicity results for the HEK-293-hSERT cells in the presence of crude *Salvia officinalis* extract (A, dark red), the aq-EtOH (B, yellow), n-hexane (C, blue) or the CHCl<sub>3</sub> SE (D, green). Bars represent mean results for three independent experiments (except CHCl<sub>3</sub> SE at 31.25 μg/ml, which was tested in 2 independent experiments), each run in duplicate. Error bars represent 95% confidence intervals. *P<0.05, significant difference compared to solvent control.

The aq-EtOH SE did not show any toxicity against HEK293-hSERT cells and values for cell viability were around 100% or above. The higher concentration of the crude *Salvia officinalis* extract (125 μg/ml) reduced the cell viability to ~80% and the lower test concentration (41.6 μg/ml) to 90%. The CHCl<sub>3</sub> SE resulted in cell viability values around 70% (31.25 μg/ml) and 80% (125 μg/ml) and the n-hexane SE reduced the cell viability to ~80% at 31.25 μg/ml and even ~50% at the highest concentration of 125 μg/ml. According to the one-sample t-test the cytotoxicity reached statistical significance with the n-hexane SE at 125 μg/ml and with the CHCl<sub>3</sub> SE at 31.25 μg/ml.
6.7. Discussion

The kinetic experiments confirmed the expression of the human 5-HTT by the HEK293-hSERT cells and that the assay was performing well. Neither the crude *Salvia officinalis* extract nor the SEs showed statistically significant inhibition of the 5-HT re-uptake. The *n*-hexane SE (at 125 µg/ml) and the CHCl₃ SE (at 31.25 µg/ml) showed significant cytotoxicity. Cytotoxic effects only have an impact on results showing inhibition, but as inhibition was not observed, these effects did not bias the results of the SSRI assay. The end point in the SSRI assay is measured by scintillation counting and not spectrophotometrically and hence the dark colour of the extracts did not impact on the outcome of the method. The re-uptake inhibition by the positive controls fluoxetine (100 µM) reached values around 25% of 5-HT re-uptake, which represents the non-specific uptake of 5-HT by the HEK293-hSERT cells. The solvent controls (1% DMSO) showed a greater variability compared to the positive controls.

A common issue with herbal extracts is the questions whether activity can be detected in vitro by testing complex crude extracts or extracts obtained by a simple solvent-solvent partitioning. A study focusing on *Cimicifuga racemosa* extracts (75% EtOH and 100% MeOH) also tested whether the extracts targeted the 5-HTT and inhibit 5-HT re-uptake in HEK293-hSERT cells. Both extracts showed no ability to inhibit the re-uptake of 5-HT. However, after fractionation of the 100% MeOH extract, N₂-methyl serotonin was isolated and exhibited 5-HT₁ receptor binding activity at picomolar levels, cAMP induction activity at nanomolar levels, and SSRI activity in the micromolar range (Powell et al., 2008). On the other hand, an aq-MeOH extract of *Hypericum perforatum* was found to inhibit 5-HT re-uptake with an IC₅₀ value of 7.9 µg/ml (Gobbi et al., 1999). The findings showed that the SSRI assay is a suitable method for the purpose of investigating the influence of herbal extracts on the 5-HT system and that possible inhibitors of the 5-HTT can be identified. However, for the *Hypericum perforatum* extract it was found afterwards that the re-uptake inhibition did not result from a blockade of the 5-HTT, as the extract did not inhibit citalopram binding in further experiments. Gobbi et al. (1999) obtained further insights with several different assays and finally concluded that other, still unknown mechanisms are possibly involved in the antidepressant effects of *Hypericum perforatum*. A study using the forced swimming test in mice also came to the conclusion that the mode of antidepressant action of *Hypericum perforatum* differs from that of SSRIs (Hirano et al., 2004).

Overall, the results presented in this chapter do not suggest that 5-HT re-uptake inhibition is involved in the anti-hot flush effect of the crude *Salvia officinalis* extract.
7. CHAPTER: Defining the estrogenic potency – Estrogen receptor α activation by the crude *Salvia officinalis* extract and its subextracts

The majority of women use treatments or products for menopausal symptoms which possess estrogenic potency. The efficacy of estrogenic anti-hot flush therapy has been shown in a large number of studies (NAMS, 2010). Classical hormone replacement therapy (HRT) and phytoestrogens stimulate estrogenic response by activating the estrogen receptors (ERα and β). For example, the anti-hot flush effect of the hop-derived compound 8-prenylnaringenin, one of the most potent phytoestrogens, has been shown to be ER mediated in a rat model, when ERα or β specific antagonists were able to block the observed effects (Bowe *et al.*, 2006).

Although phytoestrogens possess many other pharmacological activities, e.g. influencing various key enzymes of the steroid metabolism, exhibiting anti-inflammatory, anti-atherosclerotic and anti-allergic activities (Beck *et al.*, 2005), all phytoestrogens mimic endocrine estrogen action (Ososki and Kennelly, 2003). Whether this may be the only activity of relevance for the anti-hot flush effect, remains unproven. However, the activation of the ER is probably the most important target of the investigations into the anti-hot flush effect of the crude *Salvia officinalis* extract.

In this chapter the ability of the crude *Salvia officinalis* extract and the subextracts (SE) to activate the ERα was investigated. Two different in vitro assays, both based on human cell lines (MCF7 and T47D-KBluc cells), were utilised to answer this question. The ability to induce estrogenic response by activating the ERs is widely accepted as a method to determine compounds/mixtures which are estrogenic (Soto *et al.*, 2006).

7.1. Introduction to estrogenic effects

Until the ERβ receptor was identified in 1996, compounds that stimulate a physiological response similar to 17-β-estradiol (E2), or, as defined by Herzt (1985), “substances, which elicit the proliferative activity of the organs of the female genital tract”, were classified as estrogens. The stimulation of uterus growth in mice has long been the standard test for identification of estrogenic or, synonymously, uterotrophic compounds (Koehler *et al.*, 2005). However, advances in research and increasing knowledge about the ER isoforms, their distribution and signalling, have led to more detailed and complex definitions of estrogens. Nowadays, estrogenicity can be defined purely by an
affinity to the ER\(\alpha\) or \(\beta\), an ability to activate expression of estrogen-dependent genes, or a stimulation of cell proliferation of ER-competent cells (Kortenkamp, 2007b).

The ERs belong to a nuclear receptor superfamily. The existence of “an ER” is well known since the late 1950s and the ER\(\alpha\) was first isolated in 1986, before the ER\(\beta\) was isolated in 1996 (Dahlman-Wright et al., 2006). Compounds that regulate the expression of specific genes through the interaction with ERs, either bind to ERs and form an estrogen-receptor complex (which, upon dimerisation, binds to specific target sequences of DNA, the estrogen-responsive elements (ERE)), or they influence transcription of genes whose promoters do not harbor EREs (Heldring et al., 2007).

Estrogens exert effects on multiple organs and both ER\(\alpha\) and ER\(\beta\) can be detected in a broad spectrum of tissues. ER\(\alpha\) is dominant in e.g. the uterus, prostate, ovary, testes, bone, breast and various regions of the brain, whereas ER\(\beta\) can be found in e.g. the colon, prostate, testis, ovary, bone marrow, salivary gland, vascular endothelium, and certain regions of the brain (Dahlman-Wright et al., 2006). The different distribution and expression levels of each ER, the relative affinity for an ER, the ligand and cofactor availability, and interaction with other transcription factors, result in a tissue dependent effect of the ligands of the ER (Dahlman-Wright et al., 2006). Where E2 shows similar affinity to both subtypes, many plant-derived compounds, which are classified as phytoestrogens, have a higher binding affinity to ER\(\beta\) than to ER\(\alpha\) (Harris et al., 2005).

The standard test for an estrogen, the uterotrophic assay, would not identify ligands on the ERP, as ER\(\alpha\) is the predominant isoform in the uterus. Thus, in order to identify compounds with affinities to different ERs, “estrogenic effects” need to be characterised in terms of the receptor target and the response (Koehler et al., 2005).

The more general definition, that estrogens are compounds which induce a response upon binding to the ER, which is similar to the response to endogenous estrogens like estradiol, serves the overall question of the project sufficiently and will provide an answer to whether the crude \textit{Salvia officinalis} extract possesses estrogenic activity.

7.2. Estrogenic effects reported for the genus \textit{Salvia}

\textit{Salvia officinalis’} reputation as a plant with estrogenic properties, which is described in the introduction (section 1.7), is not based on strong scientific evidence. The only experimental evidence published is in the study by Kroszcynski and Bychowska from 1939. They reported a change in the histology and the size of the uterus of ovariectomised mice after treatment with ethyl acetate and petroleum ether extracts of \textit{Salvia officinalis}, which was similar to the changes after estrogen injections (Kroszcynski
and Bychowska, 1939). Since then, no other study has been published which would confirm the estrogenic activity either in vitro or in vivo. The traditional uses of *Salvia* to e.g. arrest milk production or alleviate menopause related disorders (Bartram, 1995), which may indicate estrogenic properties, have never been elucidated with an experimental approach. Furthermore, only a few studies looked into the estrogenic properties of other plants of the genus *Salvia*.

Zhao *et al.* (2007) treated female mice with various Chinese medicines including Radix *Salviae miltiorrhiza*. The effects of the serum of female mice, which were treated with Chinese medicines alone or in combination with the control estrogen diethylstilbestrol for four days, were studied with MCF7 (ER+) breast cancer cells. The serum from mice treated with radix *Salviae miltiorrhiza* increased the proliferation rate of the MCF7 cells. The serum of mice treated with radix *Salviae miltiorrhiza* in combination with diestylstilbestrol conversely inhibited proliferation. Thus, radix *Salviae miltiorrhiza* was found to exhibit estrogenic effects, when applied alone, and anti-estrogenic effects in the presence of other estrogens. Extracts and essentials oils of *Salvia lavandulifolia* have been tested in a recombinant yeast estrogen screen. Weak estrogenic responses were found with a 96% ethanolic extract at 1.25 mg/ml and for the aqueous layer, which was obtained by partitioning the 96% ethanolic extract between CHCl₃ and H₂O, at 5 mg/ml (Perry *et al.*, 2001). To our knowledge no other experiments, showing estrogentic effects of either *Salvia officinalis* or other *Salvia* species, have been published so far.

### 7.3. Methodological considerations — evaluation of estrogenicity in vitro

Defining estrogens by affinity to ERs or the ability to bind to ERs and the subsequent activation of gene transcription or cell proliferation allows various experimental approaches for the identification of estrogenic compounds in vitro. However, the handling, suitability and availability, as well as the costs, need to meet the purpose of the experiment and the questions asked, in order to give useful information and help to plan ethically justifiable in vivo experiments where needed.

Receptor binding assays, which assess direct binding/competitive binding to ERs in competition to E2, use cell extracts or isolated ERs. The extracts may derive from the uteri of different animal species (bovine and rat), from human cell lines that contain ERs (MCF7-, T47D-cells), or isolated ERs are available as recombinant receptor proteins. The measured parameter is the relative binding affinity, which describes the ability of the compound to compete with the endogenous ligand (usually radio-labeled E2). These assays are easy to perform, but cannot give an answer as to whether the
compound has the ability to initiate or inhibit gene transcription. Hence, receptor binding assays cannot distinguish agonists from antagonists, and partial agonists from full agonists. Finally, they are quite insensitive compared to reporter gene or proliferation assays (Wilson et al., 2004; Soto et al., 2006).

Cell proliferation assays are based on the principle that an estrogenic response will lead to an increased number of cells. The proliferative activity, the endpoint often described as the "biological hallmark" of estrogen action, is assessed with cells which naturally express ERs. Assays using human breast cancer cells (e.g. MCF7 cells) have been used for many years and most of the compounds defined as estrogenic by today were identified using this method (Soto et al., 2006). The E-SCREEN assay, developed by Soto et al. (1995) distinguishes between agonists and antagonists, and also between full and partial agonists or antagonists. However, mitogens may influence cell proliferation in the same way as estrogens, which reduces the specificity of the assay (Dickson and Lippman, 1995). Generally, cell proliferation assays are straightforward to use and allow determination of the estrogenic potency of compounds by their relative proliferative potency to E2 in a cost and time efficient manner.

The cell lines used in reporter gene assays have been designed to identify estrogens and the numerous cell lines include recombinant yeast strains and human breast cancer cell lines. Amongst the reporter gene assays, cell lines that already express the ER can be distinguished from those that do not naturally express the ER. In case they naturally express the ER, the cells are then transected, by transient or stable transfection, with a reporter gene that is inducible by estrogens. For example, the MVLN cell line is a MCF7 cell line, stably transected with a reporter gene whereby the vitellogenin promoter regulates the expression of luciferase. The estrogen receptor reporter gene (ERLUX) assay utilises T47D cells with an ERE-driven luciferase reporter gene and the frequently used MELN cell line (MCF7 cells) is based on a similar principle (Demirpence et al., 1993; Gagne et al., 1994; Wilson et al., 2004; Soto et al., 2006).

Other cell lines, which initially do not naturally express ER, have been obtained by double transfection; first, with a construct that allows the constitutive expression of the ER, followed by a construct containing the reporter gene. Many of these assays use yeast cells (Gaido et al., 1997), others are based on HeLa cells (HELN-ERα/HELN-ERβ) and many allow selective expression of one of the ERs (Balaguer et al., 2001). Assays based on yeast cells suffer from problems with their membrane permeability and hence the transport of compounds into the cells. Both of these factors differ from the behaviour of mammalian cell membranes. This difference can influence the observed
response and the false-negative rate is therefore fairly high in yeast-based assays (Gaido et al., 1997).

The overall sensitivity of the assays is comparable with proliferation assays. Supra-maximal responses (higher than for the control E2, set to 1) are observed by certain compounds, which sometimes results in a more complex picture when the aim is to distinguish between a partial and a full agonistic response (Soto et al., 2006). However reliable and sensitive the *in vitro* assay is, predicting the outcome of *in vivo* assays based on *in vitro* results is not straightforward. Compounds may be activated or inactivated by metabolic transformation *in vivo* or may act independently of the receptor via other pathways. Hence, whether a chemical is, or is not, to be classified as estrogenic often depends on the test system used (Beresford et al., 2000).

It was decided to investigate the estrogenic potential of the crude *Salvia officinalis* extract and the SE in a reporter gene assay, the ERLUX assay. Further, the effects of the crude extract and positive responses by the SEs were verified in the ESCREEN assay, which utilises cell proliferation as the endpoint. Both assays have the advantage of being based on human cell lines, and have the complexity of cell-based systems, for example including signalling or enzyme systems that are not present in isolated receptor binding studies or in yeast-based assays.

The ERLUX assay is based on the human adenocarcinoma breast cancer cell line (T47D), which was modified by Wilson *et al.* (2004) in order to obtain the cell line T47D-KB luc. The cells were stably transfected with a plasmid harbouring an ERE and a reporter gene (luc). Upon binding of an estrogen-receptor-complex dimer to the ERE, the luc-gene is expressed and functional enzyme (firefly luciferase) is produced via translation. The measurable luciferase activity allows quantification of the estrogenic response. Toxic effects on the T47D-KB luc cell line can result in false-negative results in the ERLUX assay. In order to control toxicity in the ERLUX assay, the mitochondrial activity of the cells was assessed using a well-established method for determination of cell-viability, the cytotoxicity assay described by Mosmann (1983).

In the ESCREEN assay, the estrogen responsive human breast cancer cell line MCF7 (MCF7-BUS) is treated with the test sample and estrogenic compounds are detected by their ability to trigger cell proliferation (Soto *et al.*, 1995).
7.4. Experimental procedure – the ERLUX assay

7.4.1. Chemicals, reagents, solutions

Steady-Glo® Luciferase Assay System was purchased from Promega UK Ltd., Southampton. 17-β-estradiol (E2; ≥98% pure) and fulvestrant (≥98% pure) were obtained from Sigma-Aldrich Company Ltd., Dorset, UK. Cell culture reagents were purchased from Invitrogen (Paisley, UK) unless stated otherwise.

All samples and controls were dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, UK).

7.4.2. Routine cell culture - T47D-KBluc cells

The cell line T47D-KBluc, used in the ERLUX assay, was obtained from the American Type Culture Collection (ATCC) via LGC Standards, Middlesex, UK.

Cells were maintained in 75 cm² cell culture flasks (Greiner, Gloucester, UK) in growth media (GM) consisting of RPMI (L-glutamine free), which contained glucose (2.5 g/l, Sigma Aldrich), HEPES (10mM), sodium pyruvate (1mM, Sigma Aldrich), insulin (0.2 U/ml), 10% foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2mM). Media was replaced every other day and cells were subcultured, when the cultures reached about 80% confluence. For sub-culturing, the cells were split by washing with 5 ml Hank's buffered salt solution (HBSS) and then disaggregating with 1 ml 0.05% trypsin solution (containing 0.02% EDTA) to achieve a single cell suspension. Cell suspensions were split into 75 cm² flasks. Cells were kept in a humidified incubator at 37°C with 5% CO₂.

7.4.2.1. Removing endogenous estrogen from the serum

Removing endogenous estrogens, such as naturally occurring steroids, from FBS is essential to ensure that this class of compounds can not contribute to the response of the cells in the ERLUX assay. The applied procedure treats the FBS, which is used for supplementing pre-assay-media and dosing media, with deionised water containing 5% activated charcoal and 0.5% dextran.

The deionised water/charcoal/dextran mixture was allowed to equilibrate for 30 min on a rolling mixer at room temperature. Supernatant was removed after centrifuging for 10 min at 2000 rpm. The FBS was added to the obtained pellets of charcoal and dextran and mixed for 60 min at room temperature. Subsequently, the serum mixture was first centrifuged at 2.000 rpm and the supernatant was then centrifuged for 30 minutes at
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20,000 rpm. The serum was sterile filtered and the resulting serum is referred to as charcoal dextran-fetal bovine serum (CD-FBS) throughout this thesis.

7.4.3. The ERLUX assay

The assay was performed as described by Wilson et al. (2004) and basic information on the Steady-Glo® Luciferase assay system can be found in the manufacturer instruction on www.promega.com (Promega). Briefly, T47D-KBluc cells were transferred into pre-assay media (PAM) one week prior to testing. PAM was identical to GM except penicillin and streptomycin were omitted and instead of 10% FBS medium it was supplemented with 10% of CD-FBS. The cells (10,000 cells in 100 μl PAM) were seeded into white, sterile, 96 well plates with lids (Greiner BIO-ONE Ltd.). Over the next 24 h the cells were allowed to attach. PAM was then removed from the plate and the samples, positive controls (E2, 1nM, maximal estrogenic response, eight replicates) and solvent controls (0.5% DMSO, eight replicates) were applied in DM. DM contained phenol-red free RPMI (without L-glutamine) supplemented as PAM except for 5% CD-FBS instead of 10% CD-FBS. It has been shown that phenol-red possesses weak estrogenic activity (Berthois et al., 1986) and media containing phenol red was therefore avoided during the assay. In order to monitor the performance of the assay, two additional controls were included on each plate. Eight replicates of solvent controls plus an antiestrogen, fulvestrant (1 μM), and eight replicates of positive control plus fulvestrant (1 μM), were used to monitor the background estrogenicity, as recommended by Wilson et al. (2004). The concentration of 1 μM for fulvestrant was chosen as this achieved the maximum inhibition of the E2 induced estrogenic response. The performance of the assay was considered to be satisfactory when the antiestrogen sufficiently blocked the estrogenic effect of the positive control. Incubation was allowed for 24 h, before 100 μl of Steady-Glo® assay reagent was added to each well. The assay reagents were mixed for 10 min on a plate shaker in the dark to allow cell lysis. The plates were loaded into the FLUOstar optima plate reader (BMG, Labtech Ltd.) and incubated for a further 10 min before the luminescence was measured. To allow constant conditions the plate reader chamber was maintained at 27°C throughout.
7.4.3.1. Data normalisation and analysis of estrogenic response in the ERLUX assay

The raw readings of luminescence were normalised by subtraction of the mean value of on-plate solvent controls \((n=8)\) and division by the mean of on-plate positive controls \((n=8, \text{ per definition}=1)\). Dose–response relationships were obtained by fitting linear regression or non-linear regression (sigmoid, 4 Hill parameter) models to the normalised data. Errors are presented as 95% confidence intervals. Plots were prepared using SigmaPlot 8.02 (2002).

7.5. Experimental procedure – the cytotoxicity assay

The MTT assay was performed as described by Mosmann (1983). Briefly, the assay works on the principle that thiazolyl blue tetrazolium bromide (MTT) salt is cleaved to form purple formazan precipitates via the succinate-tetrazolium reductase system. This pathway is found in the respiratory chain of mitochondria and is only active in metabolically intact cells. An increase in the number of living cells results in an increase in total metabolic activity that subsequently leads to a stronger colour formation. Thus, the MTT assay measures the conversion of MTT into purple-coloured MTT formazan by the redox activity of living cells, and a decrease of cellular MTT reduction could be an index for cellular damage, hence cytotoxicity (Abe et al., 2000). Mosmann (1983) used an acidic/alcohol HCl/isopropanol solution to solubilise the cells. A more recent development of this assay (Hansen et al., 1989) uses a dimethyl formamid (DMF) based solution to solubilise the formazan. The DMF based solution has a better ability to solubilise the formazan crystals than the HCl/iso mixture and was used for this assay (Kabil, 2008).

Further, the pH in the assay should be kept at 4.7, as at a pH higher than 5.5, MTT is spontaneously converted into formazan, while at a pH lower than 4, MTT formazan is converted back into MTT. A pH 4.7 prevents that a spontaneous formation of formazan or conversion of formazan into MTT (Hansen et al., 1989).

7.5.1. Chemicals, reagents, solutions

Thiazolyl blue tetrazolium bromide (MTT) and all other chemicals were purchased from Sigma-Aldrich Company Ltd., Dorset, UK unless stated otherwise.

7.5.2. Preparation of cells for the cytotoxicity assay

In order to assess the cytotoxicity of the test samples on the T47D-KBluc cells (ERLUX assay) during the screening, the cells were treated as described in section 7.4.2 :routine cell culture for T47D-KBluc cells. Experiments with different cell densities
(10,000 – 100,000 cells/well, no treatment) were conducted in order to find a cell number suitable to quantify toxic effects. 40,000 cells/well was chosen for the cytotoxicity testing as the original number of 10,000 cells/well was too low to observe mitochondrial activity by formation of purple coloured formazan (see Appendix). The cells were seeded, incubated and treated in the same manner as described for the ERLUX assay (section 7.4.3) until the final stage, the application of Steady-Glo® reagent and quantification of the estrogenic response.

7.5.3. The cytotoxicity assay

The cells were seeded and incubated as described in section 6.5.2 and the cytotoxicity assay was carried out as described by Mosmann (1983), with a few minor modifications. The use of the solubilisation solution was described by Hansen et al. (1989). Twenty-four h after the application of the treatment the DM was gently removed and replaced by 100 µl DM and an additional 20 µl of MTT solution (5 mg/ml in HBSS) per well. Cells were incubated for 4 h to allow viable cells to cleave the yellow MTT salt. The cells were lysed afterwards with 150 µl of a mixture consisting of 250 ml DMF, 250 ml deionised water, 100 g sodium dodecyl sulafate, 10 ml glacial acetic acid and 5 ml HCl (2M). The solubilisation solution was adjusted to pH 4.7 (Hansen et al., 1989). The optical density of the formazan was photospectrometrically quantified at 570 nm (Labsystem Multiscan, UK).

7.5.3.1. Data normalisation and analysis of cytotoxic effects

The results of the cytotoxicity assay were normalised by subtraction of the background absorbance of the DM (100 µl), the MTT solution (20 µl) and 40,000 cells (blank wells). The normalised raw data were then divided by the average of on-plate solvent controls (n=8) and multiplied by 100, to express the results as % of viable cells compared to solvent controls. Bar charts were prepared using Sigma Plot 8.02 (2002), error bars represent 95% confidence intervals.
7.6. Experimental procedure – the estrogen screen (ESCREEN)

7.6.1. Chemicals, reagents, solutions

E2 (≥98% pure) was purchased from Sigma-Aldrich Company Ltd., Dorset, UK. Cell culture reagents were purchased from Invitrogen (Paisley, UK) unless stated otherwise. Samples and controls were dissolved in ethanol (Fisher Scientific, UK).

7.6.2. Routine cell culture (MCF7 cells)

Human mammary carcinoma MCF7-BUS cells were a kind gift from Ana Soto of the Tufts University School of Medicine (Boston). The cells were maintained in 75 cm² flasks (Greiner, Gloucester, UK) in assay media (Dulbecco’s Modified Eagle Medium (DMEM) with Glutamax) supplemented with 5% FBS and 1% MEM-non-essential aminoacids (MEM-NEAA) in a humidified incubator at 37°C, 5% CO₂. The media was replaced every other day and the cells were sub-cultured when they reached about 80% confluence. For sub-culturing, the cells were split by washing with 5 ml HBSS followed by detachment with 1 ml 0.05% trypsin solution (containing 0.02% EDTA) to achieve a single cell suspension that was split into 75 cm² flasks.

7.6.3. The ESCREEn assay

The ESCREEn experiments were carried out following the general method established by Soto et al. (1995), with minor modifications which were undertaken by Silva et al. (2007) to adapt the method to the 96 well format. Briefly, cells were seeded in assay media at a density of 2,500 cells/well into clear 96 well plates (Falcon, BD Bioscience, UK) and allowed to attach for 24 hours. The cells were washed carefully with wash media (phenol red free DMEM, no supplements) before test and control solutions were applied. The stock solutions of samples and controls were diluted with dosing media (DM) consisting of phenol red free DMEM, 10% charcoal-dextran stripped FBS (described in 6.4.2.1), 0.05% MEM-NEAA and 0.1 mM sodium pyruvate, before application to the cells. Final maximal concentration of ethanol in the reaction volume (200 µl) was 0.5% (v/v) and the concentration of the positive control (E2) was 25 nM. The plates were incubated for 120 h. In the final stage of the assay the plates were put on ice (1 min) and the DM was removed. The cells were fixed on ice by applying 200 µl of an ice-cold 10% trichloroacetic acid solution for 20 min. The plates were washed with water afterwards and left to dry. Fifty µl of sulforhodamine B (SRB) (0.4% in 1% acetic acid) was applied for 10 min. A final wash with 1% acetic acid removed unbound SRB dye. The plates were dried in the dark, before the bound dye was solubilised with
100 μl Tris-base (10 mM, pH 10.5) and absorbance was read at 510 nm to indirectly quantify the cell number.

**7.6.3.1. Data normalisation and analysis of estrogenic response in the ESCREEN assay**

The raw data were normalised by subtraction of the average of on-plate solvent controls (n=8), before resulting values were divided by the average of the on-plate positive controls (n=8). Data were normalised on plate-by-plate basis to reduce intra- and inter-experimental variation of the results (Rajapakse *et al.*, 2004). Graphs were prepared using Sigma Plot 8.02 (2002). Best-fit curves were obtained by linear regression or non-linear regression (sigmoid) using 3 or 4 Hill parameter model (Scholze *et al.*, 2001). Error bars represent 95% confidence intervals.
7.7. Results

7.7.1. Estrogenicity in the ERLUX assay

7.7.1.1. Estrogenic response of 17-β-estradiol (E2) in the ERLUX assay

E2 was tested at eight concentrations between 0.01 and 772 pM (six-fold dilution series) in order to confirm that the assay performed well in our hands (Fig. 7.1). The EC_{50} value for E2 was 1.5 pM. Slight supra-maximal effects with E2 are introduced by the data variation in the on-plate positive control of the ERLUX assays, which are used for normalisation. 1.0 nM of E2 was used in further assays as positive control and represented maximal E2 response set to 1.0 in the ERLUX assay. The results confirmed that the performance of the assay was reliable as they agree with the results found by Wilson et al. (2004) (EC_{50} 3.0 pM).

![Figure 7.1: Dose-response curve of E2 in the ERLUX assay. Data are from three independent experiments, tested in triplicate. Filled circles represent individual data points. Means were fitted using a non-linear regression model (sigmoid, 4 parameter Hill) and error bars represent 95% confidence intervals. Controls are presented next to the y-axis, pink circles represent the positive controls (1.0 nM E2) and cyan circles represent the solvent controls (0.5% DMSO).](image)

7.7.1.2. Evaluation of solvent effects in the ERLUX assay

An evaluation of the effect of DMSO in the ERLUX assay was performed because the standard solvent for test samples used by Wilson et al. (2004) was ethanol and not DMSO. In the manufacturer instructions (www.promega.com) it was stated that DMSO increases the assay sensitivity at low concentrations. However, the increased sensitivity was demonstrated using pure firefly luciferase in medium and in the absence of cells, a
scenario which is unlike the situation in the ERLUX assay. In order to evaluate the influence of increasing DMSO concentration on the T47D-KBlue cells, the response to E2 was measured in the presence of DMSO at concentrations of 0.1%, 0.2%, 0.5% and 1.0% in the assay solution (see Appendix). The E2 concentrations also varied (0.0125 nM, 0.025 nM and 0.05 nM), which furthermore allowed comparisons of the effect of the DMSO at different test concentrations. The slightly increased response in the presence of DMSO concentrations of 0.5% and 1.0% might be due to an enhanced cell permeability in the presence of higher concentrations of DMSO. However, the effect was not considered to influence the observed effects significantly and concentrations of DMSO were subsequently kept constant at 0.5%/well for all test samples and controls throughout.
7.7.1.3. The crude *Salvia officinalis* extract and the subextracts

The crude *Salvia officinalis* extract and SEs were tested in two-fold dilution series of eight concentrations in triplicate. The concentration range chosen for the crude *Salvia officinalis* extract was between 1.2 and 153 μg/ml, that for the aq-EtOH SE between 1.2 and 150 μg/ml, the range for the n-hexane SE from 0.01 to 30.5 μg/ml and that for the CHCl₃ SE from 0.8 to 100 μg/ml (Fig. 7.2). The concentrations were chosen based on preliminary cytotoxicity tests in order to avoid testing at highly toxic concentrations (data not shown).

![Diagram](image)

Figure 7.2: ERLUX assay results for crude *Salvia officinalis* extract (red), n-hexane SE (blue), CHCl₃ SE (green) and aq-EtOH SE (yellow). Data sets were obtained in three independent experiments, each tested in triplicate. Circles represent mean values of the replicates per experiment. Aq-EtOH SE data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill); the others were fitted using linear-regression. Data represented by non-filled circles were not considered for the fits. On-plate controls, positive controls (1.0 mM E₂, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis.

The aq-EtOH SE showed maximal effects greater than the maximal effect of E₂ (per definition set to 1). The possibility of supra-maximal effects is known for the ERLUX assay (Wilson *et al.*, 2004) and was also observed in similar reporter-gene assay (Legler *et*
The EC₅₀ of the aq-EtOH SE in the ERLUX assay was 64 μg/ml. The crude *Salvia officinalis* extract and CHCl₃ SE appeared to exhibit estrogenic effects at around 10-50 μg/ml (presented as non-filled circles, Fig. 7.2). These results were single observations obtained in one experiment. The other repeats of the experiments did not show activity for the crude *Salvia officinalis* extract and the CHCl₃ SE at this concentration range, hence they did not support the observed results. The data of this single observation were therefore not included in the linear regression fits, which are shown in the Figure. The n-hexane SE did not show any estrogenic response. The crude *Salvia officinalis* extract and SE were additionally tested in the presence of 1.0 μM of the ER antagonist fulvestrant (for data see Appendix). Any previously observed estrogenic response was reduced to solvent control level and this indicates, according to Wilson *et al.* (2004), that the observed estrogenic response is mediated by the ER α.
7.7.2. Cytotoxic effects in the ERLUX assay

The crude *Salvia officinalis* extract and SE were tested for cytotoxic effects on the T47D-KBluc cells, in order to avoid false-negative results which may arise from an overlapping of toxic effects with estrogenic effects. Tested concentrations were identical to the concentrations tested for estrogenicity in the ERLUX assay: crude *Salvia officinalis* extract between 1.2 and 153 µg/ml, the aq-EtOH SE between 1.2 and 150 µg/ml, the n-hexane SE between 0.01 and 30.5 µg/ml, and the CHCl₃ SE between 0.8 and 100 µg/ml (Fig. 7.3). Results were evaluated in comparison to the solvent control (0.5% DMSO), which represented 100% viable cells.

![Graphs showing cytotoxic effects](image)

Figure 7.3: Cytotoxic effects on the T47D-KBluc cells. Bars represent the mean values of crude *Salvia officinalis* extract (red), n-hexane SE (blue), CHCl₃ SE (green) and aq-EtOH SE (yellow). Bars in cyan represent mean for 0.5% DMSO (solvent control). Each result was obtained in three independent experiments, tested in duplicate. The error bars represent 95% confidence intervals.

The cell viability observed in the cytotoxicity assay was good for most of the tested concentrations and the cell number was not affected by toxic effects. Only the highest concentrations of the crude *Salvia officinalis* extract and the CHCl₃ SE showed toxic effects on the T47D-KBluc cells. The crude *Salvia officinalis* extract reduced the number of viable cells to ~70% compared to solvent controls at a concentration of 153 µg/ml and the CHCl₃ SE reduced the number of viable cells to ~40% compared to solvent...
controls at a concentration of 100 µg/ml. All other tested concentrations or extracts did not affect the cell viability in the ERLUX assay.

7.7.3. Estrogenicity in the ESCREEN assay

7.7.3.1. The crude Salvia officinalis extract and the subextracts

In order to confirm the estrogenicity observed for the aq-EtOH SE in the ERLUX assay, the aq-EtOH SE was tested in the ESCREEN assay in a two-fold dilution series of eight concentrations. In addition, the crude Salvia officinalis extract was included. The concentration range chosen for the crude Salvia officinalis extract was between 0.6 and 76.5 µg/ml and for the aq-EtOH SE between 1.2 and 150 µg/ml (Fig. 7.4). The crude Salvia officinalis extract did not show any activity in the ERLUX assay and the ESCREEN assay confirmed the absence of estrogenic activity of the crude Salvia officinalis extract which was found in the ERLUX assay. The aq-EtOH SE induced MCF7 cell proliferation with an EC50 value of 130 µg/ml, hence the ESCREEN assay confirmed the estrogenic potential of the aq-EtOH SE.

Figure 7.4: ESCREEN assay results for Salvia officinalis and aq-EtOH SE. Data were obtained in three independent experiments, each tested in duplicate. Filled circles represent the mean and error bars (95% confidence intervals) of crude Salvia officinalis extract (A, red) and aq-EtOH SE (B, yellow). Data were fitted using linear regression (crude Salvia officinalis extract) and non-linear regression model (aq-EtOH SE, sigmoid, 4 parameter Hill). The controls are presented next to the y-axis. Pink circles represent individual positive control values (on-plate, 25 nM E2), cyan circles are solvent controls (on-plate, 0.5% EtOH).
7.8. Discussion

The results of both the ERLUX assay and the ESCREEN assay showed no clear estrogenic activity of crude \textit{Salvia officinalis} extract. Around 25-50 \(\mu\text{g/ml}\) some of the data indicated estrogenic activity in the ERLUX assay. However, these observed effects were single observations and the estrogenic activity was not confirmed by further experiments. The same applies for the single observations of the CHCl\textsubscript{3} SE (10-20 \(\mu\text{g/ml}\)). The \(n\)-hexane SE showed no estrogenic effects.

The results of the cytotoxicity assay confirmed that the absence of estrogenicity in the crude \textit{Salvia officinalis} extract, the \(n\)-hexane and the CHCl\textsubscript{3} SEs, are true observations. Toxic effects were only observed for the highest test concentration of the crude \textit{Salvia officinalis} and the CHCl\textsubscript{3} SE. The possibility of false-negative results in the ERLUX assay can therefore be excluded, as the observed estrogenic effects were not affected by toxicity and a clear estrogenic effect would have been detectable in the ERLUX assay, at least up to the non-toxic concentrations.

The aq-EtOH SE on the other hand clearly possessed estrogenic activity. One or several constituent(s) present in the extract bound and activated the ER in the ERLUX assay in a dose-dependent manner (EC\textsubscript{50} 64 pg/ml). Toxicity by the aq-EtOH SE against the T47D-KB\textsubscript{Luc} cells was not observed. The estrogenic effect observed in the ESCREEN assay resulted to an EC\textsubscript{50} value of 130 \(\mu\text{g/ml}\).

The ERLUX and ESCREEN assays are suitable methods for the identification of estrogenic compounds with respect to ER binding and activation or induction of proliferation. The results show that the crude \textit{Salvia officinalis} extract contains compound(s), which possess estrogenic activity, as its aq-EtOH SE displayed estrogenic activity. This also suggested that the estrogenic compound(s) existed in very minor concentrations in the crude \textit{Salvia officinalis} extract, but were more enriched in the aq-EtOH SE. That, however, is not a limitation of the assay itself, but rather a frequently observed phenomenon with complex mixtures such as herbal preparations/extracts. If the activity can be attributed to a single or a couple of compounds, they are often difficult to detect with \textit{in vitro} experiments on crude extracts, which was one of the reasons why the subextracts of the crude \textit{Salvia officinalis} extract were tested in parallel.

There is the possibility that toxic effects in the ERLUX assay may have been masked by the set-up chosen for the cytotoxicity assay. The toxicity experiments were carried out with a higher cell number/well than the ERLUX assay (40.000 as opposed to 10.000). The reason for that was that the sum of mitochondrial activity for cell seeding densities lower than 40.000 cells/well was too low to be detected in this assay and therefore not
suitable to detect toxic effect on the T47D-KBluc cells. However, the higher cell number, which may result in false-negative results, was accepted in order to allow identification of toxic effects on the T47D-KBluc cells in the best way possible. Toxic effects of the crude *Salvia officinalis* extract and the aq-EtOH SE on the MCF7 cells were not assessed as it is not possible to distinguish between compounds that are unable to induce proliferation of the MCF7 cells and those with weak toxic effects.

The aq-EtOH SE is the most polar fraction of the crude *Salvia officinalis* extract and is therefore rich in phenolic compounds. The species *Salvia officinalis* is not known to contain isoflavones, which are potent and well-studied plant-derived phytoestrogens. Some flavones, flavanones and flavonols (*e.g.* apigenin, kaempferol and naringenin) have also been found to activate ER-mediated signalling (Moutsatsou, 2007). For example, Van der Woudt et al. (2005) reported that the flavanol quercetin induced ER/ERE-mediated luciferase activity in U2-OS cells (human osteosarcoma cell lines) transfected with either ERα or ERβ. It further induced proliferation of MCF7 cells at low concentrations confirming that the stimulating effect of quercetin on cell proliferation is ER-dependent. In a different study, the flavonoid apigenin was found to induce weak cell proliferation of MCF7 cells (Seo et al., 2006). This, however, may indicate that phenolic compounds, *e.g.* apigenin derivates, in the aq-EtOH SE could possibly be responsible for the detected estrogenic activity.

The phytoestrogens (*e.g.* apigenin, coumestrol, daidzein, equol, genistein, kaempferol, naringenin, resveratrol and quercetin) generally show a higher affinity for the ERβ (Harris et al., 2005). Thus, the observed estrogenic response for the aq-EtOH SE may have been stronger if the extract had been tested on either cells expressing ERβ only or cells in which the expression level of ERα/β was more balanced, assuming that phytoestrogens are components of the extract. Strom et al. (2004) reported mRNA ratios of ERα:ERβ of 9:1 for the T47D cells, but they were not able to detect any endogenous ERβ protein in these cells. Hence the ERLUX assay is able to detect compounds which activate the ERα, but may miss selective ERβ activators. The MCF7 cells, used in the ESCREEN assay, also express ERα as the dominant ER. Smuc and Rizner (2009) determined the mRNA levels for the ER in MCF7 cells and found a ratio of 2.4 ERα:0.01 ERβ (relative to cyclophilin A), which confirmed that ERα is the dominant ER in the MCF7 cell line.

The study described in this thesis demonstrates an estrogenic potential of *Salvia officinalis*, which has been reported long ago, but never been confirmed in current and accepted *in vitro* methods for estrogenicity. Although the crude *Salvia officinalis* extract
did not show estrogenic responses at the concentrations tested, the compounds exhibiting the estrogenic activity in the aq-EtOH SE are present in the crude extract. Although the observed effects were weak in comparison to known estrogenic compounds such as genistein (EC$_{50}$ 30nM (Evans et al., 2010) = 0.0081 µg/ml (M, 270.24)), they merit investigation of the compound(s) responsible. The concentration of possibly responsible compounds in vivo may reach high levels, as large amounts could be consumed via plants, fruits or vegetables.

The estrogenic activity of the aq-EtOH SE was further investigated in this thesis. In order to identify the compounds responsible for the estrogenic activity the SE was subjected to an activity-guided fractionation (chapter 8). After the identification of the estrogenic compounds in Salvia officinalis, studies of their activity and affinity to the ER$\beta$ should follow.
8. Activity-guided fractionation of estrogenic principle of the aqueous ethanolic subextract

In activity-guided fractionation, biologically active constituents are isolated from a natural source by using their biological activity in *in vitro* assays to monitor the chromatographic separations. The fractions retaining the initial activity are subjected to further fractionations until the isolation of the active constituent(s) is achieved. The basic idea of activity-guided isolation of natural products assumes that the activity shown by an extract may either be due to the sum of the activities of a few individual constituents or to only one lead compound. Hence, the activity-guided fractionation should result in the isolation of compounds which are expected to exhibit a higher activity than the crude extract. This approach has already led to the identification of new drug candidates (*e.g.* taxol and artemisinin (Wani *et al.*, 1971; Anonymous, 1979)). It furthermore can provide important knowledge for standardisation of extracts to their assumed major active compound, which may result in a more predictable pharmacological activity. Activity-guided fractionation could on the other hand result in a loss of the activity. This may be explained by decomposition of the active compound(s) during the fractionation process or with synergistic effects that were responsible for the observed biological activity (summarised in (Hostettmann, 1999; Houghton *et al.*, 2007)).

The identification of active constituents is highly important in order to ensure pharmaceutical and thereby therapeutic quality of herbal extracts. Once the active compound(s) are known, further investigations of their pharmacological activity and mechanism(s) of action can be performed that are more straightforward with pure compounds than with complex herbal extracts (Nahrstedt and Butterweck, 2010). The bioassay for an activity-guided fractionation should be simple, inexpensive and rapid to allow the testing of large numbers of fractions. Furthermore, the bioassay should be sensitive enough to detect active principles which are generally present only in small concentrations in crude extracts. The selectivity of the bioassay should maintain the number of false-positives at a reasonably low level (Hostettmann, 1999).
8.1. Guidelines for the activity-guided fractionation of the aqueous ethanolic subextract

The aqueous ethanolic subextract (aq-EtOH SE) was chosen for the activity-guided fractionation based on the results described in chapter 7. The aq-EtOH SE showed estrogenic activity in the estrogen receptor reporter gene (ERLUX) assay with a half-maximal effective concentration (EC₅₀) of 64 µg/ml and the estrogenic activity was confirmed in a second assay for estrogenicity, the estrogen screen (ESCREEN) assay (described in section 7.4 and 7.6 respectively). The SE was fractionated using reverse phase (RP) vacuum liquid chromatography (VLC) and size exclusion chromatography (described in chapter 2). The obtained fractions were analysed thin layer chromatography (TLC) and proton nuclear magnetic resonance (¹H-NMR) spectroscopy and combined based on similarities. All obtained fractions were tested for estrogenic activity in the ERLUX assay (described in section 7.4). The ERLUX assay fulfilled the requirements of a bioassay for activity-guided fractionation, because the solvent (DMSO), which was used for sample preparation solubilised both more polar and less polar compounds, which were obtained by the gradient elution. Furthermore it was possible to carry out solvent controls in parallel on each plate and the set-up of the assay allowed testing of dilution series of the test solution of each fraction (CanneU, 1998a). All fractions were tested for cytotoxic effects on the T47D-KBluc cells, which were used in the ERLUX assay (as described in section 7.5).

8.1.1. Explanatory remarks

In this chapter fractions obtained from the aq-EtOH SE are numbered and abbreviated with AQ (e.g. AQ 1.1). All obtained fractions are displayed in the fractionation scheme (Fig. 8.2) together with the conditions, which were applied to obtain the fractions. The fractions highlighted in red were subjected to for further fractionation. TLC analyses of the fractions on normal phase Silica (Silica gel 60 F₂₅₄) are presented in the corresponding sections in this chapter and the solvent system, which was used to develop the TLC plate, is indicated above the presented TLC plate. The spots marked by ] absorbed light with UV₂₅₄nm and spots marked with [ showed fluorescence at UV₃₆₆nm. TLC analyses were carried out in parallel to the fractionation process. Fractions which appeared similar on the TLC plate, were pooled together throughout the fractionation process. This avoided having many fractions containing identical compounds, and resulted in a more manageable number of fractions for the bioassay.
testing. $^1$H-NMR analysis was also carried out with nearly all fractions in order to gain more insight into similarities or differences in the obtained fractions, their complexity or purity. Most of the $^1$H-NMR spectra of the fractions are presented in the Appendix.

The fractions which gave the highest estrogenic response in the ERLUX assay were those which were subjected to further fractionation (highlighted in red in Fig. 8.2). The ERLUX results of fractions without activity in the ERLUX assay are included in the Appendix.

Figure 8.1: A Individual data points of solvent controls (0.5% DMSO) in the cytotoxicity assay, the MTT assay (cyan). Data were obtained in four independent experiments (1-4), with eight replicates. Black data-points represent the individual data points of all four experiments. B Individual data points for solvent controls (cyan, 0.5% DMSO, 4 experiments, eight replicates each, reproduced from A) present normal data variation in the cytotoxicity assay. Red circles represent the average of the results for fraction AQ 0, which was obtained by two independent cytotoxicity assays, run in duplicate.

Figure 8.1.A presents results for the solvent controls (0.5% DMSO) in the cytotoxicity assay. The solvent controls of the four experiments, which were carried out to determine any cytotoxicity by the extracts AQ 0 - AQ 10 (see section 8.2.2), were
chosen for this graph. The aim was to set a standard, which defines normal data variation in the cytotoxicity assay. Eight solvent control wells were included on each plate of the four cytotoxicity experiments. An observed effect was considered cytotoxic only if the result was below the values observed for the solvent controls. Any possible influence by cytotoxic effects on the ERLUX assay results is discussed, where necessary, in the following sections. A cell viability greater than 100% can be observed in this assay. This may result from a variation in cell seeding densities across the multi-well plates or from unexpected cell growth. As the experiments were carried out with duplicate for each sample these effects should not affect the surveillance of a strong cell toxicity by the test samples. Figure 7.1.B shows the results of the cytotoxicity assay for the first fraction obtained from the aq-EtOH SE (AQ 0, see section 8.2.2). The data for the cell viability in the presence of fraction AQ 0 are within the range of the solvent controls and the fraction AQ 0 was therefore considered as not toxic.
8.2. Results of the activity-guided fractionation of the aqueous ethanolic subextract

8.2.1. Fractionation scheme

The fractionation scheme in Figure 8.2 displays all fractionation steps. The TLC analyses and ERLUX results are described in the subsequent sections in this chapter. First the aq-EtOH SE was fractionated by VLC over reversed-phase Silica gel (LiChroprep RP-18) with a step gradient from 100% H₂O to 100% MeOH in 10% increments and produced 10 fractions (TLC and ERLUX results in section 8.2.2). The four fractions which showed the highest estrogenic activity were chosen for further fractionation (AQ 4, AQ 5, AQ 6 and AQ 7).

The fraction AQ 4 was further fractionated by RP-18 VLC with a step gradient from 100% H₂O to 20% MeOH in 5% increments, followed by 100% MeOH. The fractionation yielded 8 fractions (AQ 4.1-4.8) and their TLC and ERLUX results are described in section 8.2.3.1. Of those eight fractions fraction AQ 4.4 was further fractionated by size-exclusion chromatography over Sephadex LH-20 with 70% MeOH to result in 6 fractions (AQ 4.4.1-4.4.6, TLC and ERLUX results in section 8.2.3.2). The

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Figure 8.2: Fractionation scheme of the aq-EtOH SE.

The fraction AQ 4 was further fractionated by RP-18 VLC with a step gradient from 100% H₂O to 20% MeOH in 5% increments, followed by 100% MeOH. The fractionation yielded 8 fractions (AQ 4.1-4.8) and their TLC and ERLUX results are described in section 8.2.3.1. Of those eight fractions fraction AQ 4.4 was further fractionated by size-exclusion chromatography over Sephadex LH-20 with 70% MeOH to result in 6 fractions (AQ 4.4.1-4.4.6, TLC and ERLUX results in section 8.2.3.2). The
second fraction of AQ 4, which was further fractionated, was fraction AQ 4.5. Size-exclusion chromatography over Sephadex LH-20 with 70% MeOH resulted in 5 subfractions (AQ 4.5.1-4.5.5, TLC and ERLUX results in section 8.2.3.3). The other fractions, (AQ 5, AQ 6, AQ 7) which were further fractionated (highlighted in red), were also subjected to RP-18 VLC and size-exclusion chromatography over Sephadex LH-20. Amounts of used fractions and chromatographic conditions are indicated next to the respective fractionation step in the fractionation scheme (Fig. 8.2). The TLC and ERLUX results of fractions obtained from AQ 5, AQ 6 and AQ 7 can be found in the sections 8.2.4, 8.2.5 and 8.2.6 respectively.

8.2.2. Fractions AQ 0-9

The TLC analysis of the fractions AQ 0-9 (Fig. 8.3) showed that fractions 3-7 were rich in compounds which were visualised by vanillin/sulphuric acid and absorbed light with $\text{UV}_{254\text{nm}}$ (\()\) or showed fluorescence at $\text{UV}_{366\text{nm}}$ (\()\).

The fractions AQ 0-9 were tested for estrogenic activity in the ERLUX assay (Fig. 8.4). Fractions AQ 4, AQ 5, AQ 6 and AQ 7 showed estrogenic potential. Their $\text{EC}_{50}$ values were 69 µg/ml (AQ 4), 58 µg/ml (AQ 5), 14 µg/ml (AQ 6) and 3 µg/ml (AQ 7). The fraction AQ 3 also showed estrogenic response at the highest test concentration (150 µg/ml). Fraction AQ 8 exhibited estrogenic effects around 20 µg/ml, but the estrogenic response decreased at higher test concentrations. The results of the cytotoxicity assay for AQ 8 did not indicate toxic effects on the T47D-KBluc cells and toxicity can therefore not explain the observed decrease (cytotoxicity data see Appendix). Fraction AQ 0, AQ 1, AQ 2 and AQ 9 showed no estrogenic activity in the ERLUX assay.
TLC analysis (Fig. 8.3) indicated that AQ 4 contained different types of natural compounds. The bright pink spot at the top of the TLC plate is likely to be rosmarinic acid, a caffeic acid derivative very abundant in *Salvia officinalis*. The same spot can be observed in AQ 5 and weakly in AQ 6. The most potent fraction AQ 7 does not seem to contain this compound. Fractions AQ 4, AQ 5, AQ 6 and AQ 7 were the most active fractions and were chosen for further fractionation by VLC using a step gradient with smaller increments. Results are described individually in the following sections.
Figure 8.4: ERLUX assay results for fraction AQ 0-9. Data were obtained in two independent experiments, each tested in triplicate. Data were fitted using linear regression or a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis.
8.2.3. Fraction AQ 4
8.2.3.1. Fractions AQ 4.1 - 4.8

Fractionation of AQ 4 by RP-18 VLC resulted in 8 fractions (AQ 4.1 - 4.8). TLC analysis of fraction AQ 4.5 - 4.7 (Fig. 8.5) showed the bright pink spot, which was already observed in the starting fraction AQ 4. TLC analysis against pure rosmarinic acid standard (Fig. 8.5) indicated its presence in the fractions AQ 4.4 to 4.7. Fraction AQ 4.4 seemed to contain only minor amounts of rosmarinic acid.

$^1$H-NMR experiments of fraction AQ 4.7 and rosmarinic acid standard (Fig. 8.6) confirmed the presence of rosmarinic acid. Hence, the fraction seemed to be dominated by this compound and the pink spot on TLC was rosmarinic acid.

Figure 8.5: TLC analysis of the VLC fractions AQ 4 and AQ 4.1 – 8. RA: Rosmarinic acid. Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).

Figure 8.6: $^1$H-NMR spectra of fraction AQ 4.7 (bottom) and rosmarinic acid standard (top) (MeOD, 400 MHz).

Fractions AQ 4.1 - 4.8 were tested in a 1:2 dilution series ranging from 0.6 to 75 µg/ml. Fractions AQ 4.4, AQ 4.5 and AQ 4.6 exhibited estrogenic responses in the ERLUX assay (Fig. 8.7). A maximal response of 1, which represents the effect relative to
estradiol (E2) 1 nM to which the data were normalised, was reached by fraction AQ 4.5 (EC_{50} 64 µg/ml), while AQ 4.4 (EC_{50} 47 µg/ml) exhibited a maximal response of 0.8. These fractions AQ 4.4 and AQ 4.5 were therefore further fractionated using size exclusion chromatography (results in section 8.2.3.2 and 8.2.3.3). AQ 4.6 gave a half maximal response. Individual data points for the three fractions (AQ 4.4 - 4.6) are presented in the Appendix. Fractions AQ 4.1 - 4.3 and AQ 4.7 and 4.8 did not exhibit any estrogenic response (see Appendix). As rosmarinic acid was a dominant compound in four fractions (AQ 4.4 - 4.7) the commercial rosmarinic acid standard was tested in the ERLUX assay (Fig. 8.8) at the same concentrations as the fractions of AQ 4 (0.5 - 75 µg/ml). Rosmarinic acid did not exhibit any estrogenic activity in the ERLUX assay.

Figure 8.7: Results of the ERLUX assay for fractions AQ 4.4 (green), 4.5 (pink), 4.6 (blue). Regression curves for AQ 4.4-4.6 were obtained by fitting data from one experiment, each tested in triplicate (see Appendix), by using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 4 (grey, dotted line) is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.4).

Figure 8.8: ERLUX results of rosmarinic acid standard. Circles represent individual data points obtained in one experiments. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis.
8.2.3.2. Fractions AQ 4.4.1 - 4.4.6

Fraction AQ 4.4 was further fractionated by size-exclusion chromatography over sephadex LH-20 and resulted in 6 fractions (AQ 4.4.1 - 4.4.6). The TLC analysis of fraction AQ 4.4.1 - 4.4.6 was dominated by bright yellow spots (AQ 4.4.6 was combined with AQ 4.4.7 based on TLC and 'H-NMR analyses). AQ 4.4.2 appeared as bright blue spot TLC (Fig. 8.9). The fractions obtained were tested in the ERLUX assay in a 1:2 dilution series ranging from 0.4 to 50 µg/ml.

Fractions AQ 4.4.6 (combined with AQ 4.4.7) and AQ 4.4.5 showed estrogenic activity (Fig. 8.10).

Fractions AQ 4.4.1 - 4.4.4 did not display any estrogenic effects in the ERLUX assay (see Appendix). With a response of 0.1 (relative to E2, 1nM) at the highest test concentration (50 µg/ml), the activity of AQ 4.4.5 was very weak. AQ 4.4.6 gave a maximal response of 0.3 in the ERLUX assay. With 64 µg/ml the EC₅₀ value of AQ 4.4.6 in the ERLUX assay was very similar to the EC₅₀ value of the parent fraction AQ 4 (EC₅₀ 69 µg/ml). Identification and structure elucidation of fraction AQ 4.4.6 (42.9 mg, compound 1) is described in section 8.3.
Figure 8.10: Results of the ERLUX assay for fractions AQ 4.4.5 (blue circles) and 4.4.6 (red circles). Both regression curves were obtained by fitting to data from one experiment, each tested in triplicate, by using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 4.4 (grey, dotted line) from which these sub-fractions were derived is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.7).

8.2.3.3. Fractions AQ 4.5.1 - 4.5.5

Five fractions (AQ 4.5.1 - 4.5.5) were obtained by size-exclusion chromatography of AQ 4.5 (described in Fig 8.2). The fractions were tested for estrogenicity in a 1:2 dilution series ranging from 0.4 to 50 μg/ml in the ERLUX assay. None of the fractions showed activity in the ERLUX assay even at the highest concentration. It appears therefore that the estrogenic activity of AQ 4.5 was lost during fractionation (see Appendix). The TLC analysis (Fig. 8.11) showed that most of the fractions were dominated by one single compound, which appeared as a pink spot. The absence of it in fraction 4.5.4 may be explained by insufficient concentration of this fraction applied on the TLC plate. It has been shown that fraction 4.5 (Fig. 8.5) was rich in rosmarinic acid, which did not show any estrogenic activity in the ERLUX assay (Fig. 8.8).

Figure 8.11: TLC analysis of the VLC fractions AQ 4.5 and AQ 4.5.1 - 4.5.5. Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).
8.2.4. Fraction AQ 5

8.2.4.1. Fractions AQ 5.1 - 5.9

Fractionation of AQ 5 by RP-18 VLC obtained 9 fractions (AQ 5.1 - 5.9). The TLC analysis of AQ 5.6 - 5.9 (Fig. 8.12) showed the presence of the bright pink spot, which again was likely to be rosmarinic acid, as it was already observed in the starting fraction AQ 5 (Fig. 8.3).

Figure 8.12: TLC analysis of the VLC fractions AQ 5 and AQ 5.1 - 5.9. Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).

Fractions AQ 5.1 - 5.9 were tested in a 1:2 dilution series ranging from 0.6 to 75 µg/ml. Fraction AQ 5.8 and AQ 5.9 exhibited estrogenic responses at 37.5 and 75 µg/ml in the ERLUX assay (Fig. 8.13). Fraction AQ 5.1 - 5.7 did not display any estrogenic activity in the ERLUX assay (see Appendix). The fractions AQ 5.1 - 5.9 were not subjected to further fractionation because of time constrains and their weak estrogenic potential compared to other more promising fraction AQ 6 and AQ 7.

Figure 8.13: ERLUX assay results for fractions AQ 5.8 (blue) and 5.9 (red). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 5 (grey, dotted line) is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.4).
8.2.5. Fraction AQ 6

8.2.5.1. Fraction AQ 6.1-6.13

RP-18 VLC of AQ 6 resulted in fractions AQ 6.8 - 6.13. The fractions AQ 6.1 - 6.8 were combined to AQ 6.8 based on TLC analysis (see Appendix). The TLC analysis of AQ 6.8 to AQ 6.13 is presented in Figure 8.14. Fractions AQ 6.8 – 6.13 were tested for estrogenicity in a 1:2 dilution series ranging from 0.6 to 75 μg/ml. Fractions AQ 6.9, AQ 6.10 and AQ 6.11 exhibited estrogenic response (Fig. 8.15), where AQ 6.8 (Fig. 8.15) and AQ 6.12 – 6.14 were inactive in the ERLUX assay (see Appendix).

AQ 6.10 was the fraction with the most potent estrogenic response in the ERLUX assay and it exhibited a supra-maximal response, with an EC$_{50}$ of 11 μg/ml. Both neighbouring fractions (AQ 6.9 and AQ 6.11) were also estrogenic, but with EC$_{50}$ values of 30 μg/ml (AQ 6.11) and 42 μg/ml (AQ 6.9), they were less potent than AQ 6.10. This distribution of the activity over three fractions may suggest spreading of one or more estrogenic compounds over three fractions. Fraction AQ 6.10 most likely contained the highest concentration of the compound(s) and showed the most interesting $^1$H-NMR spectrum of the three (Fig. 8.16).

Figure 8.15 suggests that AQ 6.10 is more potent than the parent fraction AQ 6 (EC$_{50}$ of 14 μg/ml). The dose-response curve of AQ 6.10 determined by the regression model was not an ideal fit for the data and the curve suggested a higher potency that the individual data points. However, even if fraction AQ 6.10 was equally potent as the parent fraction AQ 6 it was the most potent fraction obtained from AQ 6 and was therefore further fractionated.
Figure 8.15: ERLUX assay results for fractions AQ 6.8 (grey), AQ 6.9 (green), AQ 6.10 (pink) and AQ 6.11 (blue). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6 (grey, dotted line) is shown for comparison (reproduced from Figure 8.4).

Figure 8.16: 1H-NMR spectra of fractions AQ 6.9 - AQ 6.11 (MeOD, 400 MHz).
8.2.5.2. Fraction AQ 6.10.1 - 6.10.8

Fraction AQ 6.10 was further fractionated by size-exclusion chromatography. Fractions AQ 6.10.1 - 6.10.8 were tested in a 1:2 dilution series ranging from 0.3 to 35 μg/ml. Fractions AQ 6.10.5, AQ 6.10.6 and AQ 6.10.7 exhibited estrogenic effects in the ERLUX assay (Fig 8.17).

AQ 6.10.6 and 6.10.7 showed estrogenic activity at the lowest concentration (0.3 μg/ml), which could indicate that the higher test concentration caused cytotoxic effects and observed effects were false-negative results. However, at any of the higher concentrations tested these two fractions did not show cytotoxicity (see Appendix). The absence of cytotoxic effects led to the conclusion that estrogenicity was absent and not masked by cytotoxicity. It was therefore assumed that the observed estrogenic effects at 0.3 μg/ml were caused by an experimental variation. The fraction AQ 6.10.7 gave a maximal response around 0.6 (relative to E2) which was the highest estrogenic activity observed with the three fractions shown in Figure 8.17. Fractions AQ 6.10.1 - 6.10.4 were without estrogenic responses in the ERLUX assay (see Appendix).

Figure 8.17: ERLUX assay results for fractions AQ 6.10.5 (green), AQ 6.10.6 (red) and AQ 6.10.7 (blue). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis.
The AQ 6.10.8 was the most potent fraction obtained from AQ 6.10. The EC$_{50}$ of AQ 6.10.8 was 4 µg/ml (Fig. 8.19). The TLC analysis (Fig. 8.18) showed three yellow spots, which showed UV quenching activity at UV$_{254}$ nm. The other active fractions AQ 6.10.6 and 6.10.7 showed a pink spot at the top of the TLC plate, which was likely to be rosmarinic acid. This compound did not appear on the TLC chromatogram of fraction AQ 6.10.8. The fraction AQ 6.10.8 displayed a higher potency than the parent fraction AQ 6.10 (Fig. 8.15) and was subjected to further fractionation.

Figure 8.19: ERLUX assay results for fractions AQ 6.10.8 (pink). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6.10 (grey, dotted line) is shown for to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.15).
8.2.5.3. Fraction AQ 6.10.8.1-6.10.8.7

Size-exclusion chromatography of AQ 6.10.8 produced 7 further fractions. The fractions AQ 6.10.8.5 - 6.10.8.7 displayed a maximal estrogenic response of 1.0 (relative to E2) between 2.3 and 37.5 µg/ml (Fig. 8.21). Due to a limitation of isolated material the fractions were not tested for cytotoxicity and 'H-NMR spectra were not recorded.

![Figure 8.20: TLC analysis fraction AQ 6.10.8 and AQ 6.10.8.x (1-7). Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).](image)

The three fractions were more potent than the parent fraction AQ 6.10.8 and the EC50 values of all three fractions were 2.3 µg/ml. The isolated amounts were 0.74 mg for AQ 6.10.8.5, 0.63 mg for AQ 6.10.8.6 and 2.43 mg for AQ 6.10.8.7. The ERLUX results of fractions AQ 6.10.8.1 - 6.10.8.3 are presented in the Appendix. Fractions AQ 6.10.8.1 and 6.10.8.2 showed some estrogenic response and the activity of AQ 6.10.8.4 was similar to the estrogenic response by the parent fraction AQ 6.10.8 (EC50 4 µg/ml, see Appendix). Further analysis of fraction AQ 6.10.8.7 is described in section 8.3.2.

![Figure 8.21: ERLUX assay results for fractions AQ 6.10.8.5 (green), AQ 6.10.8.6 (blue) and AQ 6.10.8.7 (pink). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6.10.8 (grey, dotted line) is shown for comparison (reproduced from Figure 8.19).](image)
8.2.6. Fraction AQ 7

8.2.6.1. Fraction AQ 7.1 - 7.8

RP-18 VLC of fraction AQ 7 resulted in 8 fractions, which were tested in for estrogenicity. Figure 8.22 shows the TLC analysis of fractions AQ 7.1 - 7.8. Figure 8.23 displays the activity of fractions AQ 7.5 - 7.8 in the ERLUX assay. AQ 7.6 gave the highest estrogenic response. The estimated EC\textsubscript{50} value for AQ 7.6 of 3 \mu g/ml was identical to the EC\textsubscript{50} value of the parent fraction AQ 7. The estrogenic response of AQ 7.5 levelled off at concentrations higher than 18.8 \mu g/ml and AQ 7.7 exhibited estrogenic effects at 37.5 \mu g/ml and 75 \mu g/ml. Fraction AQ 7.8 did not show any estrogenic response in the ERLUX assay. The fraction AQ 7.3 and AQ 7.4 also showed estrogenic response in the ERLUX assay (see Appendix). The fraction AQ 7.6 was the most potent fraction and was therefore chosen for further fractionation.

Figure 8.22: TLC analysis of the VLC fractions AQ 7 and AQ 7.1 - 7.8. Stationary phase: Silica gel 60 F\textsubscript{254}, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).

Figure 8.23: ERLUX results of fractions AQ 7.5 (blue), AQ 7.6 (red), AQ 7.7 (non-filled circles) and AQ 7.8 (green). Circles represent individual data points obtained in one experiment, each tested in duplicate. Data of AQ 7.6 were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). Positive controls (E2, pink) and solvent controls (cyan) are indicated next to the y-axis. The regression curve of fraction AQ 7 (grey, dotted line) is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.4).
8.2.6.2. Fraction AQ 7.6.1 - 7.6.8

Eight fractions (AQ 7.6.1 - 7.6.8) were obtained by fractionation AQ 7.6 by size-exclusion chromatography over Sephadex. The TLC analysis (Fig. 8.24) of the fractions AQ 7.6.1 - 7.6.4 showed mainly blue-light purple compounds, whereas the other fractions (AQ 7.6.5 - 7.6.8) mainly consisted of compounds, which appeared as yellow to light pink spots on the TLC plate. Fractions AQ 7.6.4 and 7.6.6 appeared as bright blue fluorescing spot at the top of the TLC after detection with *Naturstoffreagenz-polyethyleneglycol* (NST-PEG) reagent. AQ 7.6.7 was dominated by an orange spot and displayed additional compounds, which appeared green and blue. Caffeic acid derivatives are known to appear blue with NST-PEG reagent and orange spots might indicate the presence of flavonoids.

![Figure 8.24: TLC analysis of the VLC fractions AQ 7.6 and AQ 7.6.1 - 7.6.8. Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (left, heated 1 min at 170 °C) and sprayed with NST-PEG reagent (right).](image)

Fraction AQ 7.6.7 exhibited estrogenic activity with an EC$_{50}$ value of 2 µg/ml (Fig. 8.25). The response did not reach the same maximal effect as E2 and a plateau was observed with concentrations higher than 2.3 µg/ml. AQ 7.6.6 showed a similar dose-response curve, but the estimated EC$_{50}$ value was 16 µg/ml. AQ 7.6.8 did not exhibit any estrogenic effect in the ERLUX assay and the same applied for fraction AQ 7.6.1 - 7.6.5 (see Appendix). Fraction AQ 7.6.7 was the most potent fraction and was subjected to further fractionation.
Figure 8.25: ERLUX results of fractions AQ 7.6.6 (red), AQ 7.6.7 (green) and AQ 7.6.8 (blue). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). Positive controls (E2, pink) and solvent controls (cyan) are indicated next to the y-axis. The regression curve of fraction AQ 7.6 (grey, dotted line) is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.23).

8.2.6.3. Fraction AQ 7.6.7.1-7.6.7.6

Fraction AQ 7.6.7 was further fractionated by size-exclusion chromatography over Sephadex LH-20. Obtained fractions were combined according to their appearance in TLC (Fig. 8.26) and ¹H-NMR analyses and resulted in 6 fractions (AQ 7.6.7.1-7.6.7.6).

![TLC analysis of the size-exclusion chromatography fractions AQ 7.6.7.1-6 and AQ 7.6.7.](image)

Figure 8.26: TLC analysis of the size-exclusion chromatography fractions AQ 7.6.7.1-6 and AQ 7.6.7. Stationary phase: Silica gel 60 F₂₅₄, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).

Fractions AQ 7.6.7.6 and 7.6.7.7 showed a strong estrogenic response in the ERLUX assay (Fig. 8.27). AQ 7.6.7.6 exhibited supra-maximal effects of 1.2 (relative to E2), which reached at plateau at 1.2 µg/ml. The estimated EC₅₀ value was 0.7 µg/ml. The fraction AQ 7.6.7.7 reached a plateau, with a lower maximal response around 1.0, at 2.3
μg/ml and the EC₅₀ value was calculated as 2.2 μg/ml. Fraction AQ 7.6.7.5 did not exhibit any estrogenic response in the ERLUX assay. The fractions AQ 7.6.7.1 - 7.6.7.4 showed weak estrogenic response in the ERLUX (see Appendix). Due to a limitation of isolated material the fractions were not tested for cytotoxicity and ¹H-NMR spectra were not available. The amounts of the isolated fractions AQ 7.6.7.6 and AQ 7.6.7.7 were 1.81 mg and 0.62 mg, respectively. The most estrogenic fraction AQ 7.6.7.6 was further analysed as described in section 8.3.2.

Figure 8.27: ERLUX results of fractions AQ 7.6.7.5 (green), AQ 7.6.7.6 (blue) and AQ 7.6.7.7 (red). Filled circles represent individual data points obtained in one experiment, each tested in duplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). Positive controls (E₂, pink) and solvent controls (cyan) are indicted next to the y-axis. The regression curve of fraction AQ 7.6.7 (grey, dotted line) is shown to allow comparison of the estrogenic potency between parent fraction and obtained fractions (reproduced from Figure 8.25).
8.3. Structure elucidation, identification and quantification of estrogenic compounds

8.3.1. Structure elucidation of compound 1 (AQ 4.4.6): Luteolin-7-O-β-glucuronide

Compound 1 (AQ 4.4.6, 42.9 mg) was isolated from the aq-EtOH SE of the crude Salvia officinalis extract as yellowish powder by RP-18 VLC followed by size-exclusion chromatography over Sephadex LH-20 as described in Figure 8.2.

Figure 8.28: TLC analysis of compound 1. Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).

Compound 1 quenched UV light at 254 nm on the silica TLC plate. After spraying with vanillin/sulphuric acid reagent, the compound appeared as a broad yellow spot (Fig. 8.28), which is suggestive for a flavonoid structure. The negative-mode ESI mass spectrometry [(-)-ESI-MS] spectrum (Fig. 8.29) showed a deprotonated molecule ion peak at $m/z$ 461.2 [M-H], suggesting the molecular formula C21H18O12. The molecular ion peak at $m/z$ 461.2 was subjected to collision energy of 35% of 5V (Fig. 8.30), which resulted in one fragment with $m/z$ 285.3. This fragmentation pattern suggested the cleavage of glucuronic acid (GluA) $m/z$ 176. The new peak also indicated luteolin to be the aglycone ([M-GluA-H]) (Fabre et al., 2001; Stochmal et al., 2001). The accurate mass of the compound was established as $m/z$ 463.0873 [M+H]$^+$ by high-resolution mass spectrometry (ESI-MS) (Fig. 8.31).
Figure 8.29: (-)-ESI-MS spectrum of compound 1.

Figure 8.30: (-)-ESI-MSMS spectrum of compound 1 after subjecting m/z 461.2 to collision energy of 35% of 5V showed the resulting fragment [M-GluA-H].

Figure 8.31: (+)-ESI-MS spectrum of compound 1.
Figure 8.32: $^1$H-NMR spectrum of compound 1 (DMSO-$d_6$, 500 MHz), B and C are expansions of A. Chemical structure presented is luteolin-7-O-$\beta$-glucuronide.

Figure 8.32 displays the $^1$H-NMR spectra of compound 1 in DMSO-$d_6$. The peak at $\delta$ 5.10 (H-1", d, $J = 7.4$ Hz) was characteristic for an anomeric sugar proton. It suggested the presence of a single sugar unit and the coupling constant of the annomeric proton ($J = 7.4$) suggested a $\beta$-configuration of the glycoside (reference $J = 7.8$ Hz, (Markham and Geiger, 1993)). The chemical shift of the anomeric sugar proton H-1" is indicative for a 7-O-glucuronidic nature of the compound (reference 5.1-5.18 ppm, (Markham and Geiger, 1993)).

In the downfield region of the $^1$H-NMR spectrum in DMSO-$d_6$ (Fig. 8.32 B), six aromatic signals that integrated for six protons were observed with chemical shift values of $\delta$ 7.46 (H-2', d, $J = 1.7$ Hz), $\delta$ 7.37 (H-6', dd, $J = 8.4$ Hz; 2.0 Hz), $\delta$ 6.86 (H-5', d, $J = 8.4$ Hz), $\delta$ 6.76 (H-8, d, $J = 2.0$ Hz), $\delta$ 6.70 (H-3, s) and $\delta$ 6.41 (H-6, d, $J = 2.0$ Hz). The coupling constants $J_{6,8}$ and $J_{5,6}'$ indicated meta coupling between these protons whereas
the coupling constant for $J_{5,6}$ showed these protons (H-5' and H-6') to be ortho positioned to each other. The observed doublet of doublets for H-6' is a further indicator that this proton coupled with two other protons. The meta coupling between H-6 and H-8 and the typical chemical shift values for H-6 and H-8 protons of 7-O-glycosilated or methylated 5,7-dihydroxyflavones $\delta$ 6.76 (H-8) and $\delta$ 6.41 (H-6) gave further indication on the substitution pattern of the aglycone (Markham and Geiger, 1993). The singlet at $\delta$ 6.7 is a typical H-3 signal of flavones (Lu and Foo, 2000).

In the upfield region of the $^1$H-NMR spectrum complex and overlapping signals between $\delta$ 3.13 and $\delta$ 3.37 were observed, which are typical for non-anomeric methine sugar protons. The signal for H-5'' at $\delta$ 3.67 ($J = 9.9$ Hz) is shifted downfield from the multiplet signal (Fig. 8.32 C) and represents a typical chemical shift for a H-5'' proton of glucuronides (Markham and Geiger, 1993). The loss of a fragment in (-)-ESI-MSMS with a molecular mass of m/z 176, further confirmed glucuronic acid as the sugar moiety (Stochmal et al., 2001).

Investigation of the $^{13}$C-NMR spectrum showed the presence of 20 peaks, which represented 21 carbon atoms (Fig. 8.33, A). The DEPT90° spectrum of compound 1 (Fig 8.33, B) contained ten peaks for eleven methine groups. The DEPT135° spectrum of compound 1 (Fig. 8.33, C) was identical to the DEPT90° spectrum and thus revealed the lack of methylene and methyl groups. Hence, the compound 1 had to contain ten quaternary carbons. The signal at $\delta$ 182.5 was readily assigned to a keto-function (C-4) and the signal at $\delta$ 172.0 to a carboxyl group (C-6''). The chemical shift of C-5 ($\delta$ 161.0) was indicative for a substitution with hydroxyl groups on the quaternary C-5. In addition, two hydroxylated quaternary carbons (C-3', $\delta$ 146.0, C-4', $\delta$ 150.3), three oxygenated quaternary carbons (C-2, $\delta$ 164.5, C-9, $\delta$ 157.0, C-7, $\delta$ 163.0) and one aromatic quaternary carbon (C1' $\delta$ 121.0) were observed.

Six aromatic methine groups (C-8 $\delta$ 94.5, C-6 $\delta$ 99.5, C-3 $\delta$ 102.9, C-2' $\delta$ 113.4, C-5' $\delta$ 116.1 and C-6' $\delta$ 119.0) were detected and the signal at $\delta$ 102.9 assigned to the C-3 of a flavone which was correlated by HMQC to the H-3 (Fig. 8.34) (Lu and Foo, 2000). HMQC revealed an additional methine group at $\delta$ 99.5 (C-1''). Anomeric carbon signals of O-glycosidic sugar moieties resonate around 90-112 ppm in $^{13}$C-NMR spectra, whereas signals for C-glycosides resonate around 70-80 ppm (Agrawal, 1992). Thus, the signal C-1'' at $\delta$ 99.6 confirmed an O-glycoside. The signals for C-3'' ($\delta$ 76.4), C-5'' ($\delta$
73.8), C-2" (δ 73.0) and C-4" (δ 71.9) resonate in the region of 51-86 ppm, which is typical for sugar methine signals.

Figure 8.33: $^{13}$C-NMR (A, 125 MHz) and DEPT90° (B, 125 MHz) and DEPT135° (C, 125 MHz) spectra of compound 1 (DMSO-$d_6$).
Figure 8.34: HMOC spectrum of compound 1 (DMSO-d6, 125 MHz, 32 scans)

The HMOC (Fig. 8.34) showed the connectivities between the proton and carbon atoms. It revealed connectivities between H-6 (δ 6.41) and H-1" (δ 5.10) with the peak at δ 99.5 and confirmed that it represented two carbon atoms. The second methine group at this position was linked to the anomeric sugar carbon C-1". The aromatic protons H-2', H-6', H-5', H-8, H-3 and H-6 were assigned to their corresponding carbon atoms (C-2', C-6', C-5', C-8, C-3 and C-6).

The correlations between the signal at δ 5.10 (H-1", d, J = 7.4 Hz) and C-1" at δ 99.6 confirmed the O-glycosidic nature of compound 1. The multiplet protons observed in the low field of the 'H-NMR spectrum (δ 3.13-3.37, m) correlated with the carbon signals at δ 72.0 (C-4"), δ 73.0 (C-2") and δ 76.4 (C-3"), which could be readily attributed to the sugar moiety by HMOC (Fig. 8.34). The signal for H-5" (δ 3.67) can be correlated to δ 73.81 (C-5"). The signal in the downfield region at δ 172.0 was indicative for a carboxylic group (Agrawal, 1992), hence was assigned to C-6" in glucuronic acid moiety.
The analysis of the COSY spectrum (Fig. 8.35) showed the presence of two proton spin systems within the compound. The first short spin system was composed of the aromatic protons H-2' and H-6' and H-5'. The protons in the glucuronide (H-1'' - H-5'') represented the second spin system.

An additional weak coupling between the methine protons H-8 and H-6 was also observed.
The HMBC spectrum assisted to investigate long-range correlations of protons with neighbouring carbon atoms further. The HMBC spectrum (Fig. 8.36 and Fig. 8.38) revealed a $^2J$ correlation between H-6 and C-5 ($\delta$ 161.0, s) and C-7 ($\delta$ 163.0, s); from H-8 to C-7 ($\delta$ 163.0, s) as well as H-8 to C-9 ($\delta$ 157.0, s). Further $^2J$ correlations between H-3 and C-2 ($\delta$ 164.5, s) and the carboxyl carbon C-4 ($\delta$ 181.8, s) confirmed the C-ring of the flavon (Fig. 8.37).

$^3J$ correlation between H-2' and C-3' ($\delta$ 145.95, s) as well as long-range $^3J$ correlations between H-2' and C-4' ($\delta$ 150.3, s), H-2' and C-6' ($\delta$ 119.0, d) and H-2' and C-2 (164.5, s) were observed, which confirmed the attachment of the B-ring to the C-2 position.
Additional heteronuclear coupling was observed from H-5" and H-4" to the carboxyl-carbon C-6" at δ 171.98 (s) (Fig. 8.37). The anomeric sugar proton H-1" showed correlations to C-7 (δ 162.96, s), hence verifying the attachment of the sugar unit at C-7 position of the aglycone.

The aromatic nature, the attached sugar moiety, the molecular mass and the above described ¹H-NMR and ¹³C-NMR data indicated that the isolated compound was a flavonoid glycoside. The mass of the aglycone was consistent with luteolin (m/z 286) and, as described above, the typical glucuronic acid anomeric proton signal (H-1", δ 5.1) together with the unusual fragmentation pattern were indicative of glucuronic acid as sugar moiety.

Hence, the compound isolated in the fraction AQ 4.4.6 was identified as luteolin-7-O-β-glucuronide (Fig. 8.39). This compound is a common flavonoid glycoside and has been described from numerous plant families (e.g., Asteraeae (Kim et al., 2007; Özgen et al., 2010), Fabaceae (Stochmal et al., 2001)) and several members of the Lamiaceae family.
e.g. Thymus vulgaris, Origanum majorana, Mentha piperita (Fecka and Turek, 2007; Fecka and Turek, 2008), Salvia species (Salvia palaestina, Salvia pratensis, Salvia triodea (for review see (Lu and Yeap Foo, 2002)), including Salvia officinalis (Lu and Foo, 2000; Fecka and Turek, 2007). The $^1$H-NMR- and $^{13}$C-NMR spectroscopy signals observed in this study are in agreement with previously reported data for luteolin-7-O-β-glucuronide (Lu and Foo, 2000; Özgen et al., 2010) (Tab. 8.1).

![Figure 8.39: Chemical structure of luteolin-7-O-β-glucuronide (fraction AQ 4.4.6, compound 1)](image)

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<td>100.2 $d$</td>
<td>5.10 (d, $J = 7.4$)</td>
<td>5.06 (d, $J = 7.3$)</td>
</tr>
<tr>
<td>2&quot;</td>
<td>73.0 $d$</td>
<td>73.6 $d$</td>
<td>3.13-3.37 (m)</td>
<td>3.14-3.39 (m)</td>
</tr>
<tr>
<td>3&quot;</td>
<td>76.4 $d$</td>
<td>77.1 $d$</td>
<td>3.13-3.37 (m)</td>
<td>3.14-3.39 (m)</td>
</tr>
<tr>
<td>4&quot;</td>
<td>71.9 $d$</td>
<td>72.6 $d$</td>
<td>3.13-3.37 (m)</td>
<td>3.14-3.39 (m)</td>
</tr>
<tr>
<td>5&quot;</td>
<td>73.8 $d$</td>
<td>74.5 $d$</td>
<td>3.67 (d, $J = 9.9$)</td>
<td>3.60 (d, $J = 9.9$)</td>
</tr>
<tr>
<td>6&quot;</td>
<td>172.0 $s$</td>
<td>172.5 $s$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 8.1: $^1$H-NMR- and $^{13}$C-NMR data for compound 1 (DMSO-$d_6$, 500 MHz/125 MHz). Chemical shifts $\delta$ are reported in ppm, $J$ values in Hz. * reference values for luteolin-7-O-β-glucuronide (Özgen et al., 2010).
The purity of the isolated luteolin-7-O-β-glucuronide was investigated by HPLC analysis (Fig. 8.40). The UV activity was measured in the range of 350 nm and 370 nm. The absorbance maxima of luteolin are at 252 nm and 347 nm (Stochmal et al., 2001). HPLC analysis with 10 µl (1 mg/ml) of fraction AQ 4.4.6 was analysed using a diode array detector and a reverse-phase silica column (LUNA 5 µm C18 (2), 100 Å, 250 x 4.6 mm. Gradient elution with H₂O and ACN (from 96% H₂O to 50% H₂O) confirmed 98% purity of the isolated luteolin-7-O-β-glucuronide, which was determined by calculating the areas under the corresponding chromatographic peaks with the ChemStation software.

Figure 8.40: Determination of the purity by HPLC analysis of luteolin-7-O-β-glucuronide (compound 1).
8.3.2. Identification of fraction AQ 6.10.8.7 and AQ 7.6.7.6

Fractions AQ 6.10.8.7 (2.43 mg) and AQ 7.6.7.6 (1.81 mg) were obtained from the aq-EtOH SE of the crude *Salvia officinalis* extract as yellowish powder by repeated chromatography including RP-18 VLC and size-exclusion chromatography over Sephadex LH-20 as described in the fractionation scheme (Fig. 8.2).

Fractions AQ 6.10.8.7 and AQ 7.6.7.6 were chosen for further investigation, because of their higher estrogenic activity and/or the higher amounts available compared to other active fractions.

Although fractions AQ 6.10.8.5 and AQ 6.10.8.6 appeared to be identical to AQ 6.10.8.7 on TLC (Fig. 8.20) and also exhibited the same estrogenic activity (EC50 2.3 μg/ml, Fig. 8.21) only AQ 6.10.8.7 was subjected to further analysis to identify the compound as it was with 1.81 mg the largest fraction. The three fractions were not combined, as the TLC analysis was not sufficient to ensure that the fractions were identical and 1H-NMR spectra could not be recorded.

The same applied to fraction AQ 7.6.7.7, which appeared as a single spot on TLC (Fig. 8.26) and furthermore looked purer than fraction AQ 7.6.7.6 on a silica TLC plate. However, due to a higher EC50 value (2.2 μg/ml) compared to that of fraction AQ 7.6.7.6 (0.7 μg/ml) and the small amounts that were available (0.62 mg) this fraction was not subjected to further analysis.

![Figure 8.41: TLC analysis of AQ 6.10.8.7 and AQ 7.6.7.6 (api = apigenin, lut-7-gluc = luteolin-7-O-glucoside, quer = quercetin, lut = luteolin). Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).](image-url)

Figure 8.41: TLC analysis of AQ 6.10.8.7 and AQ 7.6.7.6 (api = apigenin, lut-7-gluc = luteolin-7-O-glucoside, quer = quercetin, lut = luteolin). Stationary phase: Silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C).
The TLC analysis (Fig. 8.41) of AQ 6.10.8.7 and AQ 7.6.7.6 as well as fraction AQ 7.6.7.7, AQ 6.10.8.5, AQ 6.10.8.6 indicated that the fractions are likely to contain luteolin-7-O-glucoside. All fractions showed UV quenching activity at 254 nm. Fractions AQ 7.6.7.7, AQ 7.6.7.6 and luteolin-7-O-glucoside appeared as brown-yellow spots after spraying with the universal reagent vanillin/sulphuric acid, whereas fractions AQ 6.10.8.5, AQ 6.10.8.6 and AQ 6.10.8.7 appeared as yellow spots.

Fractions AQ 6.10.8.7 (2.43 mg), AQ 7.6.7.6 (1.81 mg) and commercial luteolin-7-O-glucoside (Extrasynthese, France) were analysed by \(^1\)H-NMR spectroscopy (Fig. 8.42). Luteolin-7-O-glucuronide (AQ 4.4.6) was also included in order to allow direct comparison of the spectra.

![Chemical structures](image)

**Figure 8.42: \(^1\)H-NMR spectra of luteolin-7-O-glucuronide (AQ 4.4.6, blue, 500 MHz), luteolin-7-O-glucoside (commercially available, green, 400 MHz), AQ 6.10.8.7 (red, 400 MHz) and AQ 7.6.7.6 (black, 500 MHz) in DMSO-\(_d_6\). Chemical structures are luteolin-7-O-glucoside (left) and luteolin-7-O-glucuronide (right).**

The \(^1\)H-NMR spectra of the fractions AQ 6.10.8.7 and AQ 7.6.7.6 showed similarities in the aromatic region (~6.5-7.7 ppm) and the anomeric proton (~5 ppm) with the \(^1\)H-
NMR spectrum of the luteolin-7-O-glucoside and the luteolin-7-O-glucuronide (AQ 4.4.6). This was a further indication that the fractions contain the same aglycone, luteolin, as the signals of the aglycone luteolin such as the protons H-2’, H-5’ and H-6’ of the B-ring and H-6 and H-8 of the A-ring are usually not affected by the sugar moiety (Markham and Geiger, 1993).

The spectra of the fractions AQ 6.10.8.7 and AQ 7.6.7.6 contained additional signals at aromatic and aliphatic regions compared to the spectra of luteolin-7-glucoside and luteolin-7-glucuronide, which indicated that they contained more than one compound and hence were not pure.

The samples were sent to the laboratory of Professor Jean-Luc Wolfender from the University of Geneva, Switzerland, for further analysis. Philippe Eugster conducted an ultra-high-pressure liquid chromatography at elevated temperature coupled to time-of-flight mass spectrometry (UHPLC-TOF-MS, as described in chapter 2) in order to confirm the assumption that both fraction AQ 6.10.8.7 and AQ 7.6.7.6 contained luteolin-7-O-glucoside. In addition capillary NMR (capNMR) experiments were carried out by Laurence Marcourt to assist with the identification of other compounds contained in the fraction (Professor Jean-Luc Wolfender’s laboratory). The positive mode UHPLC-TOF-MS spectra (Fig. 8.43) showed a protonated molecule ion peak at \( m/\zeta \) 449.1, suggesting the molecular formula \( C_{21}H_{33}O_{11} \). The \([M+H]^+\) peak at \( m/\zeta \) 449.1 of the pure compound luteolin-7-O-glucoside was observed at the same retention time and confirmed that both fractions contained luteolin-7-O-glucoside.

Figure 8.43: UHPLC-TOF-MS (ESI+) spectra of AQ 6.10.8.7 (A) and AQ 7.6.7.6 (B) and commercial luteolin-7-O-glucoside (C).
The capNMR spectra of fraction AQ 7.6.7.6 (Fig. 8.44) revealed the presence of 3 singlets around δ 6.6, which are typical for H-3 protons of flavonoids and therefore indicated that fraction AQ 7.6.7.6 contained three different flavonoids. The integration of the singlets suggested a ratio of 1.0 : 0.8 : 0.5-0.6 mol of each flavonoid. Additionally signals around δ 6.5 and δ 6.8 can be assigned to H-8 and H-6 protons of flavonoids respectively. The doublet at δ 6.9 and the two doublets δ 7.4 were assigned to the H-5' and H-6'/H-2' protons of the B-ring of luteolin (15 and 16/12 in red in Fig. 8.44). The two doublets at δ 6.95 for H-3'/H-5'and the two doublets at δ 7.9 for H-2'/H-6' are indicative for apigenin (13/15 and 12/16 in blue in Fig. 8.44) (Stochmal et al., 2001). Integration of the anomeric proton signal at δ 5.1 is 2.52 and revealed that all flavonoids are glycosides. The chemical shift of H-6 and H-8 protons are very similar, which suggests that the sugar moiety was in the same C-7 position. Gradient elution of AQ 7.6.7.6 with UHPLC-TOF-MS confirmed the presence of luteolin-7-O-glucoside with a molecular ion peaks at m/z 449 [M+H]⁺ and for apigenin-7-O-glucoside at m/z 433 [M+H]⁺ (see Appendix). The third flavonoid could not be not identified. The two additional signals around δ 7.6-7.7 are characteristic for common phthalates structures. Those and the signals around δ 3.5-4.3 and δ 1.0-1.7 were assigned to phthalates present in the AQ 7.6.7.6 (Fig. 8.44). Hence, the presence of the phthalate(s) explained the aliphatic signals, which were observed in the ¹H-NMR spectrum of AQ 7.6.7.6 (δ 0.8-1.5, Fig. 8.42), as well as the peaks at δ 4.1 and ~7.7 ppm. CapNMR analysis of fraction AQ 6.10.8.7 was not recorded because of the availability insufficient amounts of the fraction.
Figure 8.44: CapNMR spectrum of AQ 7.6.7.6 in MeOD (500 MHz)
8.3.3. Quantitative analysis of luteolin derivatives in the extracts

In order to allow conclusions about the influence of the isolated active compound luteolin-7-O-glucuronide (compound 1, AQ 4.4.6) and the luteolin-7-O-glucoside that was present in the most potent active fractions AQ 6.10.8.7 and AQ 7.6.7.6 on the overall estrogenicity of the different fractions and the crude *Salvia officinalis* extract they were quantified in the parent fractions AQ 4, AQ 6, AQ 7, in the fractions AQ 6.10.8.7 and AQ 7.6.7.6 as well as the crude *Salvia officinalis* extract and the aq-EtOH SE. Experiments were conducted by Philippe Eugster and quantification was achieved by calibration curves with a gradient UHPLC-TOF-MS (Acquity BEH RP-18 shield 50 mm x 2.1 mm, Ø 1.7 μm) (for protocol see chapter 2 and for spectra see Appendix). The isolated luteolin-7-O-glucuronide (AQ 4.4.6, section 8.3.1) was used as standard for the calibration curves, as well as the pure, commercially available luteolin-7-O-glucoside (Extrasynthese, France). The amounts of compounds in the fractions are presented as % of dried extract (m/m) (Tab. 8.2).

The crude *Salvia officinalis* extract contained 1.0% luteolin-7-O-glucoside and 6.8% luteolin-7-O-glucuronide. The aq-EtOH SE contained 0.55% of luteolin-7-O-glucoside and 6.9% of luteolin-7-O-glucuronide, whereas the subfraction AQ 4 did not contain any detectable amounts of luteolin-7-O-glucoside, but 4.8% of luteolin-7-O-glucuronide.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Luteolin-7-O-glucoside</th>
<th>Luteolin-7-O-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude <em>Salvia officinalis</em> ex.</td>
<td>1.0</td>
<td>6.8</td>
</tr>
<tr>
<td>aq-EtOH SE</td>
<td>0.55</td>
<td>6.9</td>
</tr>
<tr>
<td>AQ 4</td>
<td>n.d</td>
<td>4.8</td>
</tr>
<tr>
<td>AQ 6</td>
<td>10.1</td>
<td>12.3</td>
</tr>
<tr>
<td>AQ 6.10.8.7</td>
<td>1.5</td>
<td>n.d</td>
</tr>
<tr>
<td>AQ 7</td>
<td>7.8</td>
<td>0.3</td>
</tr>
<tr>
<td>AQ 7.6.7.6</td>
<td>23.7</td>
<td>n.d</td>
</tr>
</tbody>
</table>

* mass of compound in dried extract in %, n.d. not detected

Table 8.2: Quantities of luteolin-7-O-glucoside and luteolin-7-O-glucuronide in some of the estrogenic fractions and the *Salvia officinalis* extract.

The amounts of luteolin-7-O-glucoside (10.1%) and luteolin-7-O-glucuronide (12.3%) were similar in fraction AQ 6, but its subfraction AQ 6.10.8.7 appeared to contain only 1.5% of luteolin-7-O-glucoside.

With 1.5% the amount of luteolin-7-O-glucoside in fraction AQ 6.10.8.7 was very low. The UHPLC-TOF-MS analysis revealed only one compound, which suggested the...
presence of other compound(s) that were not detected by UHPLC-TOF-MS.
Experiments using capNMR spectroscopy (conducted by Laurence Marcourt) to
identify the additional compounds failed as the available amount of sample was too
small. Hence, the fraction AQ 6.10.8.7 contained luteolin-7-O-glucoside and one or
more unidentified compound(s).
Luteolin-7-O-glucuronide was only present in minor amounts (0.3 %) in fraction AQ 7,
whereas the content of luteolin-7-O-glucoside was 7.8%. The most active fraction of the
Salvia officinalis extract (AQ 7.6.7.6) consisted of 23.7% luteolin-7-O-glucoside.
8.3.4. Estrogenic activity of the luteolin derivatives in comparison with known estrogens

The pure commercially available luteolin-7-O-glucoside (>98% pure, Extrasynthese, France) was tested in the ERLUX assay, because it was the major compound in the most active fraction AQ 7.6.7.6. In order to compare the estrogenic potency of the identified luteolin derivatives the isolated compound luteolin-7-O-glucuronide (compound 1, AQ 4.4.6), the aglycone luteolin (>99% pure, Extrasynthese, France) and the phytoestrogen and isoflavonoid genistein (>97% pure, Alfa Aesar) were also included in the tests (as described in 7.4, Fig 8.45). The tested concentration range was 0.845 - 162.3 μM for luteolin-7-O-glucuronide, 0.217 - 278.7 μM for luteolin-7-O-glucoside, 0.00023 - 181.0 μM for luteolin, 0.00004 - 150.0 μM genistein and 0.27 x 10⁻⁹ - 0.00077 μM for E2 (reproduced from Fig 7.1). The samples were tested for cytotoxicity on the T47D-KBluc cells to identify false-negative results (as described in 7.5).

![Figure 8.45: ERLUX assay results of E2, genistein and luteolin derivatives. Individual data points (A) and regression curves only (B). Filled circles represent data points of one experiment tested in triplicate. Data were fitted using a non-linear regression model (sigmoid, 4 parameter Hill). The data and regression curve of E2 (pink) are reproduced from Figure 7.1. Data and regression curves for genistein are shown in blue (one independent experiment), luteolin-7-O-glucoside in green (two independent experiment), luteolin in red (three independent experiment) and for luteolin-7-O-glucuronide (AQ 4.4.6, three independent experiment) in dark yellow.](image-url)
Genistein and luteolin-7-O-glucoside exhibited supra-maximal effects (higher than the effect of E2 at 1 nM) with EC\textsubscript{50} values of 0.0081 µg/ml and 0.44 µg/ml respectively. The maximal effects of luteolin (EC\textsubscript{50} 1.14 µg/ml) and luteolin-7-O-glucuronide (EC\textsubscript{50} 129.3 µg/ml) were smaller than the maximal effect of E2 (EC\textsubscript{50} 4.0 x 10\textsuperscript{-7} µg/ml). The most potent plant-derived flavonoidal compound tested was genistein, followed by luteolin-7-O-glucoside, luteolin and luteolin-7-O-glucuronide (Tab. 8.3) in order of decreasing potency.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC\textsubscript{50} (nM)</th>
<th>EC\textsubscript{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-β estradiol (E2)</td>
<td>0.0015</td>
<td>4.0 x 10\textsuperscript{-7}</td>
</tr>
<tr>
<td>Genistein</td>
<td>30</td>
<td>0.0081</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside</td>
<td>1000</td>
<td>0.44</td>
</tr>
<tr>
<td>Luteolin</td>
<td>4000</td>
<td>1.14</td>
</tr>
<tr>
<td>Luteolin-7-O-glucuronide</td>
<td>280 x 10\textsuperscript{3}</td>
<td>129.3</td>
</tr>
</tbody>
</table>

Table 8.3: EC\textsubscript{50} values of pure compounds in the ERLUX assay compared to E2.

Genistein (EC\textsubscript{50} 30 nM) was ~30 times more potent than luteolin-7-O-glucoside (EC\textsubscript{50} 1000 nM) in the ERLUX assay. The estrogenic potency of the 7-O-glucoside of luteolin was four times higher than that of the luteolin aglycone and it was 280 times more potent than the glucuronide. The EC\textsubscript{50} value of luteolin-7-O-glucuronide (EC\textsubscript{50} 129.3 µg/ml) was different to that reported in section 8.2.3.2 (EC\textsubscript{50} 64 µg/ml). The reason was that the first EC\textsubscript{50} value was only based on one experiment, whereas the EC\textsubscript{50} in this section is based on three independent experiments.
The pure compounds were tested for cytotoxic effect, genistein at concentration ranging from 0.00024 to 191.8 μM, luteolin-7-O-glucoside from 0.212 to 278.78 μM, luteolin from 0.00023 to 181.0 μM and luteolin-7-O-glucuronide from 253.6 to 324.6 μM (Fig. 8.46).

The cell viability observed in the cytotoxicity assay did not show any strong toxic effects on the T47D-KBluc cells of any of the tested compounds. Luteolin-7-O-glucoside (green) and luteolin (red) showed an apparent increase in cell viability at lower concentrations. This may be due to an effect occurring at lower doses but that does not persist at higher doses, or could be an artefact introduced by, for example, variation in cell seeding densities across the multi-well plates. The same applied to genistein at around 0.7 μM. The cell number was reduced by around 10% by luteolin (~ 3.0 and 18.0 μM) and luteolin-7-O-glucoside (~27.0 μM) at the higher concentrations. Overall, the observed cell toxicity weak and should not interfere with the estrogenicity results in the ERLUX assay.
8.4. The effect of the luteolin derivatives on the estrogenic effect of the *Salvia officinalis* and aq-EtOH extracts

Herbal extracts are complex mixtures of many compounds. Identification of an active compound is always accompanied by the question whether it can explain the overall biological effect of the extract. The estrogenic activity of the crude *Salvia officinalis* extract and the aq-EtOH SE and the concentration of the most potent active compound are therefore discussed together in this section.

The crude *Salvia officinalis* extract did not show estrogenic effects, whereas the aq-EtOH SE exhibited an estrogenic response with an EC$_{50}$ of 64 µg/ml. The luteolin-7-O-glucoside contained in fractions AQ 6.10.8.7 and AQ 7.6.7.6 displayed an even higher activity (EC$_{50}$ 2.3 µg/ml and 0.7 µg/ml respectively) than the SE from which they originated AQ 6 (EC$_{50}$ 14 µg/ml) and AQ 7 (EC$_{50}$ 3 µg/ml). The isolated luteolin-7-O-glucuronide (AQ 4.4.6), however, showed a weaker estrogenic response (EC$_{50}$ 129.3 µg/ml) than its parent fraction AQ 4 (EC$_{50}$ 69 µg/ml) and the aq-EtOH SE.

Luteolin-7-O-glucoside was found to be the major compound in AQ 1.6.1.6 and in addition to that was the EC$_{50}$ value for AQ 7.6.7.6 very similar to the one for the pure commercially available luteolin-7-O-glucoside (0.7 µg/ml and 0.44 µg/ml respectively). It was therefore possible that the luteolin-7-O-glucoside may be the active principle of the fraction AQ 7.6.7.6 and it was further discussed here whether it can explain the overall effect of the extracts.

The concentration of luteolin-7-O-glucoside in the crude *Salvia officinalis* extract was found to be 1.0% (m/m) by UHPLC-TOF-MS analysis (see Tab. 8.2). Hence, an arbitrarily chosen test-concentration of 100 µg/ml of *Salvia officinalis* extract will contain 1.0 µg/ml of luteolin-7-O-glucoside. The quantitative analysis of the aq-EtOH SE showed that it contained 0.35 µg/ml of luteolin-7-O-glucoside (0.55%, m/m) at the EC$_{50}$ concentration of 64 µg/ml. These results are included in Figures 8.47 and 8.48.

The comparison of the obtained results can be used to assess the contribution of the luteolin-7-O-glucoside content to the estrogenic effect of the extracts and may help to decide whether the overall effect of the extract can be explained in terms of its luteolin-7-O-glucoside content.

The concentration of luteolin-7-O-glucoside (1.0 µg/ml, Fig. 8.47, b), which is present in the crude *Salvia officinalis* extract at 100 µg/ml (Fig. 8.47, a), should exhibit maximal estrogenic response based on the results for the luteolin-7-O-glucoside standard (Fig. 8.47, c). As the effect was not observed for the crude *Salvia officinalis* extract at this
concentration the effect of the luteolin-7-O-glucoside must have been masked in the crude extract. The concentration of luteolin-7-O-glucoside (0.35 μg/ml, Fig. 8.48, b), which is present in the aq-EtOH SE at 64 μg/ml (Fig. 8.48, a), would be expected to produce a half-maximal estrogenic response based on the results for the luteolin-7-O-glucoside standard (Fig. 8.48, c). Accordingly, the aq-EtOH SE exhibited a half-maximal estrogenic response at 64 μg/ml and thus, it appeared that the luteolin-7-O-glucoside in the SE is could be solely responsible for the observed estrogenic effect in the aq-EtOH SE.

Figure 8.47: Comparison of the estrogenic effects of luteolin-7-O-glucoside and the crude Salvia officinalis extract in the ERLUX assay. The data and regression curve of the crude Salvia officinalis extract (red) are reproduced from Figure 7.2, chapter 7 (data represented by non-filled circles were not considered for the fit) and data and regression curve for luteolin-7-O-glucoside (green) are reproduced from Figure 8.45, chapter 8. Vertical dashed line a is used to highlight the estrogenic effect of the Salvia officinalis extract at 100 μg/ml, b indicates the amount of luteolin-7-O-glucoside (1.0 μg/ml) present at a concentration of 100 μg/ml of Salvia officinalis extract and c compares the estrogenic effects at both concentrations.

Figure 8.48: Comparison of the estrogenic effects of luteolin-7-O-glucoside and the aq-EtOH SE in the ERLUX assay. The data and regression curve of the aq-EtOH SE (yellow) are reproduced from Figure 7.2, chapter 7 and data and regression curve for luteolin-7-O-glucoside are reproduced from Figure 8.45, chapter 8. Vertical dashed line a indicates the estrogenic effect of the aq-EtOH SE at 64 μg/ml (EC50 concentration), b indicates the amount of luteolin-7-O-glucoside (0.55 μg/ml) present at a concentration of 64 μg/ml of aq-EtOH SE and c compares the estrogenic effects at both concentrations.
8.5. Discussion

The activity-guided fractionation of the estrogenic aq-EtOH SE resulted in four fractions, which showed estrogenic activity in the ERLUX assay and these were further fractionated. Fractions AQ 4, AQ 5, AQ 6 and AQ 7 showed dose-response relationships and their EC_{50} values were 69 μg/ml (AQ 4), 58 μg/ml (AQ 5), 14 μg/ml (AQ 6) and 3 μg/ml (AQ 7). The estrogenic activity in AQ 4, AQ 6 and AQ 7 initially increased in all fractions with further fractionation, but fractionation of AQ 5 resulted in a loss of the activity.

The results presented in this chapter show the challenges of activity-guided fractionation. Some fractionations followed the basic idea of lead compound discovery from naturally sources, which assumes that fractions should display a higher activity than the original extract (Houghton et al., 2007), and others lost their activity during the process. The observed loss of activity in subfractions of AQ 5 is an effect often observed in natural product research and may be explained by a decomposition or chemical transformation of the active compounds during the fractionation process (Houghton et al., 2007). It is further possible that synergistic effects were responsible for the initial activity of fraction AQ 5, as synergism can cause a greater biological effect than that of the individual compounds (Williamson, 2001).

The fractionation of AQ 4 led to a marginal initial increase of the estrogenic potency from an EC_{50} value of 69 μg/ml in AQ 4 to an EC_{50} value of 47 μg/ml in AQ 4.4. However, the final fraction AQ 4.4.6 and hence the pure compound 1 showed lower potency with an EC_{50} value of 129 μg/ml. Its estrogenic activity was initially detected with an EC_{50} 64 μg/ml (section 8.2.4.1). Further experiments then showed a reduced estrogenticity, which resulted in a lower mean EC_{50} value for AQ 4.4.6. This could be indicative of a decomposition of the active compound. The isolation of sufficient amounts of compound 1 (AQ 4.4.6) allowed structure elucidation based on NMR- and MS analysis (section 8.3.1) and it was identified as luteolin-7-O-glucuronide.

Only small amounts of fractions AQ 6.10.8.7 and AQ 7.6.7.6 were available and the identification of the compounds in these fractions was approached by 1H-NMR, HPLC and UHPLC-TOF-MS and capNMR analysis (section 8.3.2).

It was possible to identify the estrogenic luteolin-7-O-glucoside as one compound present in both fractions AQ 6.10.8.7 and AQ 7.6.7.6 by UHPLC-TOF-MS analysis. Quantitative analysis of luteolin-7-O-glucoside and luteolin-7-O-glucuronide in the crude Salvia officinalis extract by positive mode UHPLC-TOF-MS showed that the amounts of luteolin-7-O-glucoside were minimal (1.0%, m/m) and the amount of...
luteolin-7-O-glucuronide was rather small (6.8%). However, the low concentration of the estrogenic luteolin-7-O-glucoside in the crude *Salvia officinalis* extract did not explain the absence of a clear estrogenic effect in the crude *Salvia officinalis* extract. With the amounts of luteolin-7-O-glucoside detected in the crude *Salvia officinalis* extract, a maximal estrogenic response in the ERLUX assay would have been expected for this extract. The lack of such an activity may be explained by antagonistic effects between compounds of the extracts, which could lead to a reduced effect from that expected (Williamson, 2001).

In the aq-EtOH SE, the amounts of the luteolin-7-O-glucoside (0.55%, m/m) could alone explain the observed estrogenic effect of the aq-EtOH (Fig. 8.48).

Fraction AQ 7.6.7.6, with an EC₅₀ value of 0.7 μg/ml the most potent fraction, was found to contain apigenin-7-O-glucoside and phthalate(s) in addition to luteolin-7-O-glucoside and another unidentified compound. Apigenin-7-O-glucoside has previously been found to exhibit estrogenic activity in an assay utilizing the MCF7/ERE luc cell line (Innocenti et al., 2007) and it could therefore have contributed to the estrogenic effect of the fraction AQ 7.6.7.6. The same could apply for the phthalate(s), although the detected estrogenic activity of phthalates was generally low (Harris et al., 1997; Shen et al., 2009). However, the activity of the commercially luteolin-7-O-glucoside was very similar to the activity of fraction AQ 7.6.7.6 which could indicate that luteolin-7-O-glucoside was the active principle of this fraction.

The commercially available aglycone luteolin, which was also tested in the ERLUX assay (Fig. 8.42 and Tab. 8.2) exhibited an estrogenic response with an EC₅₀ value of 1.14 μg/ml and although not isolated in this work, it is known to be present in *Salvia officinalis* (Lu and Yeap Foo, 2002).

Thus, also the crude *Salvia officinalis* did not exhibit estrogenic effects in the chosen cell-based assays (see section 7.7), following the estrogenicity by activity-guided fractionation revealed luteolin-7-O-glucoside and luteolin-7-O-glucuronide as estrogenic compounds within the crude extract.
9. Conclusions and final discussion

Hot flushes are a frequent problem for menopausal women. The question of what physiological event causes the hot flush has not been answered and neither has a treatment been identified that is without the burden of side effects.

This thesis investigated a *Salvia officinalis* extract, which has been shown to give relief to women suffering from hot flushes (Bioforce, 2005). In order to identify a mode of action and active principle(s) it was tested in multiple *in vitro* assays.

The assays were used to test the crude *Salvia officinalis* extract as well as its subextracts (SE), the n-hexane SE, CHCl₃ and the aqueous-ethanolic (aq-EtOH) SE, which were obtained by liquid-liquid partitioning of the crude extract. The estrogen receptor reporter gene (ERLUX) assay and the estrogen screen (ESCREEN) assay are well established and evaluated methods to test for estrogenic activity (summary of the results, Tab. 9.1). The acetylcholinesterase inhibition (AChEI) assay and the selective serotonin re-uptake inhibition (SSRI) assay were carried out according to established protocols but had to be implemented at the School of Pharmacy (summary of the results, Tab. 9.1). The ERLUX assay and the SSRI assay, which utilised the T47D-Kbluc and the HEK293-hSERT cell-lines, respectively, required the additional assessment of cytotoxic effects of the test samples in an cytotoxicity assay (MTT assay). These additional tests ensured that potential false-negative or false-positive results were identified and the cytotoxicity assay was adjusted to run in parallel to the ERLUX and SSRI assays. The potential for cytochrome P450 3A4 inhibition was evaluated using a microtiter plate format assay and inhibitory activity was found for the crude *Salvia officinalis* extract and the n-hexane SE, CHCl₃ SE (summary of results, Tab. 9.1).

Investigation of the crude *Salvia officinalis* extract and its SE with these different biological assays was followed by an activity-guided fractionation of the most significant activity, which was estrogenicity. In the ERLUX and ESCREEN assays the aq-EtOH SE showed the most significant anti-hot flush activity of all tested extracts (EC₅₀ of 64 µg/ml in the ERLUX assay). The aq-EtOH SE was therefore chosen for an activity-guided fractionation that led to the identification of two active compounds. The flavone glycosides luteolin-7-O-glucoside and luteolin-7-O-glucuronide were found to be estrogenic and present in the most active fractions of the aq-EtOH SE. Luteolin-7-O-glucuronide was isolated and found to be the active principle of fraction AQ 4. The luteolin-7-O-glucoside was identified as the major compound in the most potent fraction (AQ 7.6.7.6), which also contained apigenin-7-O-glucoside, another unidentified flavonoid and phthalate(s). The assessment of the estrogenic potency of pure
compounds, commercially available luteolin-7-O-glucoside and isolated luteolin-7-O-
glucuronide (AQ 4.4.6), resulted in an estrogenic potency of 0.44 μg/ml and 129.3
μg/ml in the ERLUX assay respectively. The observed estrogenic activity of luteolin-7-
O-glucuronide was weak, whereas the activity of luteolin-7-O-glucoside was of
interesting potency. Nevertheless, the identified estrogenic compounds cannot explain
the overall anti-hot flush effect of the crude Salvia officinalis extract. The reasons for this
conclusion are discussed in detail in the following sections.

It has to be concluded that the anti-hot flush mode of action of the Salvia officinalis
extract could not be identified and that other factors than estrogenic effects play a role
in contributing to the anti-hot flush mode of action of the Salvia officinalis extract.

The identified estrogenic compounds were of interesting potency and further
investigations of other active compounds and a synergistic potential may give crucial
information for further evaluations of the mode of action of the crude Salvia officinalis
extract.

Summary of the results in the biological assays

The crude Salvia officinalis tincture (S. officinalis ex.), the n-hexane, the chloroform (CHCl₃)
and the aqueous-ethanolic (aq-EtOH) subextracts (SEs) were tested in the listed
biological assays and the activities are briefly summarised in the following table.

<table>
<thead>
<tr>
<th>In vitro assay</th>
<th>Activity of the S. officinalis ex.*</th>
<th>Activity of the N-hexane SE*</th>
<th>Activity of the CHCl₃ SE*</th>
<th>Activity of the Aq-EtOH SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP assay</td>
<td>IC₅₀ 6.8 (0.09 - 191.25)</td>
<td>IC₅₀ 1.8 (0.03 - 75.00)</td>
<td>IC₅₀ 3.1 (0.03 - 60.00)</td>
<td>IC₅₀ 281.2 (0.28 - 625.00)</td>
</tr>
<tr>
<td>AChEI assay</td>
<td>~10% inhibition at 108.0 and 216.0</td>
<td>-</td>
<td>~5% inhibition at 120.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3.4 - 216)</td>
<td>(2.3 - 37.5)</td>
<td>(1.9 - 120.0)</td>
<td>(10.7 - 340.9)</td>
</tr>
<tr>
<td>SSRI assay</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(4.6 - 125.0)</td>
<td>(31.25 and 125.0)</td>
<td>(31.25 and 125.0)</td>
<td>(4.6 - 125.0)</td>
</tr>
<tr>
<td>ERLUX assay</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>EC₅₀ 64.0</td>
</tr>
<tr>
<td></td>
<td>(1.2 - 153.0)</td>
<td>(0.01 - 30.5)</td>
<td>(0.8 - 100.0)</td>
<td>(1.2 - 150.0)</td>
</tr>
<tr>
<td>ESSCREEN assay</td>
<td>-</td>
<td>-</td>
<td>Not tested</td>
<td>EC₅₀ 130.0</td>
</tr>
<tr>
<td></td>
<td>(0.6 - 76.5)</td>
<td>Not tested</td>
<td>Not tested</td>
<td>(1.2 - 150.0)</td>
</tr>
</tbody>
</table>

* all concentrations are μg/ml, range of concentrations tested are presented in brackets
- no activity detected

Table 9.1: Summary of the results in the biological assays
Discussion of estrogenicity as the active principle of *Salvia officinalis*

The *Salvia officinalis* extract itself did not show any estrogenic effects in the ERLUX assay. Nonetheless estrogenic compounds were present in the extract, which may therefore exhibit estrogenic effects *in vivo*. It is therefore necessary to discuss the possibility of estrogenic effects on women seeking relief from hot flushes by using the crude *Salvia officinalis* extract and, furthermore, why it was concluded that estrogenic activity cannot solely explain the pharmacological effects of the extract.

It is clear that any estrogenic activity of the crude *Salvia officinalis* extract would not be based on the activity of one compound alone, as already two compounds, luteolin-7-O-glucoside and luteolin-7-O-glucuronide, which exhibited estrogenic activity, were identified in the extract. Additionally the compound apigenin-7-O-glucoside was discovered in one of the most potent active fractions (AQ 7.6.7.6) and it has previously been shown that apigenin-7-O-glucoside also acts as an estrogen *in vitro* in a study by Innocenti *et al.* (2007).

However, if one would assume that only the most potent of these estrogenic agents, luteolin-7-O-glucoside, was responsible for the overall effect of the *Salvia officinalis* extract, and that estrogenicity is the key factor important for the amelioration of hot flushes, it becomes obvious in the following sections that estrogenicity is an implausible explanation for the anti-hot flush effects observed in the clinical study (Bioforce, 2005).

Theoretical evaluation of the estrogenic potency of the luteolin-7-O-glucoside in the *Salvia officinalis* extract *in vivo*

Houghton *et al.* (2007) discussed that if a compound/extract exhibits a desired bioactivity *in vitro* at 1.0 mg/ml the amount of compound/extract necessary to be consumed by the patient would be 16 g and thus too large to be of practical use (the plasma volume of a average adult human was assumed to be 16 L).

With an EC$_{50}$ value of 0.44 µg/ml for the commercially available luteolin-7-O-glucoside (Tab. 8.3) and a concentration of 1.0% (m/m of dried extract) of luteolin-7-O-glucoside in the crude *Salvia officinalis* extract (Tab 8.2), it would be 704 g of dried crude *Salvia officinalis* extract (7.04 g pure luteolin-7-O-glucoside) which would have to be consumed by a patient per day.

704 g of dried crude *Salvia officinalis* extract would be equivalent to 46 litres of crude *Salvia officinalis* extract (1.7% dry residue, density of 0.9), which would be an impossible amount as a daily dose many reasons e.g. the volume, cost and appearance of other pharmacological effects including the effect of the 66% alcoholic solution.
Another way to assess the potential of luteolin-7-O-glucoside to influence the endocrine system is to compare relative potencies of substances. The relative potency of a compound can be expressed relative to the activity of a reference compound (Legler et al., 1999). Relative potencies are usually used to compare observed effects of one in vitro assay with data from another in vitro assay. The potency of luteolin-7-O-glucoside relative to the estrogenic response by estradiol (E2) in the ERLUX assay can be determined with the following formula, using EC\textsubscript{50} values in nM, where the relative potency of E2 is 1 per definition:

$$\text{relative potency} = \frac{\text{EC}_{50} \text{(compound)}}{\text{EC}_{50} \text{(E2)}}$$

The EC\textsubscript{50} value of E2 was 4.0 x 10\textsuperscript{-7} µg/ml (0.0015 nM) and the EC\textsubscript{50} value of luteolin-7-O-glucoside was found to be 0.44 µg/ml (1000 nM) (Tab. 8.3). The resulting relative potency of luteolin-7-O-glucoside is roughly 6 x 10\textsuperscript{5}, compared to E2.

Two mg per day of E2 is a dose used for a classical hormone replacement therapy (HRT, e.g. Bedol® (BNF and by Joint Formulary Committee, 2011)). Although it is not possible to directly convert from relative potencies in vitro to in vivo for many reasons, the amount of pure luteolin-7-O-glucoside that would be the equivalent of the daily dose of estradiol would be 1.9 Kg. Even though only a small percentage of estradiol (~2.0%) would be biologically active (the majority is conjugated) (Sodergard et al., 1982) the amount of pure luteolin-7-O-glucoside is so large that it would be unmanageable to be consumed by a patient and it would presumably exhibit other unwanted effects.

Although both of these theoretical assessments of the estrogenic potency in vivo are rather crude, and neglect many relevant factors for in vitro to in vivo predictions, they indicate that consumption of the amounts of extract or compound, that would be necessary to exhibit a pharmacological effect, would be impossible.

**Bioavailability of the estrogenic compound in the *Salvia officinalis* extract**

Another issue which cannot be ignored while discussing the possible effect of a compound in an herbal extract are the factors of absorption and metabolism. It has been shown that flavonoid aglycones can be absorbed from the gut by passive diffusion. The flavonoid glycosides are usually hydrolysed before they are absorbed as their corresponding aglycones. Although the glycosidic form of quercetin (rutin) has been detected in the blood circulation of individuals (Spencer et al., 1999), other studies reported that they failed to detect the luteolin-7-O-glycoside in human plasma or urine after the administration of Artichoke leaf extracts (Witteimer et al., 2005). This study also failed to detect any luteolin aglycone in human plasma or urine and it was therefore
suggested that these flavonoids are metabolised in the gut wall by either glucuronidation or sulfation ((Wittemer et al., 2005) for review see (Mukinda et al., 2010)).

It is therefore obvious that it is unlikely that the necessary plasma concentration of the estrogenic luteolin-7-O-glucoside would be reached for two reasons: First, consumption of the amount of the crude Salvia officinalis extract that is needed is unrealistic and secondly, the bioavailability of the compound is poor (or absent). Hence, the luteolin-7-O-glucoside in the crude Salvia officinalis extract may be considered unlikely to exhibit any pharmacologically relevant effect in vivo at all.

It would be interesting to assess the concentration of luteolin-7-O-glucuronide that is obtained by metabolising luteolin aglycone in the plasma, as this compound did also show estrogenic activity. However, the amount of Salvia officinalis extract, which would have to be consumed to reach relevant plasma levels, even though other luteolin derivatives would contribute to the final concentration of luteolin-7-O-glucuronide, are very large.

**Relevant estrogenic potency by synergistic effects in the Salvia officinalis extract**

It is well-known that herbal extracts are complex mixtures, and contain many compounds which may affect each other in many ways. Synergistic effects would lead to an observed effect greater than for the individual compound, co-effectors might increase or reduce bioavailability, unstable compounds could be protected from decomposition, or antagonistic effects may prevent unwanted effects (Williamson, 2001; Nahrstedt and Butterweck, 2010). It is possible that the estrogenic compounds in the Salvia officinalis extract act synergistically, but this effect was not observed in vitro, as the crude Salvia officinalis extract was inactive and the activity of the aq-EtOH SE was lower than the activity of the individual compounds. Furthermore, considering the metabolism of the flavone glycosides, it seems unlikely that co-effectors present in the crude Salvia officinalis extract could influence the bioavailability positively.
9.1 Future work

In future experiments it would be of interest to identify further estrogenic compounds and investigate their estrogenic potential. Additionally it should be further assessed if the estrogenic compounds work together in a synergistic way. These experiments could then lead to evaluation of the bioavailability of the active compounds in vivo.

The fact that the crude *Salvia officinalis* tincture did not show any estrogenic effects in the in vitro methods chosen to test for estrogenicity, but its fractions did, may give rise to the question whether further fractionation of the SEs or testing the identified estrogenic compounds may have exposed other biological activities in vitro (e.g. in the SSRI assay). Luteolin, for example, has previously been found to exhibit an inhibitory potential on AChE with an IC\(_{50}\) of 25.2 \(\mu\)g/ml (Conforti et al., 2010). On the other hand, Zhao et al. (2010) reported that luteolin selectively increased serotonin (5-HT) uptake in SERT-transgenic Chinese hamster ovary (CHO) cells.

As it is unlikely that the estrogenic effects found in the fractions (chapter 8) are alone responsible for the anti-hot flush effect of the crude *Salvia officinalis* extracts, further investigations could assess which other activity may be responsible for the observed overall effect. These investigations should include testing fractions of the SEs in the SSRI assay and other assays, which investigate a possible GABA effect of the compounds in the *Salvia officinalis*, would also be of interest. Gabapentin, a GABA analogue, showed potential in the amelioration of hot flushes in prostate cancer patients (Vandecasteele et al., 2011) and the well-known *Salvia officinalis* compounds carnosol and carnosic acid, were found to influence the GABA/benzodiazepine receptor complex in brain tissue ((Kavvadias et al., 2003) for review see (Imanshahidi and Hosseinzadeh, 2006)).

The potential to inhibit the cytochrome P450 enzyme 3A4 was assessed in order to address the knowledge gap about the interactive potential of the *Salvia officinalis* extract. The crude *Salvia officinalis* extract and the less polar SE in particular (\(n\)-hexane and \(\text{CHCl}_3\) SE) displayed inhibitory activity to the CYP3A4 enzyme (Tab. 9.1). It was not the aim of this thesis to identify the potential inhibitory compounds in the extracts, but the assessment of the inhibitory potential of the SE gave useful information about the nature of the compound, which could be involved in the inhibition of CYP 3A4. Future work could include the identification of the inhibitory compounds.

The results indicated (chapter 4) that compounds of a less polar nature seem to be responsible for the CYP3A4 inhibitory activity of the crude *Salvia officinalis* extract.
Interestingly the estrogenic activity found in this study was identified in the most polar aq-EtOH SE. It may therefore be of interest in future investigations to include another herbal medicinal product also produced by Bioforce; the Menosan® tablets. The tablets consist of dried crude *Salvia officinalis* extract, which is obtained by the same procedures as the extract used in this study, but it is further concentrated by evaporation of extraction solvent in the final production step. This additional step results in the loss mainly of essential oils. Hence, Menosan tablets contain less of the compounds which are expected in the *n*-hexane SE after solvent-solvent partitioning (see chapter 3) and which seemed to be the most potent CYP3A4 inhibitors in the crude *Salvia officinalis* extract. Menosan® tablets have also been reported to have a beneficial effect on the hot flush frequency of menopausal women (Bommer et al., 2011). Further investigations of the influence of the crude *Salvia officinalis* extract on the Cytochrome P450 enzyme system could therefore include the *Salvia officinalis* extract used for the Menosan® tablets, as it may prove to have the beneficial effects of the *Salvia officinalis* extract on hot flushes without the unwanted CYP3A4 inhibitory potential.
References


References


Bioforce (2005) A randomised, double blind, placebo-controlled pilot study to investigate the efficacy of Salvia officinalis in the treatment of perimenopausal hot flushes Bioforce Clinical report, KLC0088.e01, FINAL REPORT, Study protocol No. BFGB-02322400-001.


References

Sinikka Rahile


Clarke, B.L. and Khosla, S. (2010). 'Female reproductive system and bone'. *Arch Biochem Biophys* 503 (1), 118-128.


References


cytochrome P450-mediated metabolism of marker substrates by natural products'. *Phytotherapy* 10 (4), 334-342.


Guttuso, T.J., Jr. (2000). 'Gabapentin's effects on hot flashes and hypothermia'. Neurology 54(11), 2161-2163.


Sinikka Rahve

References


Hoffmann, d.E. and Stroobant, V. (2007). Mass spectrometry. 3rd. Chichester, West Sussex: John Wiley & Sons Ltd.


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References


Kortenkamp, A. (2007a) *Breast cancer and exposure to hormonally active chemicals: An appraisal of scientific evidence*


References


References


References


References


References


References


References


Venkatakrishnan, K., von Moltke, L.L., Court, M.H., Harmatz, J.S., Crespi, C.L. and Greenblatt, D.J. (2000). 'Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches'. *Drug Metab Dispos* 28 (12), 1493-1504.


References


Appendix

Appendix chapter 7

Figure 1: Cytotoxicity experiments with T47D-KB luc cells. Cells were seeded at different cell densities (no treatment). Data were fitted using linear regression model.

Figure 2: Response of the T47D-KB luc cells to E2 in the ERLUX assay. Different test concentrations of DMSO in the assay solution (0.1%, 0.2%, 0.5% and 1.0%) were applied. Concentrations of 17-β-estradiol are 0.0125 nM (full circles), 0.025 nM (triangles) and 0.05 nM (squares). Data points represent individual experiments.
Figure 3: ERLUX experiments in the presence of an ER-antagonist. A shows data from experiments with crude *Salvia officinalis* extract (circles) and aq-EtOH SE (triangles) and B shows CHCl₃ SE (triangle) and *n*-hexane SE (circles) in combination with fulvestrant (1.0 µM, antiestrogen), each experiment in triplicate. Filled circles represent individual data points. Data were fitted using linear regression model.
Appendix chapter 8

$^1$H-NMR spectra for fractions obtained from aq-EtOH SE

Figure 4: $^1$H-NMR spectra of fractions AQ 0 - AQ 4 (MeOD, 400 MHz).
Figure 5: $^1$H-NMR spectra of fractions AQ 5 - AQ 9 (MeOD, 400 MHz).
Figure 6: $^1$H-NMR spectra of fractions AQ 4.1 - AQ 4.4 (MeOD or D$_2$O, 400 MHz).
Appendix

Figure 7: $^1$H-NMR spectra of fractions AQ 4.5 - AQ 4.7 (MeOD, 400 MHz).

Figure 8: $^1$H-NMR spectra of fractions AQ 4.4.1 - AQ 4.4.2 (MeOD, 400 MHz).
Figure 9: $^1$H-NMR spectra of fractions AQ 4.4.3 - AQ 4.4.7 (MeOD, 400 MHz).
Figure 10: $^1$H-NMR spectra of fractions AQ 4.5.1 - AQ 4.5.5 (MeOD, 400 MHz).
Figure 11: "\(^1\)H-NMR spectra of fractions AQ 5.1 - AQ 5.5 (MeOD, 400 MHz).
Figure 12: $^1$H-NMR spectra of fractions AQ 5.5 - AQ 5.9 (MeOD, 400 MHz).
Figure 13: $^1$H-NMR spectra of fractions AQ 6.8 - AQ 6.12 (MeOD, 400 MHz).
Figure 14: $^1$H-NMR spectrum of fraction AQ 6.13 (MeOD, 400 MHz).

Figure 15: $^1$H-NMR spectra of fractions AQ 6.10.1- AQ 6.10.4 (MeOD, 400 MHz).
Figure 16: $^1$H-NMR spectra of fractions AQ 6.10.5 - AQ 6.10.8 (MeOD, 400 MHz).
Figure 17: $^1$H-NMR spectra of fractions AQ 7.1 - AQ 7.5 (MeOD, 400 MHz).
Figure 18: $^1$H-NMR spectra of fractions AQ 7.6 - AQ 7.8 (MeOD, 400 MHz).

Figure 19: $^1$H-NMR spectra of fractions AQ 7.6.1 - AQ 7.6.2 (MeOD, 400 MHz).
Figure 20: $^1$H-NMR spectra of fractions AQ 7.6.3 - AQ 7.6.7 (MeOD, 400 MHz).
Appendix chapter 8

Additional ERLUX results of fractions obtained from aq-EtOH SE

Section 8.3.3: AQ 4

Figure 21: $^1$H-NMR spectrum of fraction AQ 7.6.8 (MeOD, 400 MHz).

Figure 22: ERLUX results of fractions AQ 4.1 (non-filled circles), AQ 4.2 (black), AQ 4.3 (blue), AQ 4.6 (triangle), AQ 4.7 (green) and AQ 4.8 (red). Circles/triangles represent the mean of three replicates. The regression curve of fraction AQ 4 (grey, dotted line) is shown for comparison (reproduced from Figure 6.4).

Figure 23: ERLUX results from of fraction AQ 4.4 (green), AQ 4.5 (pink) and AQ 4.6 (blue). Single data points were obtained in one experiment, tested in triplicate. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of the presented data is presented in Figure 6.7.
Figure 24: ERLUX results of fractions AQ 4.4.1 (blue), AQ 4.4.2 (non-filled circles), AQ 4.4.3 (green), AQ 4.4.4 (red) and 4.4.5 (black). Circles represent the mean of three replicates. The regression curve of fraction AQ 4.4 (grey, dotted line) is shown for comparison (reproduced from Figure 8.6).

Figure 25: ERLUX results of fractions AQ 4.5.1 (red), AQ 4.5.2 (blue), AQ 4.5.3 (non-filled circle), AQ 4.5.4 (black) and 4.5.5 (green). Filled circles represent the mean of three replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 4.5 (grey, dotted line) is shown for comparison (reproduced from Figure 8.6).
Section 8.2.4: AQ 5

Figure 26: ERLUX results of fractions AQ 5.1 (non-filled circles), AQ 5.2 (black), AQ 5.3 (red) and AQ 5.4 (blue). Circles represent the mean of three replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 5 (grey, dotted line) is shown for comparison (reproduced from Figure 8.4).

Figure 27: ERLUX results of fractions AQ 5.5 (green), AQ 5.6 (blue) and AQ 5.7 (red). Filled circles represent the mean of three replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 5 (grey, dotted line) is shown for comparison (reproduced from Figure 6.4).
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Section 8.2.5: AQ 6

Figure 28: TLC analysis (left) of the VLC fractions AQ 6 and AQ 6.1 – 6.8 and ERLUX results (right) of fractions AQ 6.12 (green), AQ 6.13 (blue) and AQ 6.14 (red). For TLC the stationary phase was silica gel 60 F254, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170 °C). ERLUX results (right) represent filled circles as the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6 (grey, dotted line) is shown for comparison (reproduced from Figure 8.4).

Figure 29: ERLUX results of fractions AQ 6.10.1 (non-filled circles), AQ 6.10.2 (green), AQ 6.10.3 (red) and AQ 6.10.4 (blue). Filled circles represent the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6.10 (grey, dotted line) is shown for comparison (reproduced from Figure 8.13).
Figure 30: ERLUX results of fractions AQ 6.10.8.1 (green), AQ 6.10.2 (non-filled circles), AQ 6.10.8.3 (blue) and AQ 6.10.8.4 (red). Filled circles represent the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 6.10.8 (grey, dotted line) is shown for comparison (reproduced from Figure 8.16).

Section 8.2.6: AQ 7

Figure 31: ERLUX results of fractions AQ 7.1 (grey), AQ 7.2 (green), AQ 7.3 (red) and AQ 7.4 (blue). Circles represent the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 7 (grey, dotted line) is shown for comparison (reproduced from Figure 8.4).
Figure 32: ERLUX results of fractions AQ 7.6.1 (blue), AQ 7.6.2 (non-filled circle), AQ 7.6.3 (red), AQ 7.6.4 (green) and AQ 7.6.5 (black). Circles represent the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 7.6 (grey, dotted line) is shown for comparison (reproduced from Figure 8.20).

Figure 33: ERLUX results of fractions AQ 7.6.7.1 (blue), AQ 7.6.7.2 (black), AQ 7.6.7.3 (non-filled circle) and AQ 7.6.7.4 (red). Circles represent the mean of two replicates. On-plate controls, positive controls (1.0 nM E2, pink) and solvent controls (0.5% DMSO, cyan), are shown as individual data points next to the y-axis. The regression curve of fraction AQ 7.6.7 (grey, dotted line) is shown for comparison (reproduced from Figure 8.22).
## Results of the cytotoxicity assay with fractions obtained from aq-EtOH SE

(Cell viability in percent compared to solvent controls (0.5% DMSO))

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Table 1: Results of the cytotoxicity assay: Fractions chapter 8
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Table 3: Results of the cytotoxicity assay: Fractions chapter 8
Figure 34: Gradient elution of luteolin with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, \( \Phi \) 1.7 \( \mu \)m, TOF-MS ESI*).

Figure 35: Gradient elution of luteolin-7-O-glucoside with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, \( \Phi \) 1.7 \( \mu \)m, TOF-MS ESI*).
Figure 36: Gradient elution of luteolin-7-O-glucuronide (fraction AQ 4.4.6) with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI*).

Figure 37: Gradient elution of crude *Salvia officinalis* extract with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI*).
Figure 38: Gradient elution of aq-EtOH SE with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI+).

Figure 39: Gradient elution of AQ 4 with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI+).
Figure 40: Gradient elution of AQ 6 with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI⁺).

Figure 41: Gradient elution of AQ 6.10.8.7 with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 µm, TOF-MS ESI⁺).
Figure 42: Gradient elution of AQ 7 UHPLC-TOF-MS (Acquity BEH RP 18 shield 50 mm x 2.1 mm, Ø 1.7 μm, TOF-MS ESI⁺).

Figure 43: Gradient elution of AQ 7.6.7.6 with UHPLC-TOF-MS (Acquity BEH RP 18 shield 100 mm x 2.1 mm, Ø 1.7 μm, TOF-MS ESI⁺).
Publications
Evans, R.M., Rahte, S. and Kortenkamp, A.
Inability to confirm estrogenicity of the heterocyclic amine PhIP in two in vitro assays.
Toxicology in vitro (2010) 24 (6), 1757-1763.

Poster presentations
Rahte S., Suter A., Kortenkamp A., Tasdemir D.
Estrogenic and Cytochrome p450 Enzyme Inhibitory Effects of Salvia officinalis Tincture and Its Subextracts
Summer School of the European University Consortium for Pharmaceutical Sciences, 26.6. - 3.7.2009, Copenhagen (Denmark)

Rahte S., Suter A., Kortenkamp A., Tasdemir D.
Estrogenic and Cytochrome p450 Enzyme Inhibitory Effects of Salvia officinalis Tincture and Its Subextracts
International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 16.8. – 20.08.2009, Geneva (Switzerland)

Rahte S., Suter A., Kortenkamp A., Tasdemir D.
Estrogenic and Cytochrome p450 Enzyme Inhibitory Effects of Salvia officinalis Tincture and Its Subextract
PhD Research Day, Internal Symposium at the School of Pharmacy, 09.10.2009, London

Rahte S., Suter A., Kortenkamp A., Tasdemir D.
Selective serotonin re-uptake and acetylcholinesterase inhibitory activities of Salvia officinalis
International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 29.8. – 2.10.2010, Berlin (Germany)

Talks
Salvia officinalis for the treatment of menopausal symptoms; a good alternative?
Exploratory studies of mode of action and potential drug-drug interactions
Project presentations, Department for Pharmacology, 19.06.2009, London

Estrogenic and Cytochrome p450 Enzyme Inhibitory Effects of Salvia officinalis Tincture and Its Subextracts
PhD Research Day, Internal Symposium at the School of Pharmacy, 24.10. 2010, London