Ane Djuv

Co-Use of Drugs and Herbal Remedies in General Practice and *In Vitro* Inhibition of CYP3A4, CYP2D6 and P-Glycoprotein by the Common Herb Aloe Vera

Thesis for the degree of Philosophiae Doctor

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Sambruk av medikamenter og urtepreparater i allmennpraksis og *in vitro* inhibisjon av CYP3A4, CYP2D6 og P-glykoprotein av en hyppig brukt urt, Aloe vera.

Det er en høy bruk av naturmidler og alternativ medisin sammen med ordinære medikamenter av ulike pasientgrupper. Det finnes per i dag få studier som har undersøkt sambruk i allmennpraksis og utsatte legemiddel-urt kombinasjoner. Sambruk av naturmidler og medikamenter kan føre til alt fra ingen til svært alvorlige bivirkninger. Det er derfor behov for grunnleggende kunnskap om mekanismene for slik interaksjoner og pasienters sambruk i allmennpraksis.

Målsetningene med denne oppgaven er todelt; 1) Kartlegge grad av sambruk av legemidler og urter (plantebaserte naturmidler) i norsk allmennpraksis og pasienters kommunikasjon om slik bruk med helsepersonell; 2) Evaluere interaksjonspotensialet til en hyppig benyttet urt, Aloe vera (*Aloe barbadensis*), for effluksproteinet P-glykoprotein (P-gp), cytokrom P-450 (CYP) enzymene, CYP3A4 og CYP2D6.

Undersøkelsen i allmennpraksis viste at blant de 381 pasientene som deltok, brukte 44 % urter. De vanligste urtene var blåbær (41%), grønn te (31%), hvitløk (27%), Aloe vera (26%) og rød solhatt (18%). Nesten hver tredje (29%) pasient benyttet urtepreparater og faste medikamenter samtidig. 255 kombinasjoner av urt og legemiddel-grupper ble registrert og 18 av disse var klinisk relevante med hensyn til interaksjoner. Omkring 40% av pasienter på antikoagulasjonsbehandling benyttet urter samtidig, hvorav hvitløk og blåbær var de hyppigst brukte. Pasienter med økt odds for sambruk var kvinner, eldre, brukere av flere urter, hadde et ønske om å behandle en sykdom med sin urtebruk, brukte smertestillende eller midler mot hudsykdom og hadde opplevd bivirkninger av urte-bruken. Kun 23% av pasientene i allmennpraksis diskuterte bruk av urter med helsepersonell.

Selv om Aloe vera er en populær og gammel medisinplante brukt både i kosmetisk og terapeutisk henseende, finnes det ingen tidligere systematiske undersøkelser av Aloe veras *in vitro* interaksjonspotensiale ved sambruk av legemidler. I all hovedsak er det tre enzymer som står for farmakokinetikken for de fleste medikamenter på markedet: effluksproteinet P-gp som transporterer medikamenter ut og CYP3A4 og CYP2D6, som metaboliserer medikamenter til mindre aktive komponenter. Disse enzymene kan påvirkes (inhiberes eller induseres) og er derfor viktige med tanke på naturmiddel-legemiddel interaksjoner.

Det ble funnet at Aloe vera juice (AVJ) ikke inhiberte P-gp mediert digoxin efflux for de undersøkte AVJ konsentrasjonene *in vitro*. Derimot ble det vist at både AVJ (10.0 mg/ml) og digoxin (≥3µM) var cytotoksiske i høye konsentrasjoner. For CYP3A4 og CYP2D6 ble to ulike juice-produkter undersøkt. Begge typene AVJ inhiberte CYP3A4 og CYP2D6 irreversibelt *in vitro,* med signifikant ulike IC_{50} verdier. Dette kan skyldes ulikt innhold av aktive stoffer i juicen. Begge IC_{50} verdiene vurderes til å være for høye til å være klinisk relevante alene. Forsiktighet bør allikevel utvise ved store inntak av AVJ, ved lav CYP2D6 aktivitet (”poor metabolisers”) eller ved bruk av medikamenter med smalt terapeutisk vindu.

Det kan konkluderes med at pasienter i allmennpraksis benytter urter og medisiner samtidig og denne sambruken kan føre til klinisk relevante bivirkninger (eks økt blødningsfare ved sambruk av hvitløk og warfarin). Eldre pasienter er mest utsatt. En av de hyppig brukte urtene i allmennpraksis, Aloe vera, ble undersøkt for *in vitro* farmakokinetiske interaksjons for P-gp, CYP3A4 og CYP2D6. En konkluderte med liten fare for klinisk relevante farmakokinetiske interaksjoner ved sambruk av Aloe vera og medikamenter, selv om det er en mulighet for klinisk relevant CYP2D6 inhibisjon ved store Aloe vera inntak for pasienter med lav CYP2D6 aktivitet samtidig med medikamenter som kodein (metaboliseres over CYP2D6). Det er behov for kliniske *in vivo* studier for å avdekke eventuelle interaksjoner hos mennesker mellom Aloe vera og medikamenter, samt for andre urter som har vist potensiale for urte-medikament interaksjoner *in vitro*. Inntil videre oppfordres leger og annet helsepersonell til å spørre alle pasienter om bruk av urter.

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Stavanger, August 2013

Ane Djuv

List of abbreviations

AV	Aloe vera
AVJ	Aloe vera juice
A-B	Apical to basolateral
B-A	Basolateral to apical
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CPM	Counts per minute
CYP	Cytochrome P-450
DMEM	Dulbecco`s modified eagles medium
DMSO	Dimethylsulphoxide
DPM	Disintegrations per minute
FLD	Fluorescent detector
FDA	U.S. Food and Drug Administration
GP	General Practitioner
HPLC	High pressure liquid chromatography
IASC	International Aloe Science Council
IC_{50}	Inhibitor concentration decreasing enzyme activity by 50 %
INR	International normalized ratio
J_{Net}	Net flux of a substrate (digoxin)
KPO	Potassium phosphate
Km	Michaelis Menten constant for a substrate
LD	Lactatedehydrogenase
LLOQ	Lower limit of quantification
NADPH	Nicotine amide diphosphate
NCTR	National Centre for Toxicological Research
NMA	Norwegian Medicinal Agency
NSAID	Nonsteroidal anti-inflammatory drugs
MDR1	Multi-Drug Resistance 1 gene
MTT	Tetrasodiumsalt
OATP	Organic Anion Transporting Polypeptide
Papp	Apparent permeability
P-gp	P-glycoprotein (MDR1)
PXR	Pregnane-X-Receptor
QC	Quality control
SD	Standard deviation
TEER	Transepithelial electric resistance
ULOQ	Upper limit of quantification
USA	United States of America
UV	Ultraviolet
WS	Working solution

Contents

Summary in English

Co-use of drugs and herbal remedies in general practice and *in vitro* inhibition of CYP3A4, CYP2D6 and P-glycoprotein by the common herb Aloe vera.

There is a widespread use of complementary and alternative medicine (CAM) and herbal remedies in particular in different patients groups, but very few are published about co-use among patients in general practice (GP) and herb-drug combinations at risk. Co-use of herbal remedies and drugs can result in none or server adverse effects. Of this reason, knowledge about the GP patients co-use and research on mechanisms of such interactions is needed.

The aims of this thesis were divided; 1) To register the co-use of drugs and herbs among GP patients in Norway and the patients communication of such use with health care professionals; 2) To evaluate the interaction potential of one of the commonly used herbs in GPs office, Aloe vera (*Aloe barbadensis*), on the P-glycoprotein (P-gp) and the cytochrome P-450 (CYP) enzymes, CYP3A4 and CYP2D6.

Among the 381 patients answering the questionnaire in the GP office, 44% used herbs. The most common herbs were bilberry (41%), green tea (31%), garlic (27%), Aloe vera (26%) and purple coneflower (18%). Almost every third (29%) patient co-used drugs and herbs. They combined 255 different drug-groups and herbs whereas 18 of these were considered to have a clinically relevant interaction potential. Close to 40% of patients on anticoagulants co-used herbs, reporting garlic and bilberry most frequently. Co-users had significantly ($p<0.05$) increased odds to be female, elderly, use herbs to treat an illness, use two or more herbs and experienced adverse effects of herbal use compared to other GP patients. Co-use was also associated with use of analgesics or dermatological drugs. Only 23% of the GP patients discussed their herb use with a health care professional.

Even though Aloe vera is a well-known, old medicine plant used both in cosmetics and as therapeutics, few or no earlier systematic research on its interaction potential has been investigated when co-used with drugs *in vitro*. Overall three enzymes accounts for the majority of the pharmacokinetics on the drugs in the market: the efflux-protein P-gp

transporting the medicinal drug out of the cell and CYP3A4 and CYP2D6, metabolizing the medicinal drugs to less active components. These enzymes can be influenced by other substances (inhibited or induced) and is therefore important regarding herb-drug interactions.

Aloe vera juice (AVJ) did not inhibit P-gp mediated digoxin efflux for the investigated AVJ concentrations *in vitro*. However, it was shown that both AVJ (10.0 mg/ml) and digoxin (≥3µM) was cytotoxic in large concentrations. Two different AVJs were used in the CYP3A4 and CYP2D6 assays. Both juices inhibited CYP3A4 and CYP2D6 irreversible *in vitro,* having significant different IC_{50} values. This can come from different concentrations of active components in the juices. Both IC_{50} values seems, however, to be too high to be clinical relevant alone. Precautions should although, be made with excessive consumption of AVJ, with poor CYP2D6 activity (”poor metabolisers”) or with use of drugs having a narrow therapeutic window.

It can be concluded that GP patients co-using drugs and herbs and that this use can give clinical relevant interactions (e.g. excessive haemorrhage when co-using garlic and warfarin). Elderly patients are most vulnerable for co-use. One of the common used herbs among GP patients, Aloe vera, was investigated for *in vitro* pharmacokinetic interactions on the enzymes P-gp, CYP3A4, CYP2D6. Although it was concluded with low possibility of clinical relevant pharmacokinetic interactions co-using Aloe vera and drugs, patients with poor CYP2D6 activity might risk interactions when co-using large quantities of Aloe vera with conventional drugs which is metabolized of CYP2D6 (e.g. codeine). Clinical *in vivo* studies are needed to reveal any interactions in humans for Aloe vera and other herbs at risk of herb-drug interactions. Until then, the GPs and other health care professionals are advised to ask all patients about herbal use.

List of Papers

This thesis is based on the following publications:

Paper I

Ane Djuv, Odd Georg Nilsen and Aslak Steinsbekk.

The co-use of conventional drugs and herbs in Norwegian general practice: a cross-sectional study. Submitted, BMC CAM, 2013

Paper II

Ane Djuv and Odd Georg Nilsen

***Aloe Vera* Juice: IC_{50} and Dual Mechanistic Inhibition of CYP3A4 and CYP2D6.**

Phytotherapy Research, 2012. 26(3): p. 445-51.

Paper III

Ane Djuv and Odd Georg Nilsen

Caco-2 Cell Methodology and Inhibition of the P-glycoprotein Transport of Digoxin by *Aloe vera* Juice. Phytotherapy Research, 2008. 22(12): p. 1623-8.

Summary of papers

Paper I

The co-use of conventional drugs and herbs in Norwegian general practice: a cross-sectional study.

The primary aim of this study was to compare patients in a general practice general practitioner's (GP) office in Norway that co-use herbal remedies and conventional drugs with those who do not, with regards to demographics, types of drugs and herbs used, reason for use and communication with health care professionals about this use. The second aim was to register the herb-drug combinations with potential clinical relevant interactions among the co-users.

A questionnaire based cross-sectional study was performed in a GP office with four GPs and one intern, situated in a middle large town in Norway. Adults >18 years who came for an office visit were invited. The questionnaire consisted of three parts; Demographics and conventional drug use, herbal use and communication about herbal use. The data was analysed with multivariable logistic regression with co-use as the dependent variable.

A total of 402 patients received and 381 completed the questionnaire. The prevalence of herbal use was 44%, with bilberry (41%), green tea (31%), garlic (27%), Aloe vera (26%) and Echinacea (18%) being the most commonly used herbs. Almost every third (29%) patient co-used drugs and herbs. They combined 255 different drug-groups and herbs whereas 18 of these were considered to have a clinically relevant interaction potential. Close to 40% of patients on anticoagulants co-used herbs. Co-users were significantly ($p<0.05$) different from other GP patients by being female (adjOR 2.0), increasing age above 50 years (adjOR 1.3 - 3.3), using herbs to defeat an illness (adjOR 4.2), using two or more herbal remedies (polyherbacy, adjOR 12.1) and having experienced adverse effects of herb use (adjOR 37.5). Co-use was also associated with use of analgesics or dermatological drugs (adjOR 5.1 and 7.9). Three out of four patients did not discuss herbal use with any health care professional.

A sizable proportion of the GP patients co-used herbs with conventional drugs, also combinations with reported interaction potential or additive effects like anticoagulants and garlic. The low disclosure of herbal use to their GP, polyherbacy and the risk of interactions in vulnerable groups like elderly and chronically ill patients, warrant increased awareness among GPs.

Paper II

Aloe vera juice; mechanistic and non-mechanistic CYP3A4 and CYP2D6 inhibition.

The main objective was to evaluate the Aloe vera juice`s (AVJ) inhibition potential towards cytochrome P-450 (CYP) enzymes 3A4 or 2D6 metabolism of testosterone or dextromethorphan *in vitro*.

A range of seven AVJ concentrations (0.01- 60.0 mg/ml) were incubated with recombinant CYP3A4 or CYP2D6 for 10 or 25 min, respectively. To assess the problem of inter-herbal differences two juice products, 'A' and 'B', were investigated. Testosterone (0.10 mM) or dextromethorphan (8 μM) were used as substrates, and ketoconazole (KTZ) (0.16 μM) or quinidine (0.24 μM) were used as positive control inhibitors. Amount of metabolites were detected by HPLC (High-performance liquid chromatography). IC_{50} values were estimated for AVJ 'A' or 'B' inhibition by non-linear regression. Time- and NADPH dependent inhibition assays were performed to investigate if the inhibition of CYP3A4 or CYP2D6 were mechanism-based.

Both AVJs showed mechanism-based inhibition on CYP3A4 and CYP2D6 *in vitro*. AVJ 'A' and AVJ 'B' had significant dissimilar IC_{50} values and this may be explained from their different concentration of juice components. These results are not considered clinical relevant, because of the high IC_{50} values. However, precautions should be made with high AVJ intake in humans presumed an additive effect of both the CYP3A4 and CYP2D6 irreversible inhibition when co-administrated with drugs metabolised by these enzymes. In addition, AVJ compounds might accumulate in the hepatocytes. Furthermore, co-administration with drugs with a narrow therapeutic window (e.g. tricyclic antidepressants) and patients with poor CYP2D6 activity ("poor metabolisers") are exposed to small changes in the drug

bioavailability. Further *in vitro* investigation of AVJ 'A' inhibition of CYP2D6 metabolism is required because of inconclusive results.

Paper III

Caco-2 Cell Methodology and Inhibition of the P-glycoprotein Transport of Digoxin by Aloe vera Juice.

The aim was to explore the Aloe vera juice`s (AVJ) inhibition potential towards P-glycoprotein (P-gp) efflux of digoxin in human colon carcinoma cells (Caco-2 cells).

Bidirectional digoxin (30 nM) fluxes across monolayers of Caco-2 cells, were determined for digoxin alone (control), in the presence of a positive inhibitor control, verapamil (100 μM), or seven AVJ concentrations (0.00001-1.0 mg/ml). The AVJ range was anticipated to cover physiological relevant concentrations. AVJ and substrate toxicity was evaluated by the tetrazolium bromide (MTT) assay, based on intracellular lactatedehydrogenase (LDH) activity. Transport linearity, transepithelial electrical resistance (TEER) and mannitol (55 μM) transport were measured to secure cell integrity and quality. Only monolayers with TEER values > 200 Ω/cm^2 and mannitol <1.0 x 10^{-6} cm/s were included. The apparent permeability coefficient (Papp (cm/s)), net Papp ($Papp_{Net}$ (cm/s)) and the net flux (J_{Net} (nmol/h/cm^2)) were determined for the digoxin transport.

AVJ did not inhibit the P-gp efflux of digoxin in the investigated AVJ concentration range, and an *in vivo* inhibition of P-gp mediated digoxin flux by AVJ might seem unlikely.
The quality and integrity (transport linearity, TEER and mannitol) of the Caco-2 cell system was satisfactory during the AVJ inhibition studies. Surprisingly, digoxin caused a linear statistically significant concentration-dependent reduction of lactatdehydrogenase (LDH) activity at concentrations ≥3 μM. Precautions should thus be made when concentrations of digoxin ≥3 μM are used in Caco-2 cell studies. AVJ showed cytotoxicity at the highest concentration applied (10.0 mg/ml).

1 Introduction

Modern medicine has evolved from the traditional folk medicine with herbs as the treatment basis. The oldest known written records are from the Sumarians 2600 BC listing hundreds of medicinal plants, although the history of herbal medicine is probably as long as the human history (Mohammad, 2006). In the work "De Materia Medica" a Greek physician known as Dioscorides (100 A.D.), described more than 600 medicinal plants recognised as an important contribution to the modern phytomedicine (Mohammad, 2006). Today, some of the well-known conventional drugs in modern medicine can still be purified from herbs i.e. digoxin from the Foxglow plant (*Digitalis purpurea*) or morphine from the opium poppy (*Papaver somniferum*).

Herbal remedies (plant derived remedies) have gained increased popularity the last decades together with other types of complementary and alternative medicine (CAM) (Eisenberg et al., 2001; Barnes et al., 2008). CAM covers a heterogeneous group of traditional to more experimental approaches that purport to prevent or treat disease and includes herbal remedies as well as treatments like homeopathy, high dose vitamins, fish oils, yoga, chiropractic care or acupuncture (Eisenberg et al., 1998; Barnes et al., 2008). In USA reported 40% of the population to use CAM and 18% reported use of herbal remedies in particular (Barnes et al., 2008). This thesis will focus on herbal remedies and preparations or products of such.

In parallel to the growth in the herbal use, case-reports and studies of possible herb-drug interactions reveal a new problem: herb-drug interactions (Williamson, 2005; Ulbricht et al., 2008; Izzo and Ernst, 2009). Drug-drug interactions are well-known in medical treatment (Cascorbi, 2012). Lately it has become more evident that the increasing consumption of herbs and dietary supplements also contains substances capable of interacting with drugs and changing the effect (e.g. co-use of sildenafil and grapefruit juice, cyclosporine and St.Johns wort) (Flanagan, 2005; Gouws et al., 2012). A number of undesired effects can appear e.g. altered drug pharmacokinetics, pharmacodynamics or bioactivation of drugs to reactive or toxic intermediates (Rodeiro et al., 2009).

Herbs and drugs may interact either pharmacokinetically or pharmacodynamically. Pharmacokinetic interactions occur at the levels of absorption, elimination, and metabolism

and determine how rapidly and for how long the drug will appear at the target organ. The main group of proteins metabolizing or transporting drugs in humans consist of cytochrome P450 (CYP) enzymes and P-glycoprotein (P-gp). Inhibition or induction of these proteins by herbal remedies increase or decrease the drugs bioavailability causing unwanted adverse effects or treatment failure (e.g. grapefruit juice inhibiting CYP3A4 and P-gp increasing sildenafil concentration). Pharmacodynamic interactions arise when the drug and herbal remedy have additive (e.g. garlic and warfarin) or antagonistic (e.g. ginseng and warfarin) effects on the body and are not so easy to detected systematically as pharmacokinetic interactions (Izzo, 2005; Cascorbi, 2012).

1.1 Use of herbal remedies

The traditional folk medicine, herbal remedies and alternative medicinal systems (e.g. Chines medicine, Ayurvedic medicine) have roots in local traditions based on available natural resources and plants (Mohammad, 2006). Through the last century and especially the last decades, increase in travelling, commercials, advertising and internet have shirked the distances between people, exchanging information and products in a faster pace. Nowadays one can order herbal remedies from all over the world and receive them by mail few days later. Thus, some of the most common herbs used by US adults like Ginkgo biloba (*Ginkgo biloba*), Garlic (*Allium sativum*), psyllium seed husks (*Plantago ovate*) does not originate from the US fauna and new herbs are continuously introduced to the growing market (Eisenberg et al., 2001; Barnes et al., 2008).

According to sale statistics for Norway in 2005, the most consumed herbs were golden root (*Rhodiola rosea*), cranberry (*Vaccinium Oxycoccus*), exstracts of soya bean (*Glycine max*), evening primrose oil /GLA (*Oenothera Biennis*) and garlic (*Allium sativum*) (Bransjerådet, 2009) while the most frequently used herbs among Norwegian cancer patients were green tea (*Camelia sinensis*), garlic, ginger (*Zingiber officinale*), noni juice (*Morinda citrifolia*) and Aloe vera (*Aloe barbadensis*) (Engdal et al., 2008). The five most popular herbs among people in Jamaica were bitter melon (*Momordica charantia L.),* life plant (*Bryophyllum pinnatum (Lam.)/ Kalanchoe pinnata*), Aloe vera, common floss flower (*Eupatorium odoratum L.*), soursop (*Annona muricata L.*) and ginger (Picking et al., 2011). In comparison, pregnant women in Great Britain used ginger, cranberry and red raspberry leaf tea (*Rubus idaeus*) (Nordeng and Havnen, 2005) while older adults in USA bought Echinacea

(*Echinacea purpurea*), garlic supplements and Ginkgo biloba (Bruno and Ellis, 2005). Thus, although the intercultural influence is increasing, still the herb used by people varies between countries, patients groups and age groups.

Studies from USA have reported extensive use (20-36%) of herbs in the general population (Martin et al., 2002; Kuo et al., 2004). Wide variability of herbal use has, however, been shown for different ethnic groups in the US were 50% of Hispanics, 50% of Asians, 41% of Whites, and 22% of African-Americans reported herbal use (Kuo et al., 2004; Feldmann et al., 2008). In comparison, an UK telephone survey from 1999 reveals, a herbal medicine use of 7% in the general population (Ernst and White, 2000). An Australian report shows an increase in herbal remedy use from nearly 10% in 1993 to above 20% in 2004 (MacLennan et al., 2006). Few up to date herbal prevalence numbers are found for the Nordic countries. Denmark had a prevalence of herbal remedies at 8% in 2000 (ViFAB, 2000) and Sweden reported CAM remedy use of 19% in 1999 (Nilsson et al., 2001). However, a recent telephone survey reported by The National Research Centre in Complementary and Alternative Medicine (NAFKAM) in Norway found a herbal use of 11% among the adult population (Fønnebø, 2012). The prevalence of herbal use has increased the latest decades and it varies between ethnic groups and cultures.

A cross-sectional study among the adult population in the USA showed that 41% used an herbal remedy sometimes or always to self-treat before seeking medical care from a physician (Martin et al., 2002). The typical herb user in the USA population (The 2002 National Health Interview Survey) were female, aged 45 to 64 years with higher education (Kennedy, 2005). About 40% of survey respondents from USA believed that taking prescription medications and herbal remedies together was more effective than taking either alone (Kuo et al., 2004)). Fifteen per cent of adults treated their children with herbs and nearly all (86%) respondents reported that they found it to be helpful or very helpful (Martin et al., 2002).

In parallel to the growing herb and supplement market, efforts has been made from the World Health Organization (WHO), European Medicines Agency (EMA) and other national and international health authorities to secure the patients safety, produce guidelines, establish rules and legislations as well as making herbal monographs on the available literature (EMA, 2012). In Norway and the other European countries herbal remedies and natural products are now regulated by quiet similar regulations (EMA, 2012). Most of the herbal remedies used in

Norway are considered as food, food supplements or cosmetics and is controlled by the Norwegian Food Safety Authority (HOD, 2003; HOD, 2004). They are bought for self-treatment without prescription in the pharmacy, shops, by mail order or collected in the nature (AESGP, 1998). For the manufacture and control of these products, the rules of Good Manufacturing Practice (GMP) are applicable (AESGP, 1998). Herbal medicine needs, however, authorization from the national health authorities on the same basis and regulations as conventional medicinal drugs (NoMA, 1999).

1.2 Co-use of herbs with conventional drugs

Herbs are perceived as safe and "natural" and are often marketed without mentioning any potential for harm (van den Berg et al., 2011). This myth has been falsified by numerous reports and papers from minor to lethal side effects or interactions with drugs (Izzat et al., 1998; Cott, 2001; Ioannides, 2002; Greenblatt and von Moltke, 2005). One of the well-known cases is transplanted patients treated with cyclosporine, an immunosuppressive drug, taking St John's wort (*Hypericum perforatum*) leading to a drop in plasma levels of cyclosporine, causing tissue rejection (Ruschitzka et al., 2000; Ioannides, 2002; Zhou et al., 2003).

It is reported a co-use of herbal remedies and drugs up to 50% in different patients groups (Smith et al., 2010; Nordeng et al., 2011; Zhang et al., 2011). An US study identified that 40% of potential adverse herb-drug interactions among the herbal users in outpatient clinics (Bush et al., 2007). Pregnant women reports to use herbal remedies (9-40%) and about 86% of these used conventional drugs concomitantly (Moussally et al., 2009; Nordeng et al., 2011). The use of herbal remedies among adults with cancer is reported to be in the interval between 30-55% (Molassiotis et al., 2005; Johansen and Toverud, 2006) and one study found that almost 40% co-use herbal remedies and chemotherapy (Engdal et al., 2008). Elderly are another group at risk and in a study were 32% of the participants defined to be at risk of having at least one possible herbal drug interaction (Loya et al., 2009). Smith and co-workers reported of warfarin interactions with herbs or vitamins for 7% of the patients (Smith et al., 2010). The American Society of Anaesthesiologists recommends to stop all herbal supplement use 2-3 weeks prior any surgery to minimize risk of increased bleedings or interactions with anaesthesia (Leak, 2000; Sabar et al., 2001). Thus, adverse effects and interactions between drugs and common herbs are a challenge today (Zhou et al., 2003).

Herbal preparations consist of multiple, often unidentified, biological active or inactive constituents (van den Berg et al., 2011). It is therefore more likely that an interaction between a single drug and a complex herbal product takes place, than towards another single drug. Furthermore, the combined effect of all constituents together in an herb may be different from the anticipated sum effect evaluated from single isolated constituents (van den Berg et al., 2011). This emphasizes the importance of performing interaction studies with crude herbal extracts as an important supplement to studies on isolated herbal fractions.

1.3 Herb use and co-use among general practice patients

Despite the large reported use of herbs and co-use of herbs and regular drugs in patients groups, few studies have been performed in primary care and general practice in particular. General practitioners (GPs, family doctors) provide the main health care to the general population in the society (Allen et al., 2005). They have the long term follow up on regular medication/chronic disease and common illnesses (i.e. diabetes, hypertension, arthritis) with a large degree of variety in the patient population (age, gender, illnesses, socioeconomic status etc.) (Gillam et al., 1989). Thus the GP is the physician to whom the patients are expected to disclose their herb use and the GP is on the other hand, expected to ask for this information in the medical history taking.

A study from Israel reported a prevalence of herbal use in family medicine practices of 36% and nearly 50 % of these co-used natural and conventional drugs usually or sometimes (Giveon et al., 2004). Another study showed herb use of 35% and a concomitantly use of CAM and conventional drugs of nearly 80% among the CAM users in Scotland (Featherstone et al., 2003). Due to the low number of studies from general practice as such, it is relevant to look at use among typical patient groups in general practice, elderly patients and patients with chronic diseases.

The elderly patients tend to go more often to their GP, have more polypharmacy problems and they are more vulnerable to interactions because of decreased health in general (hearth failure, liver failure, kidney failure etc.) (Loya et al., 2009). Considering 13-47% of elderly patients reports to consume herbs in the general population (Bruno and Ellis, 2005; Raji et al., 2005) and as many as 31- 75% of the elderly co-using herbs and prescribed drugs (Loya et al., 2009; Gonzalez-Stuart, 2011) , the risk of server interactions are high. For instance, it is reported

herbal interaction with a variety of cardiovascular drugs, which may lead to adverse alterations in drug efficacy and/or toxicity (Gurley et al., 2005; Cohen and Ernst, 2010). In addition, about 50% of the population has one or more chronic conditions and have, as the elderly, a high care rate and polypharmacy (50%) (Schoen et al., 2007). They also tend to use more herbal remedies, which increase the possibility of herb-drug interactions (Ravven et al., 2011). Thus, the risk of harmful co-use in the GP practice is present and needs to be addressed.

Only 20-45% of the population inform their physician of herb use (Giveon et al., 2003; Wheaton et al., 2005; Davis et al., 2012). In addition, the health care professionals rarely asked the patients about the use of herb or other types of use of complementary and alternative medicine (Giveon et al., 2003). ”The doctor did not ask” is the common phrase explaining the lack of communication (Saw et al., 2006). The physicians also tended to underestimate the use (Giveon et al., 2003). Thus, a lack of disclosure from the patient about herbal use is to some degree documented, particularly so among GP patients.

1.4 Aloe vera

Aloe vera (AV), *Aloe Barbadensis*, is a widely used and old medicine plant and a perennial succulent (Liliaceal) (Boudreau and Beland, 2006). It has been used traditionally for thousands of years as topical or oral therapeutic against different skin diseases and a wide range of illnesses (skin burns, antiviral, antibacterial, inflammatory bowel disease (IBD), hyperlipidaemia, diabetes, HIV etc.), as well as in cosmetic products (Reynolds and Dweck; Turner et al., 2004; Boudreau and Beland, 2006; Ahlawat and Khatkar, 2011).

The National Centre for Toxicological Research (NCTR), U.S. Food and Drug Administration (FDA), have in their report (FY 2006/2007) aimed on AVs increasing usage in both cosmetics, dietary supplement and natural medicine (Slikker, 2007). AV supplements were the top-selling botanical supplements within the natural and health foods channel in USA in 2009 (Cavaliere, 2010). The International Aloe Science Council (IASC) estimated sale of AV products for over 125 million dollars per year in 2004 (Rodriguez, 2004), and the potential market is estimated to be around 10 billion dollars. Forever Living Products sold AV products for 1.7 billion dollars in 2010 (Januszewski, 2011).

1.4.1 Pharmacological and toxicological properties of Aloe vera

The constituents of AV have biological and toxicological properties, yet the active components elude definition (Williamson et al., 2011). The AV products are made from its fleshy leaves containing aloe latex and aloe gel. The latex contains laxative anthraquinone and oral AV is documented used as a stimulant laxative (Werner C., 2007; Williamson et al., 2011). In addition, other constituents with known therapeutic activity (laxative) are barbaloin (aloin A (10S) and B (10R)) and 5-hydroxyaloin A (10S) (Farnsworth NR, 1999; Patel et al., 2012). Because of the empirically documented laxative effect, Aloe vera is defined as a medicinal herb by the Norwegian Medicines Agency (NoMA, 1999). The FDA reclassified, however, the stimulant laxative ingredient aloe from category I (monograph) to category III (more data needed) in 2002, because of lack updated safety and toxicology data (FDA, 2002; Ulbricht et al., 2008).

The aloe gel is processed to purified gels, juices or dried powder to make tablets (Ahlawat and Khatkar, 2011). The water content in AV gel is ranging from 99-99.5% (Boudreau and Beland, 2006). The remaining solid material consists mostly of polysaccharides, vitamins and minerals (Atherton, 1998; Vogler and Ernst, 1999; Ni et al., 2004; Boudreau and Beland, 2006), though its composition varies between harvest seasons and growth location (van Wyk et al., 1995). Acemannan is one of the most abundant (> 60 % of solid matter) and well-known of the polysaccharides and is believed to be important for the immunostimulating and anticancer activity attributed to AV (Zhang and Tizard, 1996; Harlev et al., 2012). A recent paper concludes of possible induction of bone formation by influence of acemannan in rats (Boonyagul et al., 2013). Other components isolated from AV are also attributed pharmacological effects in the literature, like emodin to be anti-angiogenic (Cardenas et al., 2006) and phytosterols to have hypoglycaemic activity (Tanaka et al., 2006). In addition, anti-inflammatory and thrombolytic (Vazquez et al., 1996) , anti-arthritic and antibacterial activities are reported for AV (Vogler and Ernst, 1999). This makes AV an interesting herb in regard to evaluate for *in vitro* interactions on drug metabolism and transport.

It is shown that crude AV juice (AVJ) (200 µg/ml) enhances NF-kappa B activation from human macrophages *in vitro* with 7 % (Pugh et al., 2001) and AV leave extract (1.0 ml/kg) significantly decreased high liver markers induced by lindane (Etim et al., 2006). In addition, a mixture of AV and milk thistle (*Silybum marianum*) is reported to increase the mRNA

expressions for tumor necrosis factor-alpha (TNF-alpha), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in acute hepatotoxicity in mice (Kim et al., 2009).

Human *in vivo* studies are, however, weak in study methods and inconsistency of data do not currently warrant the recommendation of oral AV for the management of diabetes mellitus, dyslipidaemia, inflammatory bowel disease, cancer, osteoarthritis, hypertension or other diseases (Williamson et al., 2011; Harlev et al., 2012). Ingestion of oral AV may give diarrhea, electrolyte imbalance or kidney dysfunction and is considered unsafe for multiple medical conditions as well as for pregnant or breastfeeding women (Ulbricht et al., 2008). Although topical application is considered more safe, episodes of contact dermatitis, erythema and phototoxicity have been reported (Farnsworth NR, 1999; Williamson et al., 2011).

1.4.2 Co-use of Aloe vera

Aloe vera is reported to be the most frequently used herb (25%) among the HIV patients using herbs in addition to be on anti-retroviral therapy in Uganda (Lubinga et al., 2012). Among osteoarthritis patients in Nigeria, 30% of the patients used herbal products and of these used nearly 30% AV concomitantly with conventional drug treatment (Obalum and Ogo, 2011). Cancer patients in Norway reported of co-use of AV and chemotherapy of 5% (Engdal et al., 2008).

Little has been known, however, about AVJs interaction potential when co-administrated with drugs. Apart from a publication (Brandin et al., 2007) indicating a minor induction of CYP1A2, CYP3A4 and MDR1 *in vitro* and a study on one single component in AV, rhein, inhibiting both CYP3A4 and CYP2D6 *in vitro* (Tang et al., 2009), no interaction data of AVJ on P-gp, CYP3A4 or CYP2D6 have been published previously to our knowledge.

1.5 Levels of interaction

The herb-drug pharmacokinetic interactions might take place on different pharmacokinetic enzymes. The cytochrome P450 enzymes (CYP) accounts for a significant part of the first pass metabolism together with the efflux protein, P-glycoprotein (P-gp) in humans (Liu et al., 2007).

1.5.1 Cytochrome P-450 (CYP) enzymes

Cytochrome P450 enzymes (CYP) are large groups of haeme-containing metabolic enzymes of great importance in xenobiotic metabolism. CYP enzymes are essential for the production of steroids, prostacyclins, cholesterol and thromboxane A_2 (Guengerich, 2008). They also are necessary for the detoxification of foreign chemicals and the metabolism of drugs. Figure 1 show the CYP catalytic cycle for substrate hydroxylation. CYP enzymes are so named because they are bound to membranes within a cell (cyto) and contain a haeme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide. Human drug metabolism are mostly performed by five of the 57 known human CYPs (Guengerich, 2008). These are CYP1A2, CYP2C9/CYP2C19, CYP2E1, CYP2D6 and CYP3A4 which metabolise 90% of drugs, with the two most significant enzymes being CYP3A4 and CYP2D6 (Lynch and Price, 2007). They are predominantly expressed in the liver, but they also occur in the small intestine (reducing drug bioavailability), lungs, placenta, and kidneys (Rendic and Di Carlo, 1997).

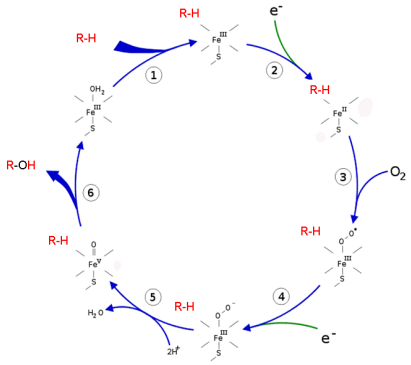

Figure 1. The substrate (R-H) binds to the CYP enzyme (1) close to the haeme-group. Depending on the substrate and enzyme involved, P450 enzymes can catalyze any of a wide variety of reactions like hydroxylation shown in this figure. In the presence of NADPH+ (2 and 4) and oxygen (3), the CYP enzyme catalyzes the metabolic reaction forming a product (R-OH) and a water molecule. After the product has been released from the active site, the enzyme returns to its original state, with a water molecule returning to occupy the distal coordination position of the iron nucleus (Figure modified from Guengerich, 2008).

1.5.1.1 CYP3A4

Isoenzyme CYP3A4 is an important metabolic enzyme and the most abounded in the intestine and liver (Liu et al., 2007; Lynch and Price, 2007).

CYPs	Hepatic expression levels [%]	Variability in expression [~ fold]	Phase-I drug reactions [% of CYP reactions]
CYP1A2	13	40	5
CYP1B1	< 1		
CYP2A6	4	30 – 100	2
CYP2B6	5	100	2 – 4
CYP2C8	5	150	1
CYP2C9	34		0
CYP2C19	7	30	5
CYP2D6	3	> 1000	20 – 30
CYP2E1	7	20	2 – 4
CYP3A	28	20	40 – 45

Figure 2. CYP isoenzymes and their relative hepatic expression levels (upper pie chart and table) and contributions to Phase-I drug reactions (lower pie chart and table). A high hepatic expression level does not necessarily correspond to a large part of the drug metabolism eg. CYP1A2 and *vice versa* (Figure from Kramer and Testa, 2008).

The CYP3A subfamily, CYP3A4 and CYP3A5 mainly, metabolizes approximately 50% of all CYP metabolized drugs, thus a heavy contributor in the drug metabolism (Rendic and Di Carlo, 1997). Furthermore, P-gp and CYP3A are frequently co-expressed in the same cells and share a large number of substrates and modulators. The disposition of such drugs is thus affected by both metabolism and transport (Liu et al., 2007).

CYP3A4 has a low degree of substrate specificity which makes it susceptible for inhibition of a variety of structurally unrelated substances e.g. antifungal agents (ketoconazole and itraconazole), antimicrobials (clarithromycin, erythromycin and ritonavir), antihypertensives (verapamil and diltiazem) and several herbal and food constituents, e.g. grapefruit juice and bergamottin (Kent et al., 2002; Liu et al., 2007; Pelkonen et al., 2008). Thus inhibition of CYP3A4 activity by AVJ seems not unlikely.

1.5.1.2 CYP2D6

Approximately 20-30% of the CYP drug metabolism is carried out by CYP2D6 enzymes (Figure 2) (Rendic and Di Carlo, 1997; Ingelman-Sundberg, 2005). Some of these drugs are commonly used and consumed in large scale as for instance cardiovascular drugs (e.g. β-adrenergic blocking agents), antidepressants and analgesics, and an interaction would therefore have large implications for the patients.

Furthermore, CYP2D6 has been studied extensively because it exhibits genetic polymorphism, meaning that distinct population differences are apparent in its expression or activity. Approximately 7 to 10 per cent of Caucasians are poor metabolisers of drugs metabolised by CYP2D6 (Cupp and Tracy, 1998). Adverse effects due to elevated drug plasma levels occur more frequently in poor metabolisers in cases where the drug clearance is dependent on CYP2D6. A lack of or inhibition of CYP2D6 enzymes reduce the effectiveness of drug therapy in cases where pro-drugs requiring activation by CYP2D6 metabolism as for tramadol and codeine (Ingelman-Sundberg, 2005). An inhibition in CYP2D6 in poor metabolisers may thus have great impact on the drugs bioavailability.

In vitro investigations have shown that extracts of *Heliopsis longipes* (A.Gray) (Rodeiro et al., 2009), isolated constituents from grapefruit juice (e.g. bergamottin) (Tassaneeyakul et al., 2000), Ginkgo biloba (ginkgolic acids I and II) (Zou et al., 2002) and ginseng (ginsenoside

Rd) (Henderson et al., 1999), can inhibit CYP2D6 metabolism. A *in vivo* study performed by Gurley et al. revealed a significant inhibition (approximately 50%) of CYP2D6 activity by goldenseal in humans, while Ginseng and black cohosh demonstrated a weak inhibitory effect on CYP2D6 metabolism of debrisoquine in the same study (Gurley, 2008). Thus, CYP2D6 metabolism seems to be vulnerable for inhibition by herbal products.

1.5.1.3 Patterns of inhibition

Inhibition of CYP3A4 and CYP2D6 can be both reversible (Figure 3a) or irreversible (mechanism-based, Figure 3b) in nature. The latter have the highest impact on the drug bioavailability (Zhou et al., 2005). A mechanism-based inactivation is anticipated to be due to a chemical modification of the haeme or protein part of the enzyme, or both, leading to the formation of reactive metabolites that bind covalently to the enzyme causing CYP inactivation (Zhou et al., 2005). A number of clinically important drugs have been identified to be mechanism-based CYP3A4 inhibitors as erythromycin, tamoxifen, midazolam and verapamil (Guengerich, 2008). In addition to some herbs as grapefruit juice, common valerian (Zhou et al., 2004; Hellum and Nilsen, 2007) and a few isolated herb constituents as bergamottin and glabridin (Kent et al., 2002). The herbs black pepper (*Piper nigrum*) (Subehan et al., 2006) and Madagascar Periwinkle (*Catharanthus roseus*) (Usia et al., 2005) have been identified as potent mechanism-based inhibitors of CYP2D6. CYP3A4 and CYP2D6 metabolism seems thus to be vulnerable for irreversible inhibition by herbal products.

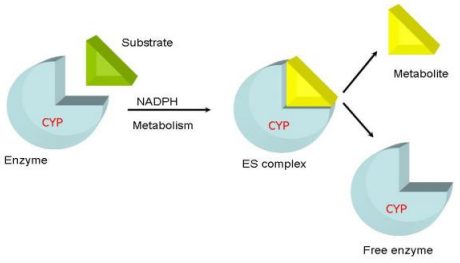

Figure 3a. Reversible inhibition of CYP enzyme binding a substrate to its active site, forming an enzyme-substrate (ES) complex. The metabolite is released and the enzyme is free to receive a new substrate.

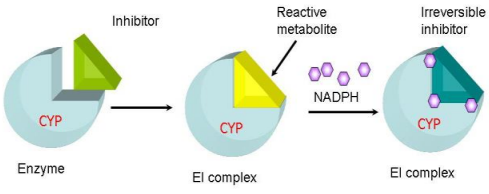

Figure 3b. Irreversible inhibition of CYP enzyme binding a substrate to its active site, forming an enzyme- inhibitor (EI) complex. The reactive metabolite binds irreversible to the enzyme using NADPH.

1.5.2 Efflux proteins

Since the discovery of the permeability-glycoprotein (P-glycoprotein/P-gp) 40 years ago the research on ATP-binding cassette proteins (ABC-proteins) have shed light on their roles in cytotoxic drug efflux in human cells and drug resistance in cancer cells (Szakacs et al., 2006). The ABC-transporters are situated in the cell-membrane and contains typically two transmembrane domains (TMDs) and two nucleotide (ATP)-binding domains (NBDs) (Taipalensuu et al., 2001; Szakacs et al., 2006). They protect the body against toxic substances including drugs, and have in general a low substrate affinity, effluxing both chemotherapeutics and naturally occurring biological compounds (Szakacs et al., 2006). In addition, the ABC-transporters are highly expressed in important pharmacological barriers as in the intestines, liver, kidneys and in the blood–brain barrier (BBB) affecting the absorption and elimination of drugs (Szakacs et al., 2006).

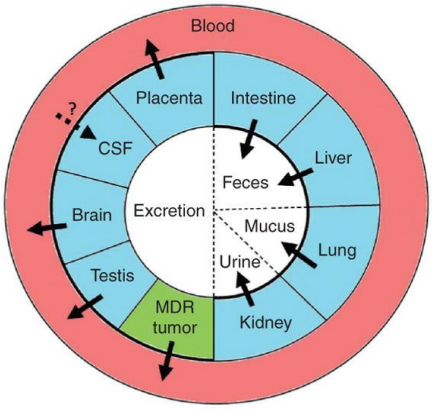

Figure 4. The distribution of P-gp in different human tissue. The bold solid arrows indicate the known direction of transport, whereas the broken-line arrow indicates unclear direction of transport. P-gp is located in the lipid bilayer (thick black line) that forms a barrier between various organs; red indicates vasculature, blue represents tissue, and white indicates excreta. CSF, cerebrospinal fluid; MDR, multidrug resistance (Figure from Kannan et al., 2009).

The human genome contains 48 genes encoding ABC-transporters divided into seven subfamilies given symbols from A to G (NCBI, 2008). P-gp is sorted as ATP-binding cassette sub-family B member 1 (ABCB1) and are the most well-known of the ABC-transporters together with the breast cancer resistance protein (BCRP, ABCG2), multidrug resistant protein 2 (MRP2, ABCC2) and the bile salt exporting pump (BSEP, ABCB11) (Taipalensuu et al., 2001).

1.5.2.1 P-glycoprotein

P-gp is located in epithelial cells in organs of excretion (Figure 4) like intestines, placenta, liver and kidneys to facilitate directed drug efflux and thus counteracting systemic accumulation of potentially toxic compounds (Zhou, 2008). Like other ABC-transporters, P-gp holds a polyspecific drug-binding site which effluxes a vast selection of different drugs (Aller et al., 2009) and is also widely expressed in cancer cells (Hochman et al., 2002). An interacting effect of AV on the P-gp transporter will influence drug pharmacokinetics and efficacies (Doherty and Charman, 2002; Lin and Yamazaki, 2003; Zhou, 2008). P-gp contributes significantly to the first pass metabolism as about 50% of all marketed drugs are reported to be P-gp substrates such as statins, antibiotics, human immunodeficiency virus (HIV) protease inhibitors, immunosuppressants, anticancer, and cardiac drugs (Endres et al., 2006; Keogh and Kunta, 2006). An alteration (inhibition or induction) of P-gp efflux by AV could therefore change the pharmacokinetics of several drugs and be of importance for patient safety in general (Lin and Yamazaki; Zhou et al., 2004).

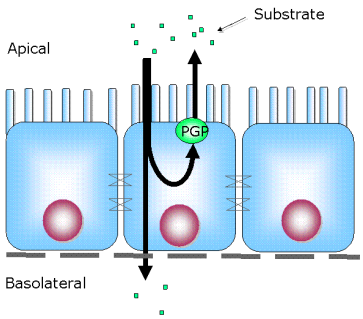

Figure 5. A schematic model of the Caco-2 and P-glycoprotein system.

Caco-2 cells derived from human colon carcinoma cells, constitute a well-established model for bi-directional P-gp transport (Figure 5) and interaction studies (Hidalgo et al., 1989; Artursson, 1991; Sun et al., 2008). This model is well tested for its suitability as an *in vitro* assay, and correlates well with *in vivo* controls (Artursson and Karlsson, 1991; Adachi et al., 2001; Collett et al., 2004). Despite this, the Caco-2 cells are a heterogeneous cell population that is exposed to different growth conditions which causes interlaboratory variabilities (Shah et al., 2006). A thorough quality control for Caco-2 cells can, however, facilitate the possibility to compare data between laboratories. Characteristics as assay linearity, transepithelial electrical resistance (TEER), mannitol transport and general cell toxicity should be obligatory (Artursson, 1991; Hidalgo, 2001; Markowska et al., 2001).

Even though a weak induction of the P-gp gene (ABCB1 gene) is reported, a P-gp inhibition by AVJ can occur at higher concentrations as reported for *Echinacea purpurea* (Hansen and Nilsen, 2008) or when given as a single dose compared with repeated dosing as shown for St.

Johns wort, grapefruit juice or garlic (*Allium sativum*) (Zhou et al., 2004). It can also be an allosteric induction or inhibition of the P-gp transport.

2 Aims

The aims of this thesis were divided; 1) To register the co-use of drugs and herbs among GP patients in Norway and the patient's communication of such use with health care professionals; 2) To evaluate the interaction potential of one of the commonly used herbs in GPs office, Aloe vera, on the P-glycoprotein (P-gp), CYP3A4 and CYP2D6 enzymes.

2.1 Co-use among GP patients

The primary aim of this study was to compare patients in a general practice in Norway that co-use herbal remedies and drugs with those who do not, with regards to demographics, types of drugs and herbs used, reason for use and communication with health care professionals about this use. The second aim was to register the herb-drug combinations and identify those with interaction potential.

To address the aims, the following questions were asked:

1. Do Norwegian GP patients use herbal remedies and if so, to what extent?
2. Are some herbs more commonly used than others?
3. Which motives do they have for use?
4. Do the patients disclose their herbal use to the GP or other health care providers?
5. Do any of the herb users also use conventional drugs on a regular basis; if so was there any difference between the drugs?
6. Which herbs and conventional drug combination can be identified, and are any of them at risk of being harmful to the patient?
7. Do they experience any adverse effects of herbal use?
8. Is there any common characteristics of those who co-use herbs and conventional, regular drugs and can the GP use them to pin-point those at risk?

2.2 Aloe vera juice in vitro studies

The aims of Paper II and Paper III were to evaluate the inhibitory potency of two different *Aloe vera* juice (AVJ) products on CYP3A4 or CYP2D6 activities *in vitro*, compare the IC_{50} inhibition values and determine if a possible AVJ inhibition of these two CYP enzymes could be mechanism-based.

The aims of Paper III were to carry out a thorough quality control setup for essential Caco-2 cell characteristics in P-gp inhibition studies and to explore whether *Aloe vera* juice (AVJ) possesses any inhibitory effect on the bidirectional transport of the P-gp substrate digoxin.

To address the objectives of these studies following questions were asked:

1. Does Aloe vera inhibit CYP3A4, if so to what extent?
2. Does Aloe vera inhibit CYP2D6, if so to what extent?
3. Does Aloe vera inhibit CYP3A4 and CYP2D6 by mechanistic inhibition?
4. Is there a difference between different commercial Aloe vera juice products in their ability to inhibit CYP3A4 and CYP2D6 mediated metabolism?
5. Is the Caco-2 cell model functioning in our laboratory as a reliable and reproducible system for evaluating P-gp transport and interactions?
6. Is Aloe vera toxic to the Caco-2 cells?
7. Does Aloe vera inhibit P-gp, if so to what extent?
8. Does Aloe vera possess a clinical relevant interaction potential towards other drugs?

3 Materials and methods

The thesis is a product of the several methods and procedures that have been used and a thoroughly described in the respective papers. An overview is given here.

3.1 Cross-sectional study

This was a questionnaire based cross-sectional study (Paper I) conducted in a GP office in a middle large town on the west coast of Norway with nearly 70 000 inhabitants (Sandnes). The demographic of the city is quiet similar to other middle large towns in Norway (Aalandslid and Østby, 2007). At the time of the data collection (2011.07.11-2011.12.15) the GP office had 6000 patients on the practice list, four GPs and one intern (me). The study was approved by the Regional Committee for Research Ethics in South-eastern Norway.

The inclusion criteria were patients 18 years old or older, having an office consultation with a GP and who were able to read and understand the questionnaire. The patients were asked if they wanted to participate by the staff when the patients contacted the reception. It was not systematically registered how many said no, but according to the staff this was about half of the patients.

The questionnaire was based on a previously questionnaire used among cancer patients in an outpatient clinic in Central Norway and adapted to fit patients in a GP office (Engdal et al., 2008). It was divided into three parts (Appendix 1). The first part contained questions about demographic data and conventional drug used. The second part asked about herbal use from a predefined list of the 24 most common herbs sold in Norway and the frequency of use (Moussally et al., 2009). Only those products defined as herbs (herbal substances, herbal preparations or herbal medicinal products) were included in the analysis (EMA, 2012). Herb users were defined as those answering that they used herbs daily, weekly, monthly, less than monthly or periodically. Non-users were defined as those answering that they used herbs earlier or never used.

In the last part of the questionnaire the communication between the patient and health care professionals, motives for use or no use, and who recommended use of herbs were obtained

(Paper I, Tables 1 and 5). In addition, they were asked about any side effects of their herbal use and approximately monthly costs.

3.1.1 Calculations and statistics

Pearson's Chi-square was used for bivariable analyses of categorical data. Multivariable analysis was conducted to disclose any associations between co-users and other variables, using binary logistic regression analysis (adjusted odds ratio, adjOR). All variables with p-values <0.2 in bivariable analysis were included in the regression analysis. P-values < 0.05 were considered as statistically significant. Tendencies were ascribed for p-values between 0.05 and 0.10. The statistics analysis was done using SPSS 19.0 (SPSS, Chicago, IL, USA).

To find the total number of consultations in the GP practice during the 5 weeks data collection period, and the age and gender distribution of these patients, a report module of the electronic health record system was used (WinMed 2.12r Statistics, CompuGroup Medical Norway AS, Lysaker, Norway).

3.2 Aloe vera juice in vitro studies

3.2.1 Preparation of Aloe vera juice

An estimated daily dose (30 ml) of AVJ was centrifuged (3000 rpm for 10 min) and the supernatant was freeze-dried (-70 ºC). The dried AVJ was resuspended in 0.8% DMSO (P-gp assays) or 1.9% ethanol (CYP assays) to make a stock solution (SS) of 270 mg/ml. The amount of DMSO or ethanol was kept to a minimum needed to dissolve the AVJ. A dilution sequence was made of the SS with HBSS or KPO buffer to make seven or eight working solutions (WS) from the AVJ. All solutions included in the assays contained the same amount of diluting reagents (ethanol or DMSO). To minimize a potential source of error from the low juice pH, each WS was pH adjusted to be >5.0.

3.2.2 P-glycoprotein *in vitro* assay

The human colon carcinoma cell line, Caco-2, express P-gp on the apical side when cultivated as monolayers. The Caco-2 cells were cultured (37 ºC, pH 7.4) in growth medium (DMEM) prior to seeding. To prevent phenotypic drift only passage 35-40 were used. The cells were

seeded onto 24-well 6.5 mm Transwell® plates with 0.4 µm Pore Polycarbonate Membrane Inserts at a density of 0.4-0.5 mill/cm^2 for transport studies. The culture medium was replaced every 2nd day. In addition, the growth medium was always replaced the day prior to the transport experiment. The seven different AVJ concentrations were pH adjusted > 5.0 pre-incubation, to minimize the confounding effects of the low juice pH on the cells (Neuhoff et al., 2005).

3.2.2.1 Cell viability

In vitro cytotoxicity of AVJ and digoxin, the P-gp substrate, were determined by the tetrasodiumsalt colorimetric assay (MTT). MTT is converted to formazan crystals by mitochondrial lactate dehydrogenases (LDH) in living cells (Figure 6). The measured absorbance of formazan at 570 nm, is thus proportional to the number of living cells (Mosmann, 1983).

Caco-2 cells were cultivated for 3 days directly in the wells. The culture medium was exchanged by the final study solutions and the cells were incubated for 90 minutes. Afterwards, the final study solutions were replaced by MTT (5 mg/ml), and incubated for 40 min. After removal of the MTT solution, the cells were washed with cold PBS buffer. DMSO was added to each well for extraction of the formazan product. The amount of crystals was analysed by spectrophotometric measurement of optical density at 570 nm.

3.2.2.2 Bidirectional transport studies of digoxin

The cells were cultured for 21-22 days before the growth medium was replaced with HBSS/HEPES buffer. The monolayer integrity was monitored by measuring TEER (transepithelial electrical resistance) before and after the transport assay, in addition to mannitol transport during the assay. The TEER values should be ≥ 200 Ω/cm^2 and mannitol $Papp_{A-B}$ values < 1.0 x 10^{-6} cm/s to be included in the results (Artursson, 1991).

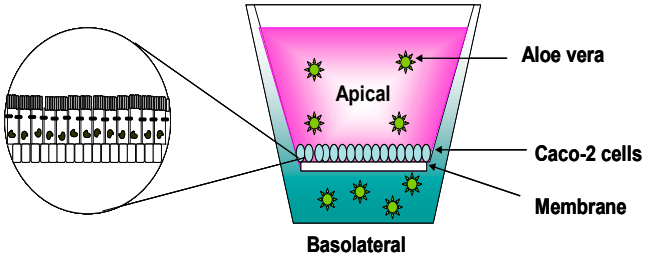

Figure 7. Caco-2 cell well with insert containing inhibitor (AVJ or verapamil) on both apical and basolateral side i.e. sink conditions. P-gp is expressed on the apical side of the Caco-2 monolayer.

The substrate, ^{3}H–digoxin, was dissolved in HBSS/HEPES buffer (30 nM, 23.5 Ci/mmol) and added to the donor side (250 µl on apical side and 500 µl on basolateral side) of the Caco-2 cell monolayer in the presence of test compounds under sink conditions (Figure 7). The digoxin transport was measured for 90 minutes in apical to basolateral (A-B) and basolateral to apical (B-A) directions. All inhibitors were added both on the donor sides and the receiver sides (sink conditions). Verapamil (100 µM) was used as a positive inhibitor control (Lin, 2003). Seven different AVJ concentrations (1.0×10^{-5} mg/ml to 1.0×10^{-5} mg/ml) were used in the AVJ inhibition assay. A 55 µM ^{14}C-mannitol solution (1.20 µCi/ml) was added to the apical side as an indicator of the monolayer integrity during the transport assay i.e. sustainable tight junctions (Artursson et al., 1994). None of the Papp $_{A-B}$ values were above 1.0×10^{-6} cm/s.

The transported amount of ^{3}H–digoxin or ^{14}C-mannitol was measured by a scintillation counter. For more detailed information see Paper III.

3.2.3 CYP enzyme in vitro assays

The CYP3A4 or CYP2D6 enzymes were in general incubated in KPO-buffer (pH 7.4) on a shaking water bath (37ºC) for 10 or 25 min., respectively. The incubation solutions contained the substrate with AVJ or without AVJ (control). Testosterone or dextrormethorphan were used as substrates for CYP3A4 or CYP2D6, respectively. Seven AVJ concentrations in the range from 0.01 mg/ml – 60.0 mg/ml were investigated. Ketoconazole (KTZ) was used as positive inhibitor control for CYP3A4 and or quinidine for CYP2D6. The final incubation concentration of ethanol was 0.04 % in all solutions to avoid ethanol influence (biphasic effect) on the CYP enzymes (Hellum and Nilsen, 2007). The CYP catalytic reactions were initiated by addition of 20 µl solution A from the NADPH-regenerating system completing a total incubation volume of 400 µl. The NADPH-regenerating system consist of two reagents, Solution A [NADP+ and glucose-6-phosphate (Glc-6-PO4)] and Solution B [glucose-6-phosphate dehydrogenase (G6PDH)]. Combined, these two reagents form an NADPH regenerating system that can be used for all NADPH requiring oxidase assays (cDNA-expressed enzymes and liver fractions).

The CYP3A4 or CYP2D6 enzyme activity was terminated after 10 or 25 min. by the addition of 200 µl ice cold methanol or ice cold acetonitrile, respectively. After centrifugation was the supernatants directly transferred to HPLC vials and the formation of metabolite (6-OH-testosterone or dextrorphan) was analysed by HPLC. For more details see Paper II.

3.2.3.1 Enzyme inhibition assays

The incubations were performed as described above. Test solutions of AVJ, KTZ or quinidine in volumes of 100 µl were added to the CYP3A4 or CYP2D6 incubations in final concentration ranges of 0.01- 60 mg/ml, 0.0064 – 2.0 µM or 0.001- 1 µM, respectively. IC_{50} values were estimated by linear regression for the CYP activity.

3.2.3.2 Time and NADPH dependent assays

The incubations were performed as described in section 3.2.3. The IC_{50} value of AVJ for CYP3A4 or CYP2D6 enzyme activity was used. In the time dependent assay was the test solutions added AVJ and preincubated for 0, 15, 30 or 45 min at 37ºC before initiation of the CYP activity (addition of solution A). To analyse whether the AVJ inhibition of the CYP3A4 or CYP2D6 enzyme was NADPH dependent, solutions with or without NADPH were pre-incubated for 45 or 30 min.

3.2.3.3 High-performance liquid chromatography (HPLC)

In general high-performance liquid chromatography (HPLC) utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules (Figure 8). Retention time varies depending on the interactions between the stationary phase, the molecules being analysed, and the solvent(s) used (Connections, 2004). The mobile phase refers to the solvent being continuously applied to the column, or stationary phase, and acts as a carrier for the sample solution. A component in the sample solution migrates according to non-covalent interactions with the column compound. This determines the degree of separation and i.e. the specific retention time to each compound.

The 6-OH-testosterone was detected by UV detector at retention time of about 20 min. with mobile phase 40 % CH_3OH/water, pH 7.4, flow rate 1 ml/min., total run time 35 min. and

injection volume 40 µl. UV detector is the most common detector and relies on the absorbance of the light by the analytes of interest which requires that the compound contain a chromophore group.

Fluorescent detectors (FLD) measure the ability of a compound to absorb and re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths (Lodder, 2009). Since only a few organic compounds are able to emit light at a different wavelength than they absorbed, is HPLC-FLD not only a highly selective detector but also a very sensitive detector, because background noise from the mobile phase is practically eliminated (Connections, 2004).

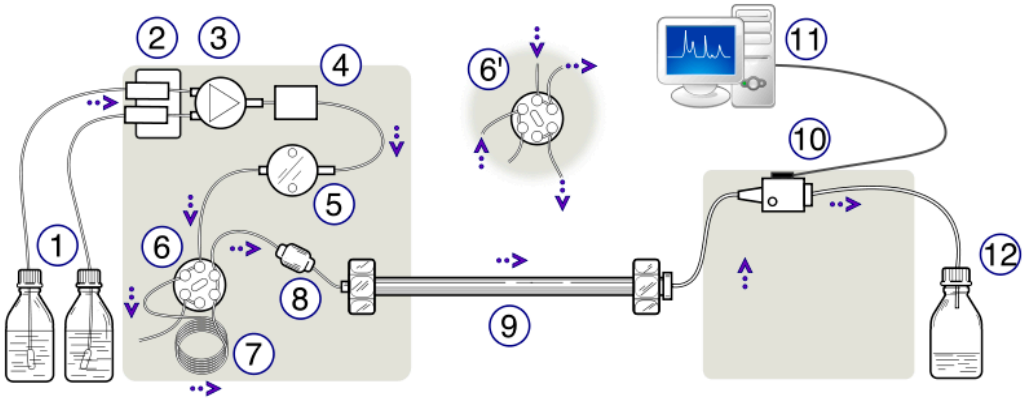

Figure 8: Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column, (9) Analytical column, (10) Detector (i.e. IR, UV, FLD), (11) Data acquisition, (12) Waste or fraction collector (From Wikipedia, 2009).

The chromatographic analysis of dextromethorphan and its metabolite was performed using a FLD at excitation and emission wavelengths of 230 and 330 nm, respectively. Mobile phase: 20 % CH_3CN and 80 % 0.01 M KPO-buffer, pH 3.4. Flow rate: 1 ml/min., total run time 25 min. and injection volume 30 µl. The registered emitted fluorescents were detected and chromatograms were printed. The retention time for the metabolite, dextrorphan, was approximately 4.3 min. and for the substrate, dextromethorphan, 21 min. Each peak in the chromatograms reflects one of the separated substances, and the area of each peak was used to estimate the amount of each substance.

3.2.3.3.1 Validation of HPLC methodologies

A seven point calibration curve of the metabolite was used to quantify the CYP3A4 or CYP2D6 activity. The standard curves were fitted to the measured peak responses. The linear correlation coefficient (r^2) had to be ≥ 0.99 for the calibration curve to be accepted. The lowest accepted concentration in the calibration curve, LLOQ, should be >5 times blank response, the peaks need to be identifiable, discrete, and reproducible with a precision < 20% and accuracy of 80-120%. Quality controls (QC) were constituted at three concentrations (low, medium and high) in three or two parallels for pre-run or in-run validation, respectively. The standards WS and QCs WS were made by two different laboratory assistants. The QCs were accepted if at least 4 of 6 QCs had a precision <15% and minimum one concentration on each level had to be accepted. In addition, the linear correlation coefficient (r^2) had to be ≥ 0.99.

The analytes precision i.e. the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of test solutions, at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. In addition, the analysis` accuracy i.e. the degree of closeness of measurements of a quantity to its actual (true) value, should be <15% of the actual value except for the LLOQ where it should not deviate >20% (FDA, 2001).

3.2.4 Calculations and statistics

The P-gp efflux was measured by digoxin transport across the cell monolayer and expressed as the apparent permeability coefficients (Papp (cm/s)), Papp ratio ($R_{B\text{-}A/A\text{-}B}$), net digoxin Papp ($Papp_{Net}$) and net digoxin flux ($J_{Net}$). Papp ratio is the Papp in basolateral to apical (B-A) direction divided with Papp in apical to basolateral (A-B) direction. The net flux (J_{Net}) expressed the net transport in B-A direction i.e. the $J_{A\text{-}B}$ subtracted from $J_{B\text{-}A}$ (nmol/h/cm^2).

The CYP3A4 or CYP2D6 activity were determined from the amount metabolite (pmol) formed during incubation divided on the total CYP amount in the incubation solution (pmol) and the incubation time (min). Enzyme activity is expressed as percentage of reference control. The IC_{50} (concentration of herb that inhibits CYP3A4 or 2D6 activity with 50%) of the investigated inhibitor (KTZ or quinidine) and herbs is estimated from non-linear

regression of inhibition plots where CYP activity is plotted against increasing inhibitor or AVJ concentrations using Sigmaplot (Sigmaplot 2008 for Windows, Version 11.0; Systat Software Inc., Point Richmond, CA, USA).

Statistical analysis was done using Microsoft Office Excel 2003 and Sigmaplot (Sigmaplot 2008 for Windows, Version 11.0; Systat Software Inc., Point Richmond, CA, USA). Differences between groups were analysed by a two-sample student's t-test. All data are given as means ± standard deviation (SD). P-values < 0.05 were considered statistically significant. Least square regression lines were considered to fit linearity at p-values < 0.05.

4 Methodological considerations

4.1 Cross-sectional study in GP office

One of the limitations of this study was that it is a cross sectional study, meaning that no causal relationship can be identified. In addition, the study took place in one GP clinic in a middle large town on the west coast of Norway. It thus might not be representative for other populations, but the patients visiting the practice are similar to other GP patients in Norway (NDH, 2012).

Although those taking part in this study were representative for all patients visiting the GP practice during the period of the survey, those using herbs might also be more positive to contribute to such a study than non-herb users. This would give an overestimation in the prevalence of herb users (selection bias). However, this would also be the same for other studies investigating herbal use, and would not hamper the comparison with these.

The patients responding had to understand the questionnaire in order to answer it correctly (information bias). To limit this bias, the patients were invited to ask the reception staff or me for help in case of questions. All data are self-reported and inaccuracies in the reported use of herbs and drugs must be taken into consideration. Still, the latter was minimized by handing out lists of the most common drugs in familiar groups with examples of the most common sales name of the different drugs (e.g. anticoagulants (Marevan™, Albyl-E ™).

The number of patients included was too low to draw any firm conclusion about herb-drug combinations at risk of giving server adverse effects among the GP patients. However, some combinations were more frequent than others and this information gives an important contribution with regard to risk of harmful adverse effects.

Although herbs were listed, patients could put other herb in the optional space in the questionnaire. However, it was not made clear in the patient information that only herbs (plant derived substances) should be included, thus, some had written fish oil or probiotics. In addition, Q10 (proteins), multivitamins and minerals were already on the list, but were excluded in the further analysis to focus on herb users. This may have influenced their response later in the questionnaire, although it did not influence the data in a significant way.

The duration or amount of herb use and the way of administration of the herb (i.e. oral, topical) was not covered in Paper I and would have given us more information whether the herb-drug interaction was clinically relevant. Aloe vera used as juice ingested orally in large daily doses has a much higher interaction potential contra Aloe vera used topically against skin burns, although dermal absorption cannot be excluded. Some of the herbs are ingested as foods like garlic and grapefruit and will in general not be a problem, unless used in excessive amounts.

During the analysis of the questionnaire some of the questions had to be merged because of quite similar options/responses were given (e.g. question 7 and 8, Appendix 1). This might have been avoided if a proper pilot-study was performed, but this seemed unnecessary since the questionnaire already had been used in an outpatient clinic in Ålesund (Engdal et al., 2008).

Working as an intern on the GP office during the data collection, my presence might have influenced some of the patients to answer or not answer the questionnaire. However, the advantages of being present (able to answer questions, give practical information or tasks) were assumed to give more value than the bias-disadvantage.

4.2 In vitro studies

4.2.1 The Caco-2 cell model

The P-gp inhibition assay was performed on polarized Caco-2 cell monolayers with an apical (A) and a basolateral (B) side. Alternatively one could have utilized LS-180 cells as an intestinal cell line model. LS-180 cells are a colorectal adenocarcinoma cell line and express human Pregnane-X-Receptor (PXR) to a higher degree than Caco-2 cells, thus making a more suitable model to show both inhibition and induction of P-gp transport (Sun et al., 2008; Fan et al., 2011). However, the experience with this cell line in P-gp studies is limited. Caco-2 cells on the contrary, are a well-known cell line and reported as a good P-gp screening model for drug-drug inhibition interactions (Markowska et al., 2001). A high interlaboratory heterogeneity has, however, been reported for the Caco-2 cells (Hidalgo, 2001). Therefore, the substrate transport at regular time intervals, demonstrating linearity, A-B and B-A

differences and effect of positive inhibitor control were performed to facilitate comparisons with other laboratories, and to show cell compatibility (Hidalgo, 2001).

Verapamil, a first generation P-gp inhibitor, was used as an inhibitory control although the Caco-2 cells also express other ABC-transporters inhibited by verapamil, and thus makes the result interpretation more inaccurate (Taipalensuu et al., 2001). Scientists all over the world are searching for selective P-gp inhibitors, but have not yet quite succeeded (Szakacs et al., 2006; Lee et al., 2013). Until then, verapamil is still used in Caco-2 cell methodology to inhibit P-gp and makes it easier to compare with other studies.

The RNA expression of multiple ABC-transporters (e.g. multidrug resistance associated protein 2 (MRP2), multidrug resistance protein1 (MDR1)) are similar to the RNA expression of the transporters in biopsies from human jejunum (Taipalensuu et al., 2001). In addition, CYP3A4 is also expressed by the Caco-2 cells, having an interrelationship in substrates and inhibitors with P-gp (Kim et al., 1999; Taipalensuu et al., 2001). In light of this and the fact that the P-gp receptor has low selectivity, finding a selective substrate for P-gp is important for the interpretation of the results of the Caco-2 assay. Digoxin is the most known selective P-gp substrate used in Caco-2 cell assays, not metabolized by CYP3A4 (Kim et al., 1999; Mease et al., 2012). However, organic anion transporting polypeptides (OATP, SLCO1A2) has been discussed to take part in the digoxin transport, but this has been falsified by a more recent report (Taub et al., 2011). Thus, digoxin has still the dominating role as substrate in P-gp inhibition assays in Caco-2 cells (Zhou, 2008; Mease et al., 2012).

4.2.2 CYP inhibition assays

The CYP assays were performed using recombinant human CYP enzymes (Supersomes™). These are commonly used models for *in vitro* single enzyme investigation, allowing investigation of the inhibitory effect on a single enzyme without any other enzymes interfering. Furthermore, are supersomes easy to use and are cost effective (Kim et al., 2006; Hellum and Nilsen, 2007). Liver microsomes and cell cultures are other *in vitro* models. They include multiple liver enzymes and co-factors and enable both induction and inhibition studies in contrast to the supersomes. Liver microsomal preparations are routinely used to predict drug interactions that can occur *in vivo* as a result of inhibition of CYP-mediated metabolism. However, the concentration of free drug (substrate and inhibitor) at its intrahepatic site of

action, a variable that cannot be directly measured, may be significantly different from that in microsomal incubation systems (Di Marco et al., 2003). Microsomes have traditionally been viewed as a more flexible system with which to study oxidative biotransformations in terms of ease of preparation from many species, long-term storage and availability. Although storage of liver prior to microsomal preparation and the nature of the preparation itself can lead to a loss in CYP activity (H.C. Rawden, 2005). Cell cultures can consist of primary human hepatocytes or immortalised cell lines e.g. HepG2 (Yoshitomi et al., 2001) or BC2 (Gomez-Lechon et al., 2001) cell lines. Primary human hepatocyte cultures are the recommended model to study both induction and inhibition of CYP enzymes *in vitro* (LeCluyse, 2001). Hepatocytes provide the most physiologically relevant model when measuring qualitative and quantitative aspects of hepatic metabolism since they contain most of the enzymes a compound is likely to encounter in the liver. Additionally, interactions with transporter proteins present in hepatocyte membranes can be key determinants of hepatic clearance (Soars et al., 2007). However, the limited accessibility on human liver tissue favours the other models like recombinant CYP enzymes.

4.2.3 Validation of HPLC methodologies

Figure 9 show typical HPLC chromatograms after incubation with CYP3A4 (A) and CYP2D6 (B). The blank (top) contained no metabolite peaks neither for CYP3A4 nor CYP2D6 assays. The metabolite peaks were not interfered by the AVJ compounds (bottom) or other compounds in the assay (middle and top). Thus there was no need to extract the metabolites before the HPLC analysis.

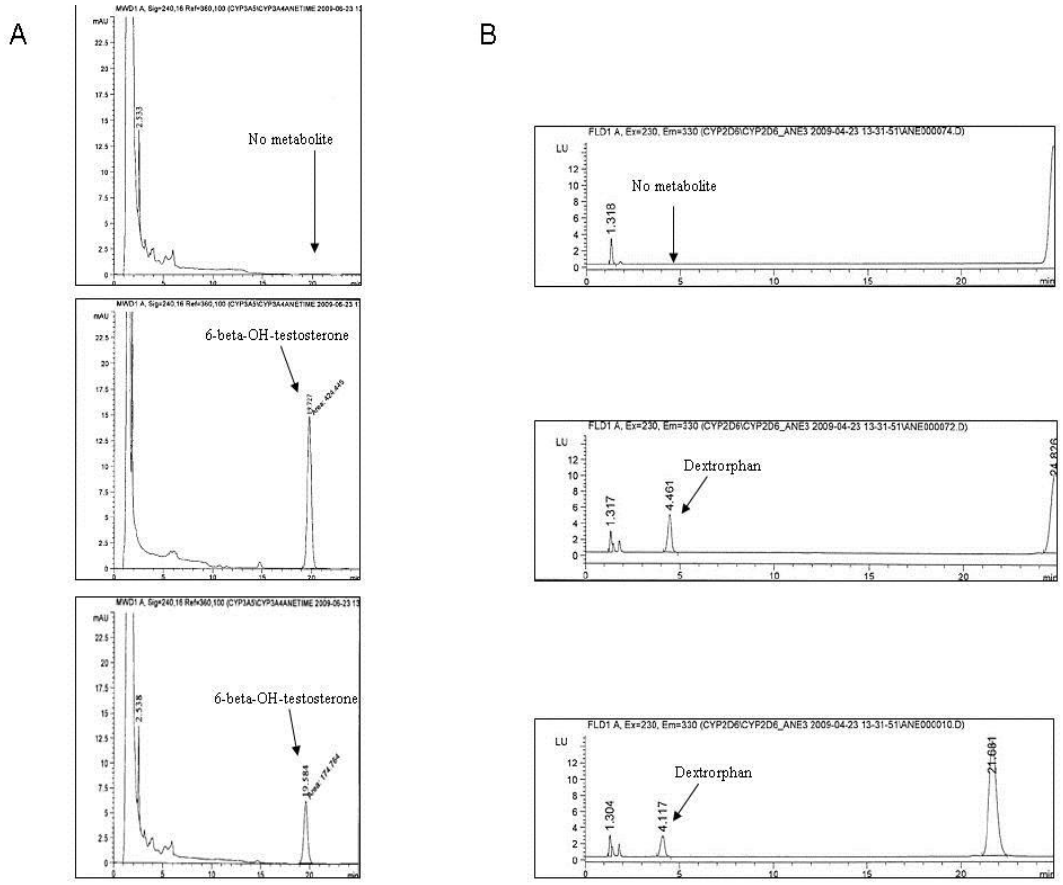

Figure 9. HPLC chromatograms for solutions incubated with CYP3A4 (A) or CYP2D6 (B) showing from the top blanks (no CYP enzymes), controls (middle) and AVJ (0.1 mg/ml) inhibitions (bottom), respectively.

CYP3A4

All points in the calibration curve correlated significantly to the curve ($r^2 \geq 0.99$). In pre-validation, the LLOQ was estimated to be 62.5 nM (CV 14.4%) and CVs of intra-day assays were all below 2.2% at low (75.0 nM), medium (3.0 µM) and high (6.0 µM) concentrations of 6-β-OH-testosterone (n=5). In addition, the inter-day CVs were all below 5.2%, for the same QCs analysed in three parallels for five days (n=15). The overall inaccuracy for all QCs was less than 10.0%. Furthermore, QCs were analysed for each HPLC run and the CVs of intra-day assays were all below 3.6% at low, medium and high concentrations of 6-β-OH-testosterone (n=2), while inter-day CVs were all <5.9%for all three concentrations (n= 6). Since the LLOQ had a precision <20%, overall accuracy of 80-120%, >5 times blank response and all QCs had a precision <15% our HPLC series were accepted.

CYP2D6

All points in the calibration curve correlated significantly to the curve ($r^2 \geq 0.99$). In pre-validation, the LLOQ was estimated to be 50 nM (CV 2.3%) and CVs of intra-day assays

were all below 2.6% at low (50.0 nM), medium (800 nM) and high (6000 nM) concentrations of dextrorphan (n=5). In addition, the inter-day CVs were all below 3.1%, for the same QCs analysed in three parallels for five days (n=15). The overall inaccuracy for all QCs was less than 14.0%. Furthermore, QCs were analysed for each HPLC run and the CVs of intra-day assays were all below 6.5% at low, medium and high concentrations of dextrorphan (n=2), while inter-day CVs were all below 9.5% for all three concentrations (n= 6). The lowest QC concentration should have been within 3 times of the LLOQ. The calibration curve contained both 10 nM and 50 nM, almost accepting the 10 nM as LLOQ. Presumably is 30 nM the correct LLOQ in our analytical runs. However, since the LLOQ had a precision <20%, overall accuracy of 80-120%, >5 times blank response and all QCs had a precision <15% our HPLC series were accepted.

5 Results and discussion

5.1 Co-use of herbal remedies and conventional drugs among GP patients.

A cross-sectional questionnaire was distributed to 402 patients in a GP office and 381 were returned (Paper I).

5.1.1 Prevalence of herbal use among patients in GP office

In addition to the likely difference between the prevalence in a GP population and the general population, the prevalence may also vary between countries and ethnic groups (Kuo et al., 2004; Raji et al., 2005) and makes comparison difficult. In addition, the prevalence in some studies may include all types of CAM (herbs, homeopathy, prayer etc.) or fish oil/vitamins and minerals, and not herbs alone (Kennedy, 2005; Barnes et al., 2008; Elolemy and Albedah, 2012) .

A total of 167 (44%) patients were currently using herbs, and nearly half of the patients used multivitamins or supplements. The prevalence of herb use is somewhat higher compared to other findings from general practice (36%) or primary care (22%) (Giveon et al., 2004; Kuo et al., 2004) and surprisingly close to the findings from a Norwegian cancer outpatient clinic where a similar questionnaire was used (46%) (Engdal et al., 2008). It is also in range of the prevalence of studies of the general population from other countries. The 2007 National Health Interview Survey (NHIS), USA, reported of herbal use in the general population of nearly 20% (Barnes et al., 2008). However, both the Czech and Saudi-Arabian population reports of higher herbal use (50-57%) compared to the US population (Elolemy and Albedah, 2012; Knotek et al., 2012). Due to the differences mentioned above, the variability in prevalence reported here and the lack of studies on herb use in GP practices, it is difficult to conclude whether or not the prevalence found in Paper I is high or low compared to other populations. However, the findings in Paper I lends support to the numerous other studies documenting a general high use of herbal remedies.

Elderly is a well-represented patient group in the GP office in addition to women, tending to go more frequently to the GP than young men (NDH, 2012). These groups are reported to be more prone to herbal use than younger patients or men and might explain our high prevalence (Barnes et al., 2008; Loya et al., 2009).

A significantly higher proportion of women were using herbs compared to men (51% herb user among women vs 29%, Table 1). In addition, polyherbacy were more frequent among women than men (80% of polyherbacy patients were women, p=0.009) and tended to be more frequent in the age group 50-59 years (31%, p= 0.059) (data not shown). For the other demographic variables there were no significant differences with regard to herb use. Multiple studies support the same tendency of higher prevalence of herb use among women compared to men (Barnes et al., 2008; Elolemy and Albedah, 2012; Knotek et al., 2012). Thus, although the prevalence seems to vary between countries it seems consistent that women use more herbal remedies than men.

Table1. Demographics of all respondents according to current herb use and co-use of conventional drugs (co-use).

		n (%)		**Current herbal user**	***p-value*[A]**	**Co-user**	***p-value*[B]**
Gender	Male	124	(33%)	29%	*<0.001*[C]	18%	*0.001*[C]
	Female	249	(67%)	51%		34%	
Age grouped	<30	50	(13%)	40%	*0.869*	20%	*0.008*[C]
	30-39	58	(16%)	45%		24%	
	40-49	52	(14%)	40%		12%	
	50-59	65	(17%)	51%		34%	
	60-69	71	(19%)	44%		38%	
	>70	76	(20%)	43%		37%	
Education	Compulsory	71	(19%)	42%	*0.943*	32%	*0.611*
	Middle level	170	(46%)	44%		28%	
	University	129	(35%)	44%		26%	
Employment	Employed/Off sick	233	(61%)	41%	*0.195*	21%	*<0.001*[C]
	Pension	129	(34%)	46%		40%	
	Unemployed/Home	12	(3%)	33%		58%	
Herbal use	Never	147	(40%)				
	Earlier	60	(16%)				
	Present	165	(44%)			66%	*<0.001*[C]

[A] P-value for comparison of current herb users with those not using herbs. Analysed with Pearson Chi-Square or Fisher exact test.
[B] P-value for comparison of co-users with non-co-users. Analysed with Pearson Chi-Square or Fisher exact test.
[C] p<0.05

5.1.1.1 Types of herbs used

A total of 28 different herbal remedies were registered used (Table 2). Bilberry (*Vacciunum myrtillus*, 41%), green tea (*Camelia sinensis,* 31%), garlic (*Allium sativum*, 27%), Aloe vera (*Aloe barbadensis,* 26%) and Echinacea (*Echinacea purpurea*, 18%) were the most commonly used herbs (Table 2). Except from bilberry, all other herbs are frequently reported by others (Bent and Ko, 2004; Barnes et al., 2008; Moussally et al., 2009; Cohen and Ernst, 2010). Bilberry is abounded of antioxidants and claimed to have anti-inflammatory activity (Karlsen et al., 2010). A sub-analysis of the reason for using bilberry revealed that it was used largely to strengthen the immune system (84% of bilberry user gave this as the reason). The use of bilberry might have been influenced by heavy marketing as a "superfood" (Renee, 2011).

Table 2 All the different herbal remedies used by the herbal user patients (n=214).

Herb	No.of users	Herb	No.of users
Bilberry	70	Ginkgo Biloba	4
Green tea	52	Soya extract	4
Garlic	46	Valeriana	4
Aloe vera	42	Nattokinase	2
Echinacea	29	Saw Palmetto	2
Cranberries	26	Broccoli extract	1
Ginger	19	Cinnamon	1
Ginseng	15	Essiac	1
Grapefruit	13	Healthy chocolate	1
Apple vinegar	11	Kan Jang	1
Golden root	10	Melissa	1
Evening Primrose oil/GLA	8	Misteltoe	1
Noni juice	5	Psyllium husk	1
Anthocyanin from bilberries	4	Rosehip	1

5.1.2 Communication about herb use

Friends or family (68%), followed by magazines or internet (32%) were common sources for recommendation or information regarding herb use (Table 3). This is in line with other studies (Martin et al., 2002; Molassiotis et al., 2005). Few patients got herbal use recommended by the pharmacy or a physician. This might be a reflection of the attitudes towards herbal remedies and CAM to be something "private", and patients trusting experiences from friends and family or anecdotes in magazines rather than scientific evidence (Verhoef et al., 2007).

Table 3: Communication with health care professionals about herbal use among current herb users and proportion of co-users of conventional drugs and herbs.

		Total *n* (%)	Proportion of co-users	*p-value*[A]
Recommended to use herbs by (n=110) [D]:	Friends or family	75 (68 %)	61 %	*1.000*
	The Physician	5 (5 %)	80 %	*0.647*
	The shop or pharmacy	32 (29 %)	59 %	*0.830*
	Read about it in Magazines or internet	35 (32 %)	69 %	*0.401*
Communication about herb use with (n=146) [C]:	Physician[D]	27 (18%)	74 %	*0.269*
	Other	10 (7%)	80 %	*0.324*
	Never discussed	113 (77%)	59 %	*0.104*
Reasons for never discussing herb use with health care professionals (n= 110):	I was never asked	50 (45%)	56 %	*0.723*
	Afraid of the response[F]	23 (21%)	65 %	
	Only my own concern/ confidential	34 (31%)	62 %	
	Uncertain of the herbal effect	3 (3%)	33 %	
Reasons for herb use (n=111) [C]:	Better life expectancies	47 (42%)	62 %	*1.000*
	Strengthen the immune system	79 (71 %)	58 %	*0.391*
	Defeat an illness	18 (16%)	89 %	*0.008*[B]
	Better than nothing	7 (6%)	29 %	*0.106*
	Pain relief	4 (4%)	50 %	*1.000*

[A] P-value for comparison of communication, motives for herbal use, adverse effects and costs between co-users of drugs and herbs and not co-users. Analysed with Pearson Chi-Square or Fisher's exact test given the number of total users were below five. [B] Significantly different with $p<0.05$. [C] Multiple answers were possible. [D] Includes GP, regular GP (family doctor) and hospital physicians.
[E] A merge of the responses «warned about the risk" and "discouraged use". [F] A merge of the responses "I was afraid of not getting acknowledgement for my choice", "I was afraid they got dissatisfied" and "I was afraid of being rejected".

Considering the literature, it was not surprising that the majority of herb users (80%) did not discuss their use of herbs with any health care professional (Table 3). The low degree of disclosure to health care professionals are similar to other studies where the most common reason for no communication about herbal use with a health care provider is; "I was never asked" (Eisenberg et al., 2001; Giveon et al., 2004). This indicates that the patients want the physician to take the initiative to disclose herbal use and this is also reported for other countries and continents (Giveon et al., 2004; Wheaton et al., 2005). One explanation why GPs do not ask about herb use might be that many GPs have a lack of knowledge about herbal remedies, adverse effects and potential interactions with conventional drugs and thus may find it difficult to give advice in such matters (Giveon et al., 2003). This might cause insecurity

among the health care providers, which seem to neither recommend nor discourage herbal use, thus a neutral response is more common as found in Paper I.

Even though the GPs might find themselves in shortage in knowledge of herb-drug interactions (Suchard et al., 2004), information about the patients herb use should be considered as "Good practise" and integrated as part of the general medical journal to promote good and safe medical treatment. The GPs needs be enabled to give advice about herb safety. Good and thorough guidelines in regard to this subject are also lacking, although some work has been initiated (EMA, 2012) more research is needed.

5.1.3 Prevalence and characteristics of co-users

Almost every third (29%) patient in this study co-used conventional drugs and herbal remedies, giving 45% co-use among those using conventional drugs regularly and 66% co-use among herbal users (Table 1). Reported co-use from GP offices in Israel in 2004 was lower (12%) (Giveon et al., 2004), however, up-to-date numbers from GP practice are lacking. Still, the co-use is in line with the co-use reported for patients groups like pregnant women (34%) (Nordeng et al., 2011) and somewhat lower than reported for the cancer patients (30-55%) (Molassiotis et al., 2005; Johansen and Toverud, 2006). Thus, our findings are in line with earlier reported co-use for patients groups.

Significant differences were seen between the gender, age and occupation groups in regard to co-use of drugs and herbs (Table 1). As earlier reported in other papers females were far more into herbal use than men (Ben-Arye et al., 2009) and were, not surprisingly, co-using herbal remedies and conventional drugs in a significant higher degree than men (Table 1). More than one of every three patients older than 50 years were co-users and this was significantly more than for younger patients ($p<0.001$). Those on disability or retirement pension (40%) co-used significantly more than those not receiving pension ($p<0.001$). The co-users also intended to defeat an illness in a significant stronger degree than non-co-users (89% vs 11%, $p=0.008$).

Based on multivariable analysis (Paper I, Table 6), the co-users had statistically significant increased odds to be female (adjOR 2.0), increasing age above 50 years (adjOR 1.3-3.3), using herbs to defeat an illness (adjOR 4.2), using two or more herbs (polyherbacy, adjOR 12.1), using analgesics or dermatological drugs (adjOR 5.1 and 7.9 respectively), and

experience adverse effects from herbs (adjOR 37.5). Being between 40 and 49 years old decreased the odds of being a co-user (adjOR 0.2).

The association between high co-use and increasing age above 50 years compared to the younger patients in our study (nearly 40% of those >70 years old were co-users), was not unexpected. Earlier studies report of co-use among elderly from 32-42% (Yoon and Horne, 2004; Shahrokh et al., 2005; Loya et al., 2009). Cohen et al. found co-use of 24% among geriatric patients, and 52% of them co-using with anticoagulants (Cohen et al., 2002). Elderly is an exposed group because of increasing poly-pharmacy, reduced general health and altered drug metabolism (Canter and Ernst, 2004; Loya et al., 2009). They have a lower tolerance for alterations in the pharmacokinetics or pharmacodynamics, which might have serious consequences (Gurley et al., 2005; Ulbricht et al., 2008; Yang et al., 2010; van den Berg et al., 2011). The strong association for co-use with increasing age, polyherbacy and having one or more medical condition, calls for concern. These patients are at high risk for clinically relevant herb-drug interactions (Abebe, 2003; Yang et al., 2010).

5.1.4 Adverse effects of herb use

Although the numbers were low, all of those who had experienced adverse effects of herbs were co-users (8 patients (7%), p=0.026). Figure 10 shows the herbs reported used by patients experiencing adverse effects. Among the co-users experiencing adverse effects, three of them were using anticoagulants, three used sedatives and two used antihypertensives. Abdominal symptoms (pain, diarrhea, emesis, 33%) or dizziness (22%) were the most common reported adverse effects. In a recent paper from Beirut as much as 60% of the co-users reported some sort of adverse effects (Efferth and Kaina, 2011; Alaaeddine et al., 2012). Although our reported prevalence is low, those reporting adverse

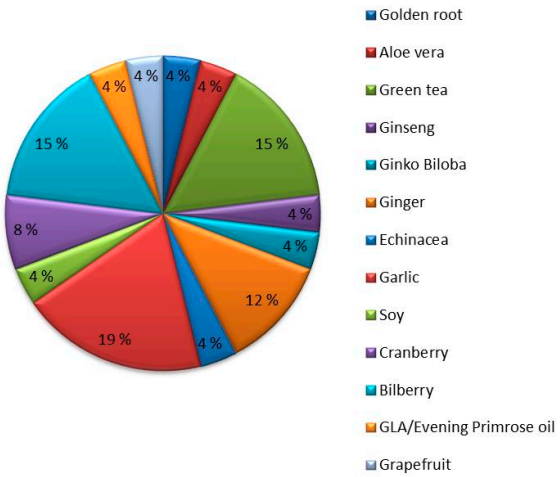

Figure 10. Relative percentage of the frequency herbs reported used among those patients experiencing adverse effects (n= 8). Garlic (19%), bilberry (15%) and green tea (15%) had the highest frequency of a total number of 26 (100%) herbal adverse effects reports.

effects were co-using herbs with reported additive effects (e.g. anticoagulants and garlic) which might cause excessive bleedings (Cohen and Ernst, 2010). Thus, the GP should consider herbal co-use with conventional drugs when patients describe adverse effects, at least when they are unexpected.

5.1.5 Herb-drug combinations used by GP patients

5.1.5.1 Herbs combined with drugs

For nearly all the types of herbs used, there were no significant difference between the types of herbs used when comparing those who co-used conventional drugs and those who did not use conventional drug (Paper I, Table 2). Bilberry (43%), green tea (31%), garlic (25%), Aloe vera (21%) and cranberry (19%) were the most frequently used herbs among the co-users in this study. In addition, the co-users tended to use more Aloe vera than not conventional drug users ($p=0.091$), and almost two of three (63%) of the polyherbacy patients were using conventional drugs concomitantly ($p<0.001$).

Except from a recent case report indicating an interaction between bilberry and warfarin, inducing rectal bleeding, few interaction data are published on bilberry (Aktas C, 2011). Thus, attention should be paid to the intake of bilberry in patients taking antiplatelet or anticoagulant drugs. Green tea has shown antioxidant properties which are mainly attributed to its polyphenolic catechins (Schonthal, 2011). Green tea and it`s compounds have also been reported to alter the pharmacokinetics of several drugs as warfarin and codeine, and may be hepatotoxic in large doses (Schonthal, 2011). Garlic might have antiplatelet activity and should thus, be used with care together with antiplatelet drugs like warfarin (Shord et al., 2009; Cohen and Ernst, 2010). Excessive bleeding has been reported in patients co-using warfarin and garlic, a patient group frequently using garlic (Chan et al., 2011). Aloe vera might cause potassium depletion or affect cardiac glycosides and is advised not to be used together with heart medication (Cohen and Ernst, 2010). However, no *in vitro* or *in vivo* clinical relevant pharmacological interactions have yet been established (Djuv and Nilsen, 2008; Cohen and Ernst, 2010; Djuv and Nilsen, 2012). Cranberry is reported to interact with warfarin, increasing International Normalized ratio (INR) values by 30% (Mohammed Abdul et al., 2008), but a randomized controlled trial concluded with minor risks for significant interactions in humans (Ansell et al., 2009). In addition, some reports state that garlic, green

tea, Aloe vera and cranberry in general seem to have a low drug interaction risk in humans (Ansell et al., 2009; Gurley et al., 2012).

5.1.5.2 Drugs combined with herbs

It was identified 255 different drug-group and herb combinations (Table 4). Of these, 18 were identified of being at risk of clinically relevant interactions (Table 4, in bold) on the basis of clinical trials, case reports or theoretical interactions extrapolated from clinical data (Cvijovic, 2009; Gurley et al., 2012). A significant higher herbal use were reported by patients using analgesics (60%, p=0.031) or anticoagulants (36%, p=0.043) among the drug users (Paper I, Table 3).

Anticoagulants (i.e. warfarin) were co-used with garlic (*Allium sativum*), cranberry (*Vaccinium oxycoccos*), ginger (*Zingiber officinale*), ginseng (*Panax ginseng*), grape fruit juice (*Citrus paradisi*) and saw palmetto (*Serenoa repens*) (Table 4), all interacting with anticoagulants increasing the risk of adverse effects (e.g. increased haemorrhage) (Izzo, 2005; Mohammed Abdul et al., 2008; Ulbricht et al., 2008; Smith et al., 2010; Chan et al., 2011; van den Berg et al., 2011). Anticoagulants have a high potential of causing harmful adverse effects when co-used with herbs (Smith et al., 2010; Chan et al., 2011) and , thus, the Norwegian Medicines Agency (NoMA) has warned against interactions between certain types of herbs and warfarin (NoMA, 2013). In general co-use of these herbs with anticoagulants or other cardiovascular drugs should be discouraged or closely monitored for adverse effects / INR (Cohen and Ernst, 2010; Chan et al., 2011). Co-use should especially be closely monitored or even discouraged among the elderly (Gurley et al., 2005).

Table 4. Concomitantly use of herbs and conventional drug-groups.

	Against gastrointestinal conditions	Analgesics	Antibacterial, antifungal and antiviral	Anticoagulants	Antidepressants	Antidiabetics	Antihistamines	Antihyperlipidemic agents	Antihypertensives and diuretics	Anti-menopausal and anticonceptives	Antirheumatic	Antiseizure, triptanes and central stimulating drugs	Chemotherapeutic drugs	Dermal drugs	Drugs used against bladder, prostate disorders and impotence	Ocular drugs	Respiratory drugs	Sedatives and Antipsychotics	Strong analgesics	Thyroids and antithyroids	Vasodilators and cardiac glycosides	Other drugs	Number of combinations
Aloe vera	**3**[A]	6		1	2	**2**	5	5	7	3	2	2		2	2	2	4	1		**4**	1	2	19
Anthocyanin		2				1	1	2	2	1	1	2								1			9
Apple vinegar	1	3	1		1	1	1	1	3			1				1	1			2		2	13
Bilberry	4	15	1	18	5	5	4	2	24	1	5	4	2	3	1	2	4	8		6	2	4	21
Cinnamon		1				1	1	1	1		1									1			7
Cranberry	2	9	1	**1**	2	1	2	2	5	1	1	3		1	3	1	4	4	1	3		1	20
Echinacea	2	4	1	1	3	1	3	5	5	2	1	1		1		1	1			4		2	17
Garlic	2	**8**	**1**	**9**	3	4	3	7	11	2	3	3		2		1	3	6		2		2	18
Ginger	1	2		**1**	3		2	1	3	1	1					1	2	1		2		1	14
Gingko Biloba	**2**	1					2				1					1	2			2		1	8
Ginseng	**1**	1	1	**1**	**2**	**1**	1	3	**2**		2	1					1	1		2		1	15
Evening Primrose oil/ GLA		1	1		1		1	1	1		1	1								1			9
Golden root	2	4	1	1	2	1	1	2	3		2	1				1	1	2		2		2	16
Grapefruit	1	3	1	**1**		2	1		**2**			1				1	2	1		1		2	13
Green tea	4	11	1	7	5	5	5	8	11	4	5	3	2	2	1	1	3	6		4		6	20
Healthy chocolate																				1			1
Kan Jang								1	1	1		1											4
Nattokinase		1								1													2
Noni juice						1		1	1														3
Rosehip				1				1	1	1								1					5
Saw Palmetto	1	1		**2**				2	1						1	1							7
Soya extract		1		1	1	1	1	2	2		1									1			9
Valeriana		1			**2**			1	2					1									5
Number of combinations	**13**	**19**	**10**	**13**	**13**	**14**	**16**	**19**	**20**	**11**	**14**	**13**	**2**	**7**	**5**	**12**	**12**	**10**	**1**	**17**	**2**	**12**	**255**

[A] Bold numbers: Clinical relevant interactions documented in clinical trials, case reports or theoretical interactions extrapolated from clinical data.

Antihypertensives and diuretics were the largest drug categories in regard to number of combinations with different herbal remedies in Paper I (Table 4), having interaction potential with ginseng or grapefruit juice (Ulbricht et al., 2008). Ginseng is also reported to interact with antidiabetics, cardiac glycosides, antidiarrheal agents and antidepressants (Ulbricht et al., 2008; Cvijovic, 2009). Anti-constipation drugs or antidiabetic agents should not be consumed with Aloe vera because of additive effects and the same has been shown for valeriana (*Valeriana officinalis*) co-used with antidepressants (Carrasco et al., 2009; Cvijovic, 2009).

Co-use of garlic with NSAIDs (e.g. acetylsalicylic acid (Aspirin™), ibuprofen (Ibux™) etc.), anti-retroviral therapy or antidepressants have also been reported to give clinically relevant interactions (Ulbricht et al., 2008; Cvijovic, 2009). In addition to garlic, NSAIDs is known to interact with many herbs (i.e. ginkgo, ginger, bilberries, and ginseng). Another analgesics, paracetamol, showed decreased *in vitro* metabolism when co-used with *Coriolus versicolor* used in traditional Chinese herbal medicine (Abebe, 2002; Ulbricht et al., 2008; Yeung and Or, 2012). Keeping in mind that nearly half (44%) of the co-users used two or more herbs (polyherbacy), the risk of interactions or additive effects are present.

5.1.6 Clinical consequences'

Although there are some characteristics of the co-users (female, elderly etc.) no specific variables can unfortunately be used by the GP to pin-point co-users. This is also the case for the comparable study (Giveon et al., 2004). The large portion of elderly patients and patients on anticoagulants co-using herbs is alarming since these groups are vulnerable to interactions (Loya et al., 2009). In addition the strong association for co-use with increasing age, polyherbacy and having one or more medical condition, calls for concern. These patients are at high risk for clinically relevant herb-drug interactions. Given the under-communication with GPs about co-use, it is difficult to prevent unwanted adverse effects and interactions. In order to monitor co-use, all GPs should ask their patients routinely to disclose their use of herbs.

5.2 In vitro studies

5.2.1 Herb selected

AVJ is an old and a well-known herb and is approved by Norwegian Medicines Agency as herbal medicine against constipation (NoMA, 1999). In contrast to some other studies extracting an assumed active component from the herb (Ohta et al., 2002; Girennavar et al., 2006), a "whole herb" extract from the AVJ was made to resemble the *in vivo* situation as much as possible. The AVJ was kept cold and dark to avoid any harmful degradation of the juice components during storage and was centrifuged to avoid aloe fibres. The supernatants were freeze dried to avoid degradation, and redissolved in water and a small amount of DMSO or ethanol as possible to solute the AVJ compounds. Ethanol were used in the CYP

assays because DMSO may inhibit CYP3A4 and CYP2D6 metabolism even at low concentrations (Busby et al., 1999).

In Paper II two different AVJs were investigated because of the unlike compositions of the juice components depending on soil, harvest season, processing etc. The two juices, juice 'A' and 'B', and some of their compounds are illustrated in figure 11. It should be notice that the concentration of the presumed active components was different in the two commercially available products.

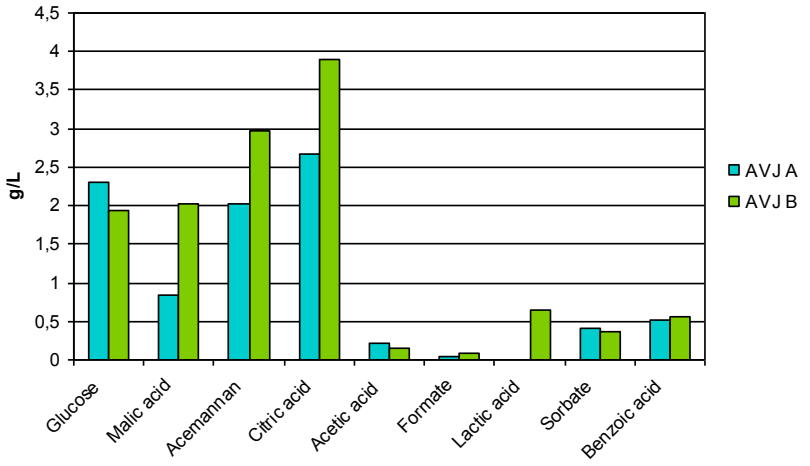

Figure 11. Concentrations of some components in AVJ 'A' and 'B'. Data from The Norwegian National Food authorities (Falch JB, 2000).

5.2.2 CYP inhibition

In Paper II we investigated the inhibition potential of two different AVJs, AVJ 'A' and AVJ 'B', towards recombinant CYP3A4 or CYP2D6 metabolism of testosterone or dextromethorphan, respectively. KTZ or quinidine was used as positive inhibitor controls, respectively (McLaughlin et al., 2005; Zhou et al., 2005). The potential of AVJ to participate in a mechanistic based inhibition of CYP3A4 or CYP2D6 metabolism was explored.

Apart from an Swedish report finding an small induction of CYP3A4 *in vitro* (Brandin et al., 2007) and a publication of one single component in AV, rhein, inhibiting both CYP3A4 and CYP2D6 *in vitro* (Tang et al., 2009) no inhibition data have been published neither of other

single constituents or of more complex AVJ extracts on CYP3A4 or CYP2D6 activities. Still, rhein is just one of several compounds in AVJ and whole herb AVJ extracts were investigated further for other inhibition patterns.

Both AVJ 'A' and AVJ 'B' inhibited the CYP3A4 metabolism of testosterone with IC_{50} values of 8.35±0.72 and 22.4±5.4 mg/ml, respectively. The IC_{50} values were, however, high and the AVJ 'B'´s IC_{50} value was approximately 2-fold higher than for AVJ 'A'. The difference was considered significant as no overlap was found in their 95% confidence intervals. This might be due to different levels of inhibitory compound(s) in the two AVJ extracts. The assumed main active constituent in AVJ, acemannan, is however, present in 1.5 as high concentration in AVJ 'B' as in AVJ 'A', 2.98 and 2.03 mg/ml (Falch and Lorås, 2000). Thus acemannan is probably not involved significantly in the CYP3A4 inhibition observed. The levels of rhein in AVJ 'A' or AVJ' B' are not known.

Furthermore, the AVJs IC_{75}/IC_{50} ratios were much lower than compared to the inhibitor controls` IC_{75}/IC_{50} ratios indicating a steeper decrease in the AVJ inhibition curve witch might suggest a different inhibition mechanism of AVJ on CYP3A4 or CYP2D6 than by KTZ or quinidine, which are reversible CYP3A4 or CYP2D6 inhibitors (Bertelsen et al., 2003; Zhou et al., 2005).

5.2.2.1 Mechanism-based inhibition

To reveal if the AVJ inhibition of CYP3A4 and CYP2D6 is mechanism-based, NADPH-, and time-dependent characteristics must be fulfilled (Zhou et al., 2005). As AVJ 'A' was identified as the most potent inhibitor of both CYP3A4 and CYP2D6 activities, this product was used in the mechanism-based inhibition assays.

AVJ caused a time-dependent decrease of both CYP3A4 and CYP2D6 activities when pre-incubated in the incubation mixture for increasing time intervals before substrate was added. A strong increase in CYP3A4 inhibition was seen after 15 min. of pre-incubation which gives a high initial inhibition velocity with a decline thereafter (exponential curve). The CYP2D6 showed however a linear decline in activity when preincubated with AVJ. This might implicate different affinities of a reactive metabolite or constituent from AVJ for the two different enzymes (CYP apoprotein and/or haem) or different reactive metabolite/constituent

inhibiting either CYP3A4 or CYP2D6. The drug ethynylestradiol has been documented to modify both the haem and the apoprotein of CYP3A4 (Lin et al., 2002). Thus this might be the explanation of the rapid decline (90% inhibition) in CYP3A4 activity after 15 min preincubation with AVJ.

Pre-incubation with NADPH gave a significant decrease in both CYP3A4 and CYP2D6 activities. The conversion of the inhibitor, AVJ, to a reactive metabolite thus requires NADPH. Irreversible inhibitors require at least one CYP catalytic cycle to form a reactive metabolite which results in irreversible modification of the haem, the apoprotein or both, and thereby decreasing the amount of working CYP enzymes (Zhou et al., 2005). Hence, AVJ pattern of inhibition is consistent with mechanism-based inhibition (irreversible) for both CY3A4 and CYP2D6. In addition, significant reductions in CYP activity were also seen for both CY3A4 and CYP2D6 without the presence of NADPH. These might result from CYP degradation or inhibition of some other components formed in the incubation solution during the pre-incubation not requiring NADPH.

5.2.3 P-gp inhibition

In paper III, AVJ was evaluated for its inhibition potential on the efflux of digoxin (30 nM) by P-gp. As mentioned previously, an alteration in P-gp expression or efflux rate may change the drug bioavailability (Kannan et al., 2009). Another perspective to the inhibition studies is the increasing trend to optimize pharmacokinetics and drug delivery by identification of P-gp inhibitors as part in the identification of new effective cancer treatment (Varma et al., 2006). Such an inhibitor might be important in future chemotherapy and might as well as a drug, be an herbal remedy.

AVJ did neither influence the A-B nor the B-A transport of digoxin at 90 min., thus no IC_{50} value was achieved on P-gp transport of digoxin for this herb (Paper III). The Papp values for digoxin (control) at 90 min for the B-A transport was 6.5 x 10^{-6} cm/s and A-B transport 1.5 x 10^{-6} cm/s corresponding earlier reported values (Xu et al., 2003). Digoxin is claimed to be a specific P-gp substrate (Fromm et al., 1999; Kim et al., 1999). However, organic anion transporting polypeptides (OATP, SLCO1A2) has lately been discussed to take part in the digoxin transport, but this has been falsified by a more recent report (Taub et al., 2011). Thus, digoxin has still the dominating role as substrate in P-gp inhibition assays on Caco-2 cells

(Zhou, 2008; Mease et al., 2012). Furthermore, was the digoxin transport inhibited by 80-90% by verapamil, the positive inhibition control, which was in agreement with most of the earlier reported values (Keogh and Kunta, 2006). Our Caco-2 cell system thus fulfilled the demands made by Hildalgo et al. (Hidalgo, 2001) and should hence make interlaboratory comparisons feasible.

In addition, cell viability, integrity and toxicity screening were tested by TEER measurements, mannitol transport experiments and MTT-assay. Thorough quality screening of the Caco-2 cells as such has been emphasized by several authors (Artursson, 1991; Hidalgo, 2001; Shah et al., 2006). TEER was measured also immediately after ended transport assay and 88 % of the wells had TEER values > 200 Ω/cm^2 also after the termination of the transport assay. These wells were included in the analysis. Although there are some interlaboratory variations in TEER measurements, our measurements were in line with other measurements preformed under similar conditions (Hidalgo et al., 1989; Markowska et al., 2001). TEER measurements after the assay in addition to pre-incubation measurements secures a better quality control of the assay, and should be implemented as a standard control in all Caco-2 cell assays.

Surprisingly, a statistically significant linear reduction in lactatdehydrogenase (LDH) activities was seen for digoxin concentrations ≥3 μM. Thus, a concentration of 30 nM could safely be applied on these cells. This has not to our knowledge been described previously. In addition gave AVJ a statistically significant decrease (38%) in LDH activity at 10.0 mg/ml compared to control, resulting in exclusion of AVJ concentrations above 1 mg/ml in the transport assay.

5.2.4 Clinical consequences'

The *in vitro* AVJ 'A' or AVJ 'B' IC_{50} values on CYP2D6 or CYP3A4 metabolism are presumably too high to imply any clinical drug metabolic interactions *in vivo*. However, intracellular inhibitor accumulations are shown (Di Marco et al., 2003), making an *in vivo* interpretation somewhat difficult. Since the most potent juice, AVJ 'A', had an IC_{25} value of 4.7 mg/ml towards the CYP3A4 activity this might imply a possible *in vivo* metabolic interaction potential in the small intestines as the daily recommended intake of AVJ is as high

as 50 ml (Appiah-Opong et al., 2008). This can have implications on drug metabolism of CYP3A4 substrates like methadone (analgesics) or clopidogrel (anti-platelet agent). Clinical significant inhibition of CYP2D6 metabolism might occur in CYP2D6 poor metabolisers at high AVJ concentrations (> 10.0 mg/ml), leading to *in vivo* interactions, especially with drugs that have narrow therapeutic index e.g. tricyclic antidepressants (TCAs). However, these hypothetical interactions require a very high AVJ consumption and are not likely to take place *in vivo*.

Our findings from a validated Caco-2 cell system showed no statistically significant inhibitory effects of AVJ on the P-gp mediated transport of digoxin, even though a wide range of anticipated relevant physiological concentrations of AVJ were tested. This might indicate that AVJ in clinically relevant concentrations, do not possess any significant inhibitory potency towards the P-gp mediated efflux transport of digoxin and similar P-gp substrates.

6 Summary of the results

The questions in aims (Chapter 2) can now be answered.

6.1 Co-use among GP patients

The following questions were asked in regard to the study among GP patients in Norway:

1. Do Norwegian GP patients use herbal remedies and if so, to what extent?
    - *Yes*, 44% of the GP patients used herbal remedies at the time.
2. Are some herbs more commonly used than others?
    - *Yes*, bilberry, green tea, garlic, Aloe vera and Echinacea were the most commonly used herbs.
3. Which motives do they have for use?
    - Over 70% did report to use herbs to improve the immune system.
4. Do the patients disclose their herbal use to the GP or other health care providers?
    - *No*, few (25%) reported to use herbal remedies to their physician or other health care providers.
5. Do any of the herb users also use conventional drugs on a regular basis; if so was there any difference between the drugs?
    - *Yes*, two of three herb users also used regularly conventional drugs (co-users).
    - Yes, a significant higher herbal use was reported by patients using analgesics (60%) or anticoagulants (36%).
6. Which herbs and conventional drug combination can be identified, and are any of those at risk of being harmful to the patient?
    - *Yes*, 255 herb-drug-group combinations were identified and 18 these were considered to be clinically relevant in regard to interactions. Co-use of herbs like garlic, cranberry, ginseng, grapefruit and saw palmetto with anticoagulants or other cardiovascular drugs should be discouraged or closely monitored for adverse effects/INR.
7. Do they experience any adverse effects of herbal use?
    - The 8 patients (7%) who reported adverse effects of herbs were all co-users ($p=0.026$). Abdominal symptoms (pain, diarrhea, emesis, 33%) or dizziness (22%) were the most common reported effects.

8. Is there any common characteristics of those who co-use herbs and conventional, regular drugs and can the GP use them to pin-point those at risk?
    - *Yes*, there were statistically significant increased odds for co-users to be female, increasing age above 50 years, using herbs to defeat an illness, polyherbacy, experience adverse effects of herb use and use of analgesics or dermatological drugs.
    - *No,* high co-use were also seen for other groups, and thus, the GPs are advised to ask all patients about herbal use.

6.2 AVJ in vitro studies

The questions addressed in the aims section for *in vitro* studies can now be answered:

1 Is the Caco-2 cell model functioning in our laboratory as a reliable and reproducible system for evaluating P-gp transport and interactions?
    - *Yes*, our Caco-2 cells are in line with other laboratories with regards to transport linearity and cell integrity (mannitol and TEER).

2 Is Aloe vera toxic to the Caco-2 cells?
    - *No*, AVJ is not toxic to the Caco-2 cells unless in extreme doses (>10 mg/ml).
    - Interesting finding was digoxin toxicity ≥3 μM.

3 Does Aloe vera inhibit P-gp, if so to what extent?
    - *No*, P-gp was not inhibited by AVJ in the range investigated *in vitro*.

4 Does Aloe vera inhibit CYP3A4, if so to what extent?
    - *Yes*, the AVJ inhibited CYP3A4. The IC_{50} values were, however, too high to imply any clinical AVJ-drug interactions in humans.

5 Does Aloe vera inhibit CYP2D6, if so to what extent?
    - *Yes*, the AVJ inhibited CYP2D6. The IC_{50} values were, however, too high to imply any clinical AVJ-drug interactions in humans.

6 Does Aloe vera inhibit CYP3A4 and CYP2D6 by mechanistic inhibition?
    - Yes, AVJ inhibited both CYP enzymes mechanistically.

7 Is there a difference between different commercial Aloe vera juice products in their ability to inhibit CYP3A4 and CYP2D6 mediated metabolism?
    - *Yes*, the AVJ'A' and AVJ 'B' showed statistically significant different inhibition potential towards both CYP3A4 and CYP2D6.

8 Does Aloe vera possess a clinical relevant interaction potential towards other drugs?

- *No*, the AVJ must be considered as a weak inhibitor of both CYP3A4 and CYP2D6 because of the high IC_{50} values. AVJ co-use with drugs metabolised of both CYP3A4 and CYP2D6 with narrow therapeutic window (e.g. antidepressants) or a high AVJ consumption in CYP2D6 PMs may, however, have clinical implications. Even so, such extrapolations should be made with reservations.

7 Conclusions

It can be concluded that GP patients co-using drugs and herbs and that this use can give clinical relevant interactions (e.g. excessive haemorrhage when co-using garlic and warfarin). Elderly patients are most vulnerable for co-use. One of the common used herbs among GP patients, Aloe vera, was investigated for *in vitro* pharmacokinetic interactions on the enzymes P-gp, CYP3A4, CYP2D6. Although it was concluded with low possibility of clinical relevant pharmacokinetic interactions co-using Aloe vera and drugs, patients with poor CYP2D6 activity might risk interactions when co-using large quantities of Aloe vera with conventional drugs which is metabolized of CYP2D6 (e.g. codeine). Clinical *in vivo* studies are needed to reveal any interactions in humans for Aloe vera and other herbs at risk of herb-drug interactions. Until then, the GPs and other health care professionals are advised to ask all patients about herbal use.

8 Further perspectives

One of the outcomes of this thesis could have been advices to GPs about how they could identify co-users in a simple way. In paper I it was concluded that this was difficult because of a general high co-use in all groups, and although women and elderly were more associated with co-use than others, these groups are to indistinct to be of any practical value. Because of the high prevalence of herb use and co-use of herbs and conventional drugs, the GP needs to ask all patients about herbal use.

The number of included patients in paper I was too low to conclude with regard to any harmful herb-drug combinations. A larger multicentre cross-sectional questionnaire based study is thus needed to identify the most problematic herb-drug combinations as well as identify patient groups prone to co-use. The herb-drug combinations should again be investigated further both *in vitro* and *in vivo* for clinically relevant herb-drug interactions.

As more and more of herbal remedies are investigated for *in vitro* drug interaction, the urge for *in vivo* studies is still present. Few clinical trials have been performed on herb-drug interactions in humans and most of them are of poor scientific value (small and not randomized). Aloe vera seems overall safe in regard to *in vitro* drug interaction from Paper II and III, although few other interaction studies have been made. The result of this thesis thus support that Aloe vera can be classified as possibly safe for oral administration for a brief period of use. However, a clinical trial with Aloe vera juice and conventional drugs (e.g. warfarin) should be performed to exclude any human clinically relevant pharmacodynamic or pharmacokinetic interactions.

9 References

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10 Appendix

Senter: | 0 | 2 | Pasient nr: | | | | |

Bruk av naturmidler blant pasienter på et allmennlegekontor.

Praktisk om spørreskjemaet.

Spørreskjemaet består av tre deler. I første del ønsker vi å få vite noe bakgrunnsinformasjon om deg. I del to spør vi om du bruker eller har brukt naturmidler. I siste del ønsker vi å vite litt om hvordan du og legen din forholder dere til naturmidler og hvorfor du bruker eller ikke bruker naturmidler.

Spørreskjemaet du har fått utdelt kan du enten fylle ut med en gang eller hjemme hvis det passer deg bedre. Skjemaet behandles konfidensielt, men er spørsmålene vanskelige å forstå vil vi være behjelpelige med en forklaring.

Når du er ferdig med skjemaet leverer du det i luka eller til legen. Vil du fylle ut skjemaet hjemme, kan du få med deg ferdig frankert konvolutt. Spørreskjemaet ditt vil bli bearbeidet av Ane Djuv ved Det medisinske fakultet i Trondheim.

Vennlig hilsen

Ane Djuv

DEL 1:
Bakgrunnsinformasjon om deg:

Skjemaet ble utfylt:

Dato		Måned		År			

1. Kjønn:

   1 ☐ Mann
   2 ☐ Kvinne

2. Hvor gammel er du?
   ☐☐ År

3. Hvilken utdanning er den høyeste du har fullført? (Bare ett kryss)

   1 ☐ Grunnskole 7-10 år, framhaldsskole, folkehøgskole
   2 ☐ Realskole, middelskole, yrkesskole, 1-2 årig videregående skole
   3 ☐ Artium, gymnas, allmennfaglig retning i videregående skole
   4 ☐ Høgskole/universitet, mindre enn 4 år
   5 ☐ Høgskole/universitet, 4 år eller mer

4. Hva slags arbeidssituasjon har du nå? (Bare ett kryss)

   1 ☐ I arbeid
   2 ☐ Sykemeldt
   3 ☐ Alderspensjon
   4 ☐ Uførepensjon
   5 ☐ Arbeidsledig, permittert
   6 ☐ Hjemmearbeidende/værende

5. Bruker du faste medisiner (eks. tabletter, stikkpiller, salver, plaster etc.)?

[1] ☐ Ja
[2] ☐ Nei
[3] ☐ Vet ikke

6. Hvis du svarte ja i spørsmålet over (nr 5) bruker du noen av følgende medisiner nå (krysse av en eller flere):

☐ Astma og/eller KOLS medisiner (eks. Seretide, Serevent, Symbicort, Atrovent, Ventolin)
☐ Allergimedisiner (eks. Cetrizin, Livostin, Eurax, Flutide, hydrokortison, Locoid)
☐ Blodtrykksenkende (eks. Selo-Zok, Metoprolol, Captopril, Amlodipin, Emconcor)
☐ Blodfortynnende (eks. Marevan, Albyl-E, Plavix)
☐ Mot høyt blodsukker (eks. Insulatard, Glucophage, Humalog, Actrapid, Metformin)
☐ For stoffskifte (eks. Levaxin, Euthyrox, Liothyronin)
☐ For reumatiske og/eller autoimmune plager (eks. Confortid, Prednison, Metotrexat)
☐ Mot mage-tarm sykdommer (eks. Somac, Duphalac, Lactulose, Afipran)
☐ Mot migrene, epilepsi, ADHD (eks. Imigran, Anervan, Zomig, Ritalin)
☐ Medisin mot kreftsykdom (eks cellegift, Herceptin, Megace, Zoladex, Casodex)
☐ Kolesterolsenkende (eks Simvastatin)
☐ Hjertekramper/ angina (eks Nitroglycerin, Monoket)
☐ Smerter (eks. Ibux, Paracet, Paralgin Forte, Voltaren, Napren-E, Nobligan, Tramadol)
☐ Sterke smerter (Kodein, Dolcontin, Temgesic, Norspan plaster)
☐ Overgangsalder (eks. Ovesterin, Activelle, Trisekvens, Eviana, Novofem)
☐ Hudsykdom/plager (eks Betnovat, Daivonex, Neotigason, Diprosalic, Skinoren, Epiduo)
☐ Øyedråper mot øyesykdom (eks. Pilokarpin, Betoptic, Spersallerg)
☐ Antibakteriell-, antiviral-, antifungal behandling (eks. Indinavir, antibiotika, Canesten)
☐ Beroligende og søvndyssende (eks. Vival, Valium, Sobril, Stesolid, Imovane, Stilnoct)
☐ Anti-depressiva (eks. Cipramil, Cipralex, Seroxat)
☐ Anti-psykotika (eks.Haldol, Risperdal, Nozinan, Clozapine, Trilafon, Cisordinol)
☐ Antikonsepsjon (eks. P-piller (Yasmin, Microgynon), minipiller (Cerazette), p-ring)
☐ Urinveis- og eller blæreplager (eks Proscar, Hiprex, Detrusitol, Vesicare)
☐ Impotens (eks Viagra, Sildenafil)
☐ Vanndrivende/ diuretika (eks. Furix, Centyl, Cozaar, Diovan)
☐ Annet:_____

DEL 2:

7. Bruker du eller har du tidligere brukt noen naturmidler, generelt eller i forbindelse med sykdom?

Kryss av for om du bruker naturmidlene nå (siste 7 dager), om du har brukt naturmidler tidligere (ved sykdom) eller om du ikke bruker. Før på flere hvis det er nødvendig. Bruk gjerne baksiden hvis det er nødvendig.

	Jeg bruker nå (siste 7 dager)	Jeg bruker ikke nå, men har brukt tidligere	Jeg bruker ikke nå, men har brukt tidligere (ved sykdom)	Jeg bruker ikke
	1	2	3	4
Natto K2	☐	☐	☐	☐
Agaricus	☐	☐	☐	☐
Misteltein	☐	☐	☐	☐
Noni juice	☐	☐	☐	☐
Rosenrot	☐	☐	☐	☐
Johannesurt	☐	☐	☐	☐
Valeriana	☐	☐	☐	☐
Haibrusk	☐	☐	☐	☐
Aloe vera	☐	☐	☐	☐
Grønn te	☐	☐	☐	☐
Essiac	☐	☐	☐	☐
Ginseng	☐	☐	☐	☐
Ginko biloba	☐	☐	☐	☐
Ingefær	☐	☐	☐	☐
Solhatt	☐	☐	☐	☐
Hvitløk	☐	☐	☐	☐
Soyaekstrakt	☐	☐	☐	☐
Tranebær	☐	☐	☐	☐
Blåbær	☐	☐	☐	☐
Epleeddik	☐	☐	☐	☐
Q10	☐	☐	☐	☐
GLA/nattlysolje	☐	☐	☐	☐
Grapefrukt	☐	☐	☐	☐
Multikosttilskudd/vitaminer *	☐	☐	☐	☐
Annet:	☐	☐	☐	☐

*(eks.VitaePro, AvantGarden, Immunoplex, B17)

8. Om du bruker naturmidler nå, hvor ofte tar du naturmidlene?

Kryss av for hvor ofte du bruker naturmidlene fra spørsmål 6: daglig, flere ganger i uken, ukentlig, ikke hver uke, men minst hver 14.dag, ikke hver 14. dag, men minst en gang i måneden, sjeldnere enn en gang i måneden eller periodevis.

Naturmiddel:	Daglig 1	Flere ganger i uka 2	Ukentlig 3	Minst hver 14. dag 4	Minst en gang i måneden 5	Sjeldnere 6	Periodevis 7
Natto K2	☐	☐	☐	☐	☐	☐	☐
Agaricus	☐	☐	☐	☐	☐	☐	☐
Misteltein	☐	☐	☐	☐	☐	☐	☐
Noni juice	☐	☐	☐	☐	☐	☐	☐
Rosenrot	☐	☐	☐	☐	☐	☐	☐
Johannesurt	☐	☐	☐	☐	☐	☐	☐
Valeriana	☐	☐	☐	☐	☐	☐	☐
Haibrusk	☐	☐	☐	☐	☐	☐	☐
Aloe vera	☐	☐	☐	☐	☐	☐	☐
Grønn te	☐	☐	☐	☐	☐	☐	☐
Essiac	☐	☐	☐	☐	☐	☐	☐
Ginseng	☐	☐	☐	☐	☐	☐	☐
Ginko biloba	☐	☐	☐	☐	☐	☐	☐
Ingefær	☐	☐	☐	☐	☐	☐	☐
Solhatt	☐	☐	☐	☐	☐	☐	☐
Hvitløk	☐	☐	☐	☐	☐	☐	☐
Soyaekstrakt	☐	☐	☐	☐	☐	☐	☐
Tranebær	☐	☐	☐	☐	☐	☐	☐
Blåbærkapsler	☐	☐	☐	☐	☐	☐	☐
Epleeddik	☐	☐	☐	☐	☐	☐	☐
Q10	☐	☐	☐	☐	☐	☐	☐
GLA/nattlysolje	☐	☐	☐	☐	☐	☐	☐
Grapefrukt	☐	☐	☐	☐	☐	☐	☐
Multikosttilskudd/vitaminer *	☐	☐	☐	☐	☐	☐	☐
Annet:	☐	☐	☐	☐	☐	☐	☐

*(eks.VitaePro, AvantGarden, Immunoplex, B17)

DEL 3:
Vi ønsker også å vite om du har snakket med legen eller annet helsepersonell om naturmidler. Deretter ønsker vi å vite hvorfor du bruker/ikke bruker naturmidler og grunnen til at du begynte med disse.

Er det liten plass under kategorien "annet" kan du fylle ut mer på baksiden av arket.

9. Har du snakket med noe helsepersonell om å bruke naturmidler, i så tilfelle, hvem? (Sett kryss ved det eller de utsagn som passer best)

1 ☐ Fastlegen
2 ☐ Annen allmennpraktiserende lege
3 ☐ Lege på sykehus eller annen helseinstitusjon
4 ☐ Sykepleier
5 ☐ Fysioterapeut/kiropraktor
6 ☐ Tannlegen
7 ☐ Annet helsepersonell:_____.
8 ☐ Jeg diskuterte det ikke med noen

10. Hvordan var responsen fra helsepersonalet? (Bare ett kryss):

1 ☐ Oppmuntret meg til å bruke naturmidler
2 ☐ Rådet meg mot å bruke naturmidler
3 ☐ Advarte meg mot risikoen ved bruken av naturmidler
4 ☐ Var nøytral/likegyldig
5 ☐ Annet:_____.
6 ☐ Ikke aktuelt da jeg ikke har diskutert dette med noen

11. Hvis du ikke snakket med noe helsepersonell om å bruke naturmidler, hva var årsaken? (Bare ett kryss)

1 ☐ Jeg ble aldri spurt
2 ☐ Jeg var redd for ikke å få forståelse for mitt valg
3 ☐ Jeg var redd for at de kom til å bli misfornøyd
4 ☐ Det er mitt valg, ingen andre trenger å vite noe
5 ☐ Jeg var redd for å bli avvist
6 ☐ Jeg vet ikke om naturmidlene har noen effekt
7 ☐ Annet:_____.

12. Hvorfor bruker du ikke naturmidler (Sett kryss for det eller de utsagn som passer best.)

[1] ☐ Jeg har aldri vurdert det
[2] ☐ Jeg tror ikke det fungerer
[3] ☐ Legen min har sagt det ikke fungerer
[4] ☐ Jeg er fornøyd med den behandlingen jeg får
[5] ☐ Annet:_____.

Hvis du ikke bruker naturmidler kan du nå avslutte spørreskjemaet og levere det som beskrevet på første side.

Bruker du naturmidler kan du fylle ut resten av spørreskjemaet.

13. Hvorfor valgte du å begynne med naturmidler? (Sett kryss ved det eller de utsagn som passer best)

[1] ☐ Jeg fikk det anbefalt av venner/familie
[2] ☐ Jeg fikk det anbefalt av lege
[3] ☐ Jeg fikk det anbefalt i helsekostbutikk
[4] ☐ Jeg fikk det anbefalt i apotek
[5] ☐ Jeg leste om det i ukeblad/presse
[6] ☐ Jeg leste om det på internett
[7] ☐ Jeg fikk det forskrevet av en alternativ behandler
[8] ☐ Annet:_____.

14. Diskuterte du bruken av naturmidler med den/de du krysset av for under spørsmål 12? (Bare ett kryss)

[1] ☐ Ja
[2] ☐ Nei
[3] ☐ Ikke aktuelt

15. Hva ønsker du å oppnå med å bruke naturmidler? (Sett kryss ved det eller de utsagn som passer best.)

1 ☐ Jeg håper det skal gi bedre leveutsikter
2 ☐ Jeg prøver å bedre immunforsvaret
3 ☐ Jeg har sykdom jeg ønsker å behandle
4 ☐ Bedre enn ingenting
5 ☐ Det hjelper mot smertene
6 ☐ Det hjelper mot depresjon
7 ☐ Annet: _____.

16. Har du opplevd ubehag som du tror kan skyldes naturmidlene du bruker? (Bare ett kryss)

1 ☐ Ja
2 ☐ Nei
3 ☐ Ikke det jeg har lagt merke til

17. Hvis du svarte ja på spørsmål 16, kan du beskrive det ubehaget du har opplevd?

_____
_____
_____
_____
_____
_____
_____
_____
_____
_____
_____

18. Omtrent hvor store utgifter har du på naturmidler?

I løpet av siste måned: _____ Kr
Gjennomsnitt pr måned: _____ Kr

Du er nå ferdig med å svare på spørreskjemaet og kan levere det som beskrevet på første side. **Tusen takk for hjelpen!**

Paper I

The co-use of conventional drugs and herbs in Norwegian general practice: a cross-sectional study.

Ane Djuv, Odd Georg Nilsen and Aslak Steinsbekk.

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Title

The co-use of conventional drugs and herbs among patients in Norwegian general practice: a cross-sectional study

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Keywords

Herb-drug interaction, general practice, safety, herbal use, disclosure, complementary therapies, co-use

Abstract

Background

Different patient groups are known to use herbal remedies and conventional drugs concomitantly (co-use). This poses a potential risk of herb-drug interaction through altering the drug's pharmacokinetics or pharmacodynamics. Little is known about co-use among patients in general practice. The primary aim of this study was to compare patients in general practise that co-use herbal remedies and conventional drugs with those who do not. The secondary aim was to register the herb-drug combinations with potential clinical relevant interactions among the co-users.

Method

A questionnaire based cross-sectional study conducted in the autumn 2011 in a general practice office with four general practitioners (GPs) and one intern in Western Norway. Adults >18 years who came for an office visit were invited. The questionnaire asked about demographics, herbal use, conventional drug use and communication about herbal use. Multivariable logistic regression was used to compare co-users to the other patients.

Results

Of the 381 patients who completed the questionnaire, the prevalence of herbal use was 43%, with bilberry (41%), green tea (31%), garlic (27%), Aloe vera (26%) and purple coneflower (18%) as the most frequently used. Among those using conventional drugs regularly, 108 (45%) co-used herbs. Close to 40% of patients on anticoagulants co-used herbs, with garlic and bilberry as the most frequent herbs. Compared to all other patients, co-users had significantly ($p<0.05$) increased odds to be female (adjOR 2.0), age above 70 years (adjOR 3.3), use herbs to treat an illness (adjOR 4.2), use two or more herbs (polyherbacy, adjOR

12.1) and having experienced adverse effects of herbal use (adjOR 37.5). Co-use was also associated with use of analgesics or dermatological drugs (adjOR 5.1 and 7.9 respectively). Three out of four patients did not discuss herbal use with any health care professional.

Conclusion

A sizable proportion of the GP patients co-used herbs with conventional drugs, also combinations with reported interaction potential or additive effects like anticoagulants and garlic. The low disclosure of herbal use to their GP, polyherbacy and the risk of interactions in vulnerable groups like elderly and chronically ill patients, warrant increased awareness among GPs.

Background

In the last two decades there has been a considerable increase in the herbal remedy market [1, 2]. Interactions between herbal remedies and drugs have been put on the agenda and received increased attention [3, 4]. Both serious and less serious adverse interactions have been reported e.g. between the drug cyclosporine and St. Johns wort (*Hypericum perforatum*), and between drugs like warfarin or aspirin which are reported to interact with a range of herbs like garlic (*Allium sativum*), cranberry (*Vaccinium oxycoccos*), *Ginkgo Biloba*, ginger (*Zingiber officinale*) and grape fruit (*Citrus paradisi*) [5-9]. Co-use of herbs and drugs might alter the drug's pharmacokinetics and/or pharmacodynamics, hence causing unexpected adverse effects of the drug [10-13].

Studies have reported extensive use (40-56%) of herbs in the general population [14-16]. The 2007 National Health Interview Survey, USA, reported that nearly 20% of the general population were using herbs [17]. The typical herb user was female, aged 30 to 69 years, with higher education or hospitalized in the last year [17]. Forty-one per cent of USA adults reported the use of herbal remedies to self-treat before seeking medical care from a physician [14].

Only 50% of herb users inform their physician about it [14]. In addition, the health care professionals rarely ask the patients about the use of herbs or other types of complementary and alternative medicine [18]. "The doctor did not ask" is the common phrase explaining the lack of communication [19]. The general practitioners (GPs) also tend to underestimate the use [18]. It is therefore important to have knowledge about the characteristics of herb users in general and co-users in particular to make health professionals more aware.

It is reported that up to 40% in various patient groups co-administrate herbal remedies and drugs [20-22]. One study found that 40% of pregnant women used herbal remedies and about 85% of these co-used conventional drugs [21]. The use of herbal remedies among adults with cancer is reported to be between 30-55% [15, 23] and one study found that almost 40% co-use herbal remedies and chemotherapy [24]. Elderly patients have more poly-pharmacy problems and are more vulnerable to interactions because of altered pharmacokinetics and decreased health in general [25]. Considering that 13-47% of elderly patients report to consume herbs [26, 27] and 31- 75% of these co-use herbs and prescribed conventional drugs [28, 29], the risk of adverse interactions might be high. About 50% of the general population have one or more chronic conditions and as the elderly, they have a high care rate and poly-pharmacy (50%) [30]. They also tend to use more herbal remedies, which increase the possibility of herb-drug interactions [31].

Despite the large reported use of herbs and co-use of herbs and conventional drugs in the general population and in various patient groups, few studies have been performed among patients in primary care and general practice in particular. About 40% of the patients in primary care clinics in USA believed that taking prescription medications and herbal remedies together was more effective than taking either alone and nearly 50% of the herb users co-used drugs [32]. An Israeli study on co-use among patients in general practitioner's offices, reported 36% of herbal use and approximately 30 % were co-users [33]. GPs are the first medical contact within the health care system, dealing with all health problems both acute and chronic [34]. Given the nature of general practice, the few studies are somewhat surprising.

The primary aim of this study was to compare patients in a general practice in Norway that co-use herbal remedies and drugs with those who do not, with regards to demographics, types of drugs and herbs used, reason for use and communication with health care professionals about this use. The second aim was to register the herb-drug combinations with potential clinical relevant interactions among the co-users.

Materials and Methods

This was a questionnaire based cross-sectional study. The survey took place in a general practitioners office with four GPs and one intern physician situated in the city centre of a middle sized town with nearly 70 000 inhabitants on the west coast of Norway. About 6000 patients were on the GPs list at the time of the data collection. The data collection took place during 5 weeks in the autumn 2011 (2011.07.11-2011.12.15). The study was approved by the Regional Committee for Research Ethics in South-eastern Norway.

Participants and recruitment

The inclusion criteria were patients 18 years old or older, having an office consultation with a GP and who were able to read and understand the questionnaire.

The questionnaire was first made available to the patients in the waiting area for self-inclusion, but after a short time the recruitment was done by the staff in the reception. The reception staff was instructed to consecutively ask the patients who contacted them when they prior or after the GP consultation whether they would be interested in taking part in the survey and gave the questionnaire to those who said yes. It was not systematically registered how many said no, but according to the staff this was about half of the patients. The first page of the questionnaire informed the patient about the project, its objectives and the handling of their information. In addition, information was given on wall posters in the waiting area. The reception staff assisted the participants with the questionnaire whenever needed. The patients were asked to return the questionnaire to the reception or by mail in pre-addressed and pre-paid envelopes. Their answers were anonymous. A completed questionnaire was interpreted as informed consent.

Questionnaire

The questionnaire included questions about herbal use, drug use and communication about herbal use and was based on a questionnaire previously used among cancer patients in an outpatient clinic in Central Norway [24].

The questionnaire was divided into three parts. The first part contained questions about demographic data (Table 1) and about conventional drugs used regularly from a predefined list of 25 drug-categories with possibilities to add other drugs (Table 3). The drug categories covered most of the regularly prescribed drugs based on data from the Norwegian Institute of Public Health and were exemplified with common Norwegian sales name to make them recognizable for the patients [35].

The second part asked about herbal use from a predefined list of the 24 most common herbs sold in Norway and the frequency of use [36] (Table 2). In addition, supplements (i.e. multivitamins) and an extra space for other herbs were also included. Only those products defined as herbs (herbal substances, herbal preparations or herbal medicinal products) were included in the analysis [37]. Herb users were defined as those answering that they used herbs daily, weekly, monthly, less than monthly or periodically. Non-users were defined as those answering that they used herbs earlier or never used.

In the last part of the questionnaire the communication between the patient and health care professionals, motives for use or no use and who recommended use of herbs were obtained (Tables 1 and 5). In addition, they were asked about any side effects of their herbal use and approximately monthly costs.

Statistics

To find the total number of consultations in the GP practice during the 5 weeks data collection period, and the age and gender distribution of these patients, a report module of the electronic health record system was used (WinMed 2.12r Statistics, CompuGroup Medical Norway AS, Lysaker, Norway).

Pearson's Chi-square was used for bivariable analyses of categorical data, like the differences between users and non-users of conventional drugs. In analyses that included less than 5 cases in a cell, the Fisher exact test was used. For multivariable analysis to disclose any associations between co-users and other variables, binary logistic regression analysis (adjusted odds ratio, adjOR) was used. All variables with p-values <0.2 in bivariable analysis were included in the regression analysis. In addition a separate multivariable logistic regression model were used to compare co-users with drug only users including variables with a p-value <0.2 in the bivariable analyses of variables that both co-users and drug only users answered (from table 1 and 3). P-values < 0.05 were considered as statistically significant. Tendencies were ascribed for p-values between 0.05 and 0.10. The statistics analysis was done using SPSS 19.0 (SPSS, Chicago, IL, USA).

Results

The total number of patients having consultation in the GP office during the five weeks of data collection was 1652. Fifty-seven per cent of these were females. The average age was 54.5 years, with 25% being 70 years old or above. The other age groups, grouped as shown in table 1, were evenly distributed in the range 13-17% of the total number patients.

Four-hundred and two questionnaires were distributed and 381 were returned. Of the 381 respondents, 67% were females, the average age was 52.5 years (SD=18.11, range 18-92) and 20% were 70 years old or above (Table 1). About 35% had higher education and 61% were employed or on sick leave (off sick). Nearly two out of three (63%) used conventional drugs regularly.

Nearly half of the patients used multivitamins or supplements (data not shown). A total of 164 (43%) patients were currently using herbs, and there was a significantly higher proportion of women using herbs compared to men (51% of all female patients vs. 29% of all male patients, $p<0.001$). Elderly above 70 years old, had a significant higher herbal use with 91% using herbs alone or co-using with drugs compared to the youngest patients ($p<0.001$). A total of 74 (20%) of the patients were using two or more herbs (polyherbacy) and about 80% of those were women ($p<0.001$, data not shown). For the other demographic variables there was no significant differences with regard to herb use (data not shown).

Among those using conventional drugs, 108 (45%) also used herbs (co-users). Significant differences were seen between the genders, age and occupational groups in regard to co-use of drugs and herbs (Table 1). Compared to men, females co-used significantly more drugs and herbs (18% vs. 34%, $p=0.001$). More than one of every three patients older than 50 years were

co-users and this was significantly more than for younger patients (p=0.008). Those employed co-used significantly less than those not employed (p<0.001).

Friends or family were those most frequently recommending herbal use (68%), followed by magazines or internet (32%), the shop or pharmacy (29%, Table 1.). Significantly more of the co-users than non-co-users, used herbs with the intention to treat an illness (89% vs 11%, p=0.008). The most common reasons for no use were "Never considered it" (39%) and "Do not believe in it/ Seems unsafe" (32%).

«TABLE 1 APROX HERE»

Among those who used herbs, bilberry (*Vacciunum myrtillus*, 41%), green tea (*Camelia sinensis,* 31%), garlic (*Allium sativum*, 27%), Aloe vera (*Aloe barbadensis,* 26%) and Echinacea (*Echinacea purpurea*, 18%) were the most commonly used herbs (Table 2). For nearly all the types of herbs used, there were no significant difference between the types of herbs used when comparing those who co-used conventional drugs and those who did not use conventional drug. Among the five most commonly used herbs (18% or more of the users), those who co-used conventional drugs tended to use more Aloe vera than not conventional drug users (p=0.091). In addition, almost two of three (63%) of the polyherbacy patients were also using conventional drugs (p<0.001).

«TABLE 2 APROX HERE»

For nearly all types of conventional drugs used there were no significant differences between herb users and non-users (Table 3). The only significant difference was higher use of herbs

among those using analgesics (60% used herbs vs 40% did not, p=0.031) or anticoagulants (36% used herbs vs 64% did not, p=0.043).

«TABLE 3 APROX HERE»

A total of 256 different herb-drug combinations were registered (Table 4). Of these, 18 were identified of being at risk of clinical relevant interactions (in bold, Table 4.). Antihypertensives and diuretics were the largest drug categories in regard to number of combinations with different herbs (n=21) followed by analgesics (n=19), antihyperlipidemic agents (n=19) and thyroid- or antithyroid hormones (n=17) (Table 4.). Bilberry (n=21), green tea (n=20) and cranberry (n=20) were the herbs with the highest number of combinations with drugs. The most common combinations were seen between bilberry and antihypertensives (n=24), anticoagulants (n=18) or analgesics (n=15) (Table 4). Green tea and garlic had also high number of co-use for these drugs.

«TABLE 4 APROX HERE»

Nearly 80% of the herb users did not discuss herbal use with any health care professional. The majority (80%) of those were co-users (p=0.104) (Table 5.). The most common health care professional the patients discussed their herbal use with was the GP (15%, data not shown). Of those discussing herbal use with their GP, about 80% were co-using conventional drugs (p=0.156). The response from the GP on disclosure of co-use differed from encouraging continued use (32%), neutral response (32%) and discouraged (14%) herbal use (p=0.815, data not shown).

Only non-co-users had been warned about risks with herb use (data not shown), while 83% of those being encouraged to continued use were co-users (p=0.463). The most common reason for no communication was "I was never asked" (45%, p=0.723).

All of those who had experienced adverse effects of herbs were co-users (7%, n=8, p=0.020). The herbs most frequently used by those experiencing adverse effects were garlic (n=5), bilberry (n=4), green tea (n=4) and ginger (n=3). The most common drugs co-used with herbs of this group were anticoagulants (33%) sedatives (33%) and antihypertensives (22%). Abdominal pain, diarrhea and emesis (33%) or dizziness (22%) was the most common reported effects.

«TABLE 5 APROX HERE»

Multivariable analysis

A total of 17 variables were included in the binary logistic regression analysis comparing co-users to all other patients. Of these, seven variables were significantly ($p<0.05$) associated with co-use of herbs and conventional drugs (Table 6), with an increased odds for co-users to be female (adjOR 2.0), above 70 years (adjOR 3.3), wanting to treat an illness (adjOR 4.2), using several herbs (polyherbacy, adjOR 12.1) and experience adverse effects (adjOR 37.5). Increased levels of co-use were also associated with use of analgesics or dermatological drugs (adjOR 5.1 and 7.9 respectively). Being between 40 and 49 years old decreased the odds of being a co-user (adjOR 0.2).

«TABLE 6 APROX HERE»

In the sub-analysis of co-user vs. drug only users, the model included gender, and use of anticoagulants, analgesics and dermal drugs (data not shown). Those who co-used drugs and

herbs tended ($p<0.100$) to be female (adjOR 1.9) and use analgesics (adjOR 1.7) compared to only drug users.

Discussion

A total of 29% of GP patients in this study co-used herbs and conventional drugs. The co-use was associated with female gender, increasing age above 50 years, using herbs to treat an illness, polyherbacy, use of analgesics or dermatological drugs and having experienced adverse effects from herbs.

Strengths and limitations

One of the limitations of this study was that it is a cross sectional study, meaning that no causal relationship can be identified. In addition, the study took place in one GP clinic in a middle sized town on the west coast of Norway. It thus might not be representative for other populations, but the patients visiting the practice are similar to other GP patients in Norway [38]. Although those taking part in this study were representative for all patients visiting the GP practice during the period of the survey, those using herbs might also be more positive to contribute to such a study than non-herb users. This would give an overestimation in the prevalence of herb users. However, this would also be the same for other studies investigating herbal use, and would not hamper the comparison with these. All data are self-reported and inaccuracies in the reported use of herbs and drugs must be taken into consideration. Still, the latter was minimized by handing out lists of the most common drugs in familiar groups with examples of the most common sales name of the different drugs.

Herbal use

The prevalence of herbal use of 44% is somewhat higher compared to other findings from general practice/family doctors (22-36%) [32, 33]. Our prevalence is surprisingly close to the findings from a Norwegian cancer patient clinic where a similar questionnaire was used (46%) [24]. It is also in range of the prevalence of studies of the general population from other countries. The 2007 National Health Interview Survey, USA, reported of nearly 20% herbal use in the general population [17]. However, both the Czech and Saudi-Arabian population reports of higher herbal use (50-57%) compared to the USA population [29, 39]. Thus, the prevalence might vary between countries and ethnic groups [27].

Few patients were recommended herbal use by the pharmacy or a physician. As reported by other papers, friends or family are the common sources for herbal recommendation or information [14, 23].

Bilberry, green tea, Aloe vera, garlic and Echinacea were the most commonly used herbs among the patients. Except from bilberry, all other herbs are also frequently reported by others [11, 17, 40, 41]. A sub-analysis of the reason for using bilberry revealed that it was used largely to strengthen the immune system (84% of bilberry user gave this as the reason). The use of bilberry might have been influenced by heavy marketing as a "super-food" [42].

Overall, every third patient in this study co-used drugs and herbal remedies. Reported co-use from GP's offices in Israel in 2004 was lower (12%) [33], however, up-to-date numbers from GP practice are lacking. The co-use is in line with the co-use reported for patient groups like pregnant women (34%) and somewhat lower than reported for the cancer patients (30-55%)

[15, 21, 23]. Thus, our findings are in line with earlier reported co-use for patient groups, and the prevalence of co-use seems to be similar across different populations.

Characteristics of co-users

Based on the high co-use of drugs and herbs, drug users are at high risk of clinically relevant interactions [3, 43]. As expected, increasing age above 50 years was associated with a higher co-use compared to the younger patients in our study (nearly 40% of those >70 years old were co-users). Earlier studies report of co-use among elderly from 32-42% [25, 44, 45]. Cohen et al. found co-use of 24% among geriatric patients, and 52% of them co-using with anticoagulants [46]. Elderly patients are an exposed group because of increasing poly-pharmacy, reduced general health and altered drug metabolism [25, 47]. They have a lower tolerance for alterations in the pharmacokinetics or pharmacodynamics, which might have serious consequences [3, 4, 8]. In addition, females, or those taking two or more herbs, were both significantly associated with co-use in this study. Females are reported in several other papers as the most common user of herbal remedies, thus not in particular as co-users [48].

The most frequent co-use of drugs in this study was with bilberry, green tea, garlic, Aloe vera and cranberry. Bilberry is abounded of antioxidants and has been reported to have anti-inflammatory activity [49]. A recent case report indicates an interaction between bilberry and warfarin that induces rectal bleeding[50], however, few interaction data are published on this herb. Thus, attention should be paid to the intake of bilberry in patients taking antiplatelet or anticoagulant drugs. Garlic might have antiplatelet activity and should thus, be used with care together with antiplatelet drugs like warfarin [11, 51]. Excessive bleeding has been reported in patients co-using warfarin and garlic, a patient group frequently using garlic [52]. Aloe vera might cause potassium depletion or affect cardiac glycosides and is advised not to be

used together with heart medication [11]. However, no *in vitro* or *in vivo* pharmacological interactions have yet been established [11, 53, 54]. Cranberry is reported to interact with warfarin, increasing International Normalized ratio (INR) values by 30% [9], but an randomized controlled trial concluded with minor risks for significant interactions in humans [55]. Some reports state, however, that garlic, green tea, Aloe vera and cranberry in general seem to have a low drug interaction risk [12, 55].

Those on regular analgesics or dermal drugs were significantly associated with co-use. NSAIDs (i.e. Aspirin) is known to interact with many herbs (i.e. ginkgo, garlic, ginger, bilberry, ginseng) and a recent study shows decreased *in vitro* metabolism of paracetamol when co-used with *Coriolus versicolor* used in traditional Chinese herbal medicine [8, 56, 57]. Keeping in mind that nearly all of the co-users used two or more herbs, the risk of interactions or additive effects are present.

In the present study, herbal adverse effects were only reported by co-users (7%). In a recent paper from Beirut as much as 60% of the co-users reported some sort of adverse effects [58, 59]. Although our reported prevalence is low, those reporting adverse effects were using herbs with reported additive effects (i.e. anticoagulants and garlic) [11]. Still, the numbers are too low to draw any firm conclusions.

Herb-drug interactions at risk

There were identified 256 different drug-group and herb combinations (Table 4). Of these, 18 were identified of being at risk of clinically relevant interactions (in bold, Table 4.) on the basis of clinical trials, case reports or theoretical interactions extrapolated from clinical data [12, 60]. Anticoagulants (i.e. warfarin) were co-used with garlic (*Allium sativum*), cranberry

(*Vaccinium oxycoccos*), ginger (*Zingiber officinale*), ginseng (*Panax ginseng*), grape fruit juice (*Citrus paradisi*) and saw palmetto (*Serenoa repens*), all interacting with anticoagulants increasing the risk of adverse effects (i.e. increased haemorrhage) [6, 8, 9, 13, 20, 52]. Antihypertensives and diuretics were the largest drug categories in regard to number of combinations with different herbal remedies in the present study, having interaction potential with ginseng or grapefruit juice [8]. Ginseng is also reported to interact with antidiabetics, cardiac glycosides, antidiarrheal agents and antidepressants [8, 60]. Co-use of garlic with NSAIDs, anti-retroviral therapy or antidepressants have also been reported to give clinically relevant interactions [8, 60]. In general co-use of these herbs with anticoagulants or other cardiovascular drugs should be discouraged or closely monitored for adverse effects / INR [11, 52]. Co-use should especially be closely monitored or even discouraged among the elderly [61]. Anti-constipation drugs or antidiabetic agents should not be consumed with Aloe vera (*Aloe barbadensis*) because of additive effects and the same has been shown for valeriana (*Valeriana officinalis*) co-used with antidepressants [60, 62].

The duration or amount of herb use and the way of administration of the herb (i.e. oral, topical) was not covered in this study and would have given us more information whether the herb-drug interaction was clinically relevant. Aloe vera used as juice ingested orally in large daily doses has a much higher interaction potential contra Aloe vera used topically against skin burns, although dermal absorption cannot be excluded. Some of the herbs are ingested as foods like garlic and grapefruit and will in general not be a problem, unless used in excessive amounts.

GPs needs to ask all patients

The majority of herb users did not discuss their use of herbs with any health care professional and only 15% discussed herbal use with their GP. For those on conventional drugs, having a chronic illness and thus having a closer relationship to their GP, one should expect a higher willingness to share information about their herbal use. As the most common reason for not communicating about the subject is "I was never asked", there are strong indications that patients are waiting for the GPs to be the one to take initiative in these matters. Although there are some characteristics of the co-users (female, elderly, use of certain drug groups etc.), there are unfortunately no specific variable that in our opinion can be used by the GP to pin-point co-users. The GP should therefore routinely ask all their patients about use of herbal remedies in order to identify potential harmful co-use.

Conclusion

The high percentage of herbal co-use among patients using conventional drugs in general practice, and the relation between increasing co-use with increasing age and comorbidity, makes general practice an arena where co-use should be discovered. Given the under-communication with GPs about co-use, it is difficult to prevent unwanted adverse effects and interactions. In order to monitor co-use, all GPs should ask their patients routinely to disclose their use of herbs.

List of abbreviations used

GP= general practitioner

INR= International normalized ratio

NSAID= Nonsteroidal anti-inflammatory drugs

RCT= randomized controlled trial

USA= United States of America

Competing interests

No financial or non-financial competing interests are given.

Authors' contributions

AD has made substantial contributions to conception and design of the study, did the data collection, performed the statistical analysis and interpretation of data, and the drafting and revising of the manuscript. OGN has been given final approval of the questionnaire and been involved in revising the manuscript critically for important intellectual content. AS has made contributions to conception and design, the statistical analysis and interpretation of data and was involved in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Table1. Demographics of all respondents according to herb and drug use; comparison of conventional and non-conventional drug users among herbal users and non-herbal-users, and comparison of co-users with non-co-users (N=381).

				Yes			No			Co-user
				Regular drug user			Regular drug user			
				Yes	No		Yes	No		
		n (%)		(Co-user)	(Only herb user)	*p-value*[A]	(Only drug user)	(Non-user)	*p-value*[A]	*p-value*[C]
Gender	Male	124	(33)	18 %	11 %	*0.551*	39 %	32 %	*0.085*	0.001[B]
	Female	249	(65)	34 %	16 %		33 %	16 %		
Age grouped	<30	50	(13)	20 %	20 %	*<0.001*[B]	18 %	42 %	*<0.001*[B]	*0.008*[B]
	30-39	58	(15)	24 %	21 %		24 %	31 %		
	40-49	52	(14)	12 %	29 %		37 %	23 %		
	50-59	65	(17)	34 %	15 %		34 %	17 %		
	60-69	71	(19)	38 %	4 %		35 %	23 %		
	>70	76	(20)	37 %	7 %		54 %	3 %		
Education	Compulsory	71	(19)	32 %	8 %	*0.178*	46 %	13 %	*0.010*[B]	*0.611*
	Middle level	170	(45)	28 %	15 %		29 %	27 %		
	University	129	(34)	26 %	18 %		36 %	20 %		
Employment	Employed/Off sick	233	(61)	21 %	20 %	*<0.001*[B]	30 %	29 %	*<0.001*[B]	*<0.001*[B]
	Disability or retirement pension	129	(34)	40 %	5 %		47 %	9 %		
	Unemployed/Home	12	(3)	58 %	17 %		8 %	17 %		
Herbal use	Never	152	(40)				63 %	37 %	*0.533*	
	Earlier	60	(16)				58 %	42 %		
	Present	164	(43)	66 %	34 %	*0.450*				
Recommended to use herbs by (n=110) [D]:	Friends or family	75	(68)	61 %	39 %	*1.000*				
	The Physician	5	(5)	80 %	20 %	*0.647*				
	The shop or pharmacy	32	(24)	59 %	41 %	*0.830*				
	Read about it in Magazines or internet	35	(6)	69 %	31 %	*0.401*				
	The alternative therapist	7	(32)	43 %	57 %	*0.424*				
	Other	3	(6)	33 %	67 %	*0.152*				
Reasons for herb use (n=111) [D]:	Better life expectancies	47	(3)	62 %	38 %	*1.000*				
	Strengthen the immune system	79	(42)	58 %	42 %	*0.391*				

	Defeat an illness	18	(71)	89 %	11 %	*0.008*[B]			
	Better than nothing	7	(16)	29 %	71 %	*0.106*			
	Pain relief	4	(6)	50 %	50 %	*1.000*			
	Other	**3**	(4)	100 %	0 %	*0.280*			
Reasons for not using herbs (n=177):	Never considered it	69	(3)				51 %	49 %	0.002[B]
	No need/satisfied with the treatment I get	52	(39)				58 %	42 %	
	Do not believe in it/ Seems unsafe	56	(29)				80 %	20 %	

[A] P-value for comparison of conventional drug user with not conventional drug user. Analysed with Pearson Chi-Square or Fisher exact test.
[B] p<0.05.
[C] P-value for comparison of co-users with non-co-users. Analysed with Pearson Chi-Square or Fisher exact test.
[D] Multiple answers were possible.

Table 2. Types of herbs used and proportion of co-users of conventional drugs and herbs (*N*=164).

Herbs [E]	Total use *n* (%)	Proportion of co-users	*p-value*[A]
Aloe vera[F]	42 (26)	55%	*0.091*
Apple vinegar	10 (6)	70%	*1.000*
Bilberry[G]	68 (41)	68%	*0.740*
Cranberry	26 (16)	77%	*0.261*
Echinacea	29 (18)	55%	*0.200*
Essiac	1 (1)	0%	*0.341*
Garlic	44 (27)	61%	*0.578*
Ginger	18 (11)	50%	*0.186*
Ginkgo Biloba	4 (2)	100%	*0.300*
Ginseng	15 (9)	67%	*1.000*
GLA/Evening Primrose oil	8 (5)	50%	*0.447*
Golden root	10 (6)	80%	*0.497*
Grapefruit	13 (8)	46%	*0.135*
Green tea	51 (31)	65%	*0.860*
Misteltoe	1 (1)	0%	*0.341*
Nattokinase	2 (1)	100%	*0.548*
Noni juice	5 (3)	20%	*0.047*[B]
Soy	4 (2)	75%	*1.000*
Valeriana	4 (2)	75%	*1.000*
Others in total[C]	15 (9)	67%	*0.480*
- Other: Anthocyanin[D]	4 (2)	75%	*1.000*
- Other: Saw Palmetto	2 (1)	100%	*0.548*

[A] P-value for comparison of co- users with herb users alone. Analysed with Pearson Chi-Square or Fisher exact test.
[B] Significantly different with p<0.05
[C] Herbs added by the respondent to the open question about other herbs they used
[D] Anthocyanin extracted from outer layers of bilberry and blackcurrant.
[E] Herbs included in the questionnaire with no users: Shark cartilage and St. Johns wort.
[F] May include either topical or oral Aloe vera use.
[G] May include both bilberry (V. myrtillus) and/or blueberry (V.cyanococcus) due to confused with one another.

Table 3. Total number and proportion of herb users for the different drug categories (*N*= 239).

Drugs (ATC group)	Total *n* (%)	Proportion of co-users	p-value[A]
Against gastrointestinal conditions (A01-09)	15 (6)	60%	*0.288*
Analgesics (M01A, N02B)	55 (23)	58%	*0.031*[B]
Antibacterial, antifungal and antiviral (G01, J01-05)	2 (1)	50%	*1.000*
Anticoagulants (B01)	88 (37)	36%	*0.043*[B]
Antidepressants (N06)	20 (8)	50%	*0.815*
Antidiabetics (A10)	23 (10)	52%	*0.515*
Antihistamines (R06)	25 (10)	32%	*0.204*
Antihyperlipidemic agents (C10)	81 (34)	47%	*0.784*
Antihypertensives and diuretics (C02-C03, C07-09)	107 (45)	45%	*1.000*
Anti-menopausal and anticonceptives (G01-03)	21 (9)	48%	*0.823*
Antirheumatic (L01, L04, M01-04)	28 (12)	39%	*0.550*
Antiseizure, triptanes and central stimulating drugs (N02C, N03, N06B)	20 (8)	45%	*1.000*
Chemotherapeutic drugs (L01-04)	5 (2)	60%	*0.660*
Dermal drugs (D01-11, C05)	14 (6)	64%	*0.171*
Drugs used against bladder and prostate disorders and impotence (G04)	11 (5)	55%	*0.551*
Ocular drugs (S01)	9 (4)	44%	*1.000*
Respiratory drugs (R01-05, 07, H02)	20 (8)	45%	*1.000*
Sedatives and Antipsychotics (N05)	39 (16)	49%	*0.726*
Strong analgesics (N02A, N07BC)	3 (1)	33%	*1.000*
Thyroids and antithyroids (H03)	26 (11)	54%	*0.406*
Vasodilators and cardiac glycosides (C01)	5 (2)	60%	*0.660*
Other drugs total	14 (6)	57%	*0.413*

[A] P-value for comparison of herbal user or non-user for each drug category, analysed with Pearson Chi-Square or Fisher's exact test if the number of total users was below five.
[B] Significantly different with $p<0.05$

Table 4. Concomitantly use of herbs and conventional drugs.

	Against gastrointestinal conditions	Analgesics	Antibacterial. antifungal og antiviral	Anticoagulants	Antidepressants	Antidiabetics	Antihistamines	Antihyperlipidemic agents	Antihypertensives and diuretics	Anti-menopausal and anticonceptives	Antirheumatic	Antiseizure. triptanes and central stimulating drugs	Chemotherapeutic drugs	Dermal drugs	Drugs used against bladder, prostate disorders and	Ocular drugs	Respiratory drugs	Sedatives and Antipsychotics	Strong analgesics	Thyroids and antithyroids	Vasodilators and cardiac glycosides	Other drugs	Number of combinations
Aloe vera	**3**[A]	6		1	2	**2**	5	5	7	3	2	2		2	2	2	4	1		**4**	1	2	19
Apple vinegar	1	3	1		1	1	1	1	3			1				1	1			2		2	13
Bilberry	4	15	1	18	5	5	4	2	24	1	5	4	2	3	1	2	4	8		6	2	4	21
Cinnamon		1				1	1	1	1		1									1			7
Cranberry	2	9	1	**1**	2	1	2	2	5	1	1	3		1	3	1	4	4	1	3		1	20
Echinacea	2	4	1	1	3	1	3	5	5	2	1	1		1		1	1			4		2	17
Garlic	2	**8**	**1**	**9**	3	4	3	7	11	2	3	3		2		1	3	6		2		2	18
Ginger	1	2		**1**	3		2	1	3	1	1					1	2	1		2		1	14
Gingko Biloba	**2**	1					2				1					1	2			2		1	8
Ginseng	**1**	1	1	**1**	**2**	**1**	1	3	**2**		2	1					1	1		2		1	15
GLA/Evening Primrose oil		1	1		1		1	1	1		1	1								1			9
Glucosamin									1														1
Golden root	2	4	1	1	2	1	1	2	3		2	1				1	1	2		2		2	16
Grapefruit	1	3	1	**1**		2	1		**2**			1				1	2	1		1		2	13
Green tea	4	11	1	7	5	5	5	8	11	4	5	3	2	2	1	1	3	6		4		6	20
Kan Jang								1	1	1		1											4
Medox		2				1	1	2	2	1	1	2								1			9
Nattokinase		1								1													2
Noni juice						1		1	1														3
Rosehip				1				1	1	1								1					5
Saw Palmetto	1	1		**2**				2	1						1	1							7
Soy		1		1	1	1	1	2	2		1									1			9
Valeriana		1			**2**			1	2					1									5
Xocai chocolate																				1			1
Number of combinations	**13**	**19**	**10**	**13**	**13**	**14**	**16**	**19**	**21**	**11**	**14**	**13**	**2**	**7**	**5**	**12**	**12**	**10**	**1**	**17**	**2**	**12**	**256**

[A] Bold numbers: Clinical relevant interactions documented in clinical trials, case reports or theoretical interactions extrapolated from clinical data.

Table 5: Communication with health care professionals, adverse effects and monthly costs of herbs among current herb users and proportion of co-users of conventional drugs and herbs.

		Total *n* (%)	Proportion of co-users	*p-value*[A]
Communication about herb use with (n=146): [C]	Physician[D]	27 (18)	74%	*0.269*
	Other	10 (7)	80%	*0.324*
	Never discussed	113 (77)	59%	*0.104*
The health care providers response to herb use (n=167):	Not discussed	134 (80)	63%	*0.463*
	Encouraged use	12 (7)	83%	
	Discouraged use[E]	7 (4)	57%	
	Neutral/indifferent	14 (8)	71%	
Reasons for never discussing herb use with health care professionals (n= 110):	I was never asked	50 (45)	56%	*0.723*
	Afraid of the response[F]	23 (21)	65%	
	Only my own concern/ confidential	34 (31)	62%	
	Uncertain of the herbal effect	3 (3)	33%	
Experienced adverse effects of herbs? (n=120)	Yes	8 (7)	100%	*0.026*[B]
	No	112 (93)	61%	
Costs of herb use per month (Euro[G])	Mean (SD, range)	36.6 (29.0, 0.4-205)	40.4 (34.8, 0.4-205)	*0.337*
Cost range, NOK (Euro) (n=88)	1-199 (0.1-27.2)	24 (27)	71%	*0.330*
	200-399 (27.3-54.5)	47 (53)	57%	
	400-599 (54.6-81.8)	9 (10)	56%	
	>600 (>82.0)	8 (9)	88%	

[A] P-value for comparison of communication, motives for herbal use, adverse effects and costs between co-users of drugs and herbs and not co-users. Analysed with Pearson Chi-Square or Fisher's exact test given the number of total users were below five.
[B] Significantly different with $p<0.05$.
[C] Multiple answers were possible.
[D] Includes GP, regular GP (family doctor) and hospital physicians.
[E] A merge of the responses «warned about the risk" and "discouraged use".
[F] A merge of the responses "I was afraid of not getting acknowledgement for my choice", "I was afraid they got dissatisfied" and "I was afraid of being rejected".
[G] Converted from NOK to Euro. Exchange rate retrieved 23.11.2012 at 09.12 AM (1 Euro= 7.32 NOK).

Table 6. Adjusted odds ratio (adjOR) with 95% confidence intervals (95%C.l.) from multivariate regression models for co-use of herbal remedies and conventional drugs.

			95% C.I.		
		adjOR	Lower	Upper	*p-value* [A]
Gender	Female vs male	2.0	1.0	4.0	*0.043* [B]
Age grouped	Age<30 vs:				*0.000* [B]
	- 30-39	0.8	0.3	2.6	*0.715*
	- 40-49	0.2	0.0	0.9	*0.034* [B]
	- 50-59	1.3	0.4	3.8	*0.665*
	- 60-69	2.8	1.0	8.3	*0.058*
	- >70	3.3	1.2	9.3	*0.023* [B]
Reasons for herb use	Treat an illness	4.2	1.3	13.4	*0.015* [B]
Drugs	Analgesics	5.1	2.4	10.7	*0.000* [B]
	Dermatological drugs	7.9	2.0	30.8	*0.003* [B]
Adverse effects of the herbal remedy	Yes vs No:	37.5	2.8	503.4	*0.006* [B]
Polyherbacy	None or one herb vs >2 herbs:	12.1	5.8	25.4	*0.000* [B]

[A] P-value for multivariable logistic regression with co-use as the dependent variable. Analyzed with regression analysis and Forward method in SPSS.
[B] Significantly different with $p<0.05$.

Paper II

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Aloe Vera Juice: IC_{50} and Dual Mechanistic Inhibition of CYP3A4 and CYP2D6

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The aim of this study was to evaluate the inhibitory potency (IC_{50} values) of ethanol extracts of two commercially available aloe vera juice (AVJ) products, on CYP3A4 and CYP2D6 activities *in vitro* and to determine if such inhibitions could be mechanism-based. Recombinant human CYP3A4 and CYP2D6 enzymes were used and the activities were expressed by the metabolism of testosterone and dextromethorphan with ketoconazole and quinidine as positive inhibitor controls, respectively. The formed metabolites were quantified by validated HPLC techniques. Time- and NADPH- dependent inhibition assays were performed to evaluate a possible mechanism-based inhibition. One of the AVJ extracts showed about twice the inhibitory potency towards both CYP enzymes over the other with IC_{50} values of 8.35 ± 0.72 and 12.5 ± 2.1 mg/mL for CYP3A4 and CYP2D6, respectively. The AVJ was found to exert both CYP mediated and non-CYP mediated inhibition of both CYP3A4 and CYP2D6. This dual mechanistic inhibition, however, seems to be governed by different mechanisms for CYP3A4 and CYP2D6. Estimated IC_{50} inhibition values indicate no major interference of AVJ with drug metabolism in man, but the dual mechanistic inhibition of both enzymes might be of clinical significance.

Keywords: aloe vera juice; CYP3A4; CYP2D6; interaction; *in vitro*; IC_{50}.

INTRODUCTION

The use of natural health products have increasing popularity in the population and are often considered safe and natural. In general about 30–40% of the population is using alternative medicine at any time and the large majority (50–90%) does not report their herbal use to their physician (Bruno and Ellis, 2005; Giveon *et al*., 2003). As many patients co-administrate prescribed drugs and natural health products (Bruno and Ellis, 2005) drug–herb interactions must be considered a challenge, especially as several metabolic interactions between drugs and common herbs are reported (Ulbricht *et al*., 2008).

Aloe barbadensis (Miller), *Aloe vera* (AV), is an old and common medicine plant used as a topical or oral therapeutic agent against a variety of diseases as well as a constituent in cosmetic products (Boudreau and Beland, 2006). The majority of AV products are produced from the inner leaf gel of AV and purchased as tablets (dried aloe gel), juices (AVJ) and purified gels. It is claimed to have medical effects such as immunostimulating, antitumor, antiinflammatory and antidiabetic (Vogler and Ernst, 1999). The inner gel of AV consists mainly of water and the remaining solid material consists of polysaccharides, vitamins and minerals (Boudreau and Beland, 2006). Acemannan, one of the most abundant polysaccharides (>60% of solid matter), is believed to be important for the immunostimulating activity attributed to AV (Zhang and Tizard, 1996). In addition, several other constituents of AV have been identified and claimed to have healing effects, i.e. Aloe emodin and rhein (Boudreau and Beland, 2006: Tang *et al*., 2009). Quantitative variations, however, are found for these constituents due to differences in harvest seasons and growth location (Boudreau and Beland, 2006). Such inter-herbal differences are also demonstrated for *Echinacea purpurea* (Hansen and Nilsen, 2008; Modarai *et al*., 2007) and *Rhodeola rosea* (Hellum *et al*., 2010). Thus, more than one AV extract from different producers should be included in drug interaction studies.

According to a report (FY 2006/2007) from The National Centre for Toxicological Research (NCTR) in the U.S. Food and Drug Administration (FDA), increased attention is needed to the escalating use of AV both in cosmetics, dietary supplements and natural medicines (Slikker, 2007). The International Aloe Science Council (IASC) reports a multi-million dollar industry in growth (Rodriguez, 2004), and the potential market is estimated to be around 10 billion dollars. Thus, a heavily growing market is assumed for this herb.

Apart from a Swedish report showing a small induction of CYP3A4 *in vitro* (Brandin *et al*., 2007) and a publication of one single isolated constituent of AV, rhein, inhibiting both CYP3A4 and CYP2D6 *in vitro* (Tang *et al*., 2009), no inhibition data have been published on CYP3A4 or CYP2D6 activities either for other single constituents or for more complex AVJ extracts. Data from isolated herbal constituents, however, are often not relevant for the inhibition potential of a more complex extract from the whole herb as shown, for instance, for grapefruit juice (Guengerich and Kim, 1990).

Cytochrome P450 (CYP) enzymes are predominantly expressed in the liver, but also in the intestines, lungs, placenta and kidneys. Although this class has more than 50 enzymes, six of them metabolize about 90% of all

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drugs, the two most significant enzymes being CYP3A4 and CYP2D6 (Lynch and Price, 2007) contributing to about 40–45% or 20–30% of all phase-I drug reactions, respectively (Ingelman-Sundberg, 2005; Lynch and Price, 2007). Large variations in the CYP2D6-metabolism are found, however, due to its genetic polymorphism (Ingelman-Sundberg, 2005).

CYP enzymes can be inhibited or induced by drugs, resulting in clinically significant drug–drug metabolic interactions that can cause adverse reactions or therapeutic failures (Pal and Mitra, 2006). The intake of herbs during drug treatment has also been shown significantly to modulate drug metabolism and pharmacokinetics (Lynch and Price, 2007). However, our knowledge in this area is still insufficient.

Inhibition of CYP3A4 and CYP2D6 can both be reversible or irreversible (mechanism-based) in nature (Zhou *et al*., 2005). A mechanism-based inactivation is anticipated to be due to a formation of reactive metabolites that bind covalently to the enzyme causing irreversible CYP inactivation (Zhou *et al*., 2005). Some herbal products have been reported as mechanism-based CYP inhibitors, e.g. grapefruit juice and medical plants such as cinnamon (*Cinnamomum burmani)*, fennel (*Foeniculum vulgare)*, true ginger (*Zingiber cassumunar)* and a few isolated herb constituents such as bergamottin and glabridin (Kent *et al*., 2002a) of CYP3A4; Greater galangal/Thai ginger (*Alpinia galangal)*, black pepper (*Piper nigrum*) (Subehan *et al*., 2006) and Madagascar Periwinkle (*Catharanthus roseus*) (Usia *et al*., 2005) of CYP2D6. Thus CYP3A4 and CYP2D6 enzymes seem to be vulnerable to irreversible inhibition by herbal products and special interest should be paid to herbs with high sale volumes.

The aim of this study was to evaluate the inhibitory potency of ethanol extracts of two different AVJ products on CYP3A4 or CYP2D6 activities *in vitro* and to compare IC_{50} inhibition values with those reported for potent drug inhibitors. Furthermore, to determine if a possible AVJ inhibition of these two CYP enzymes could be mechanism-based.

MATERIALS AND METHODS

Chemicals and enzymes. CYP2D6 supersomes™ (cat. no. 456217, lot no. 73755), CYP3A4 supersomes ™ (cat. no. 456202, lot no. 66311), NADPH-regenerating system including Solution A [31 mM NADP+, 66 mM glucose-6-phosphate and 66 mM $MgCl_2$ in H_2O] (cat. no. 451220, lot no. 71909/85424) and Solution B [40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate] (cat. no. 451200, lot no. 73022/85423) were obtained from BD Biosciences (Woburn, MA 01801, USA). 6β-OH-Testosterone (purity ≥ 97%, cat. no. H2898, lot no. 22406011), ketoconazole (purity 98%, cat. no. K-1003, lot no. 121H0524), testosterone (purity ≥ 98%, cat. no. T-1500, lot no. 1166233 22406011), dextromethorphan (purity ≥ 99%, cat. no. D2531-10G, lot no. 044KI020), dextrorphan (purity ≥ 99%, cat. no. UC205-10G, lot no. 065K3257) and quinidine (purity ≥ 98%, cat. no. 22600, lot no. 1295350) were obtained from Sigma-Aldrich (St Louis, MO63178, USA). 0.1 M Potassium phosphate buffer (KPO-buffer, pH 7.4) was made from KH_2PO_4 and K_2HPO_4 (Jansen Chimica B-2440).

Herbal products. The *Aloe vera* juice (AVJ) 'A' (99% pure *Aloe vera* containing ascorbic acid and potassium sorbate. Batch no. 805) was a gift from Lusitania, Primavida LDA (8501–915 Portimão, Portugal). AVJ'B' (99.5% *Aloe vera* juice made from the concentrate of freeze-dried AVJ containing erythorbic acid, citric acid, sodium benzoate, potassium sorbate. Batch no. 7863) was manufactured by American quality Aloe ™ (Missouri 65757, USA) and purchased in a local shop.

Herbal preparation. Tubes containing 30 mL AVJ'A' or AVJ'B' were centrifuged (3000 rpm/805 × **g**) for 10 min. The supernatants were freeze-dried (−70°C), weighed and resuspended in appropriate volumes (2.314 mL or 3.589 mL, respectively) of 1.9% ethanol solution to make stock solutions (SS) of 270 mg/mL. The ethanol concentration used for dissolving AVJ was kept to a minimum and the pH was adjusted by potassium hydroxide (KOH) to minimize a potential source of incubation error from the low juice pH. Eight working solutions (WS) [0.04, 0.40, 4.00, 20.00, 40.0, 80.0, 160.0 and 240.0 mg/mL] were made from each SS by further dissolving in 1.9% ethanol.

CYP3A4 assay. The CYP3A4 assay was performed as described earlier by Engdal and Nilsen with some modifications (Engdal and Nilsen, 2009). Incubations were performed in KPO-buffer (pH 7.4, 0.1 mM), to which was added CYP3A4 enzyme (160 μL, 50 nM), testosterone (50 μL, 0.8 mM), solution B (20 μL, 8 U/mL) and herb solutions (100 μL AVJ extracts in six different concentrations). Incubations were performed at 37°C and initiated by adding 20 μL solution A after a pre-incubation period of 5 min. All incubation solutions contained 0.4% ethanol and 0.98% acetonitrile (for dissolution of testosterone) and a total incubation volume of 400 μL (pH 7.4). Ketoconazole (KTZ) (100 μL, 0.64 μM/0.34 μg/mL) was used as a positive inhibitor control. After 10 min incubation, ice cold methanol (200 μL) was used to stop the reaction and the solutions were placed directly on ice. All solutions were centrifuged at 3000 rpm/805 × **g** for 10 min and the supernatants were transferred to HPLC vials for analyses.

CYP2D6 assay. The CYP2D6 assay was performed as described earlier by Hellum and Nilsen (2007). Incubations were performed in KPO-buffer (pH 7.4, 0.1 mM), to which was added CYP2D6 (80 μL, 50 nM), dextromethorphan (32 μL, 0.1 mM), solution B (20 μL, 8 U/mL) and herb solutions (100 μL AVJ extracts in six different concentrations). Incubations were performed at 37°C and initiated with solution A after a pre-incubation period of 5 min. All incubation solutions contained 0.4% ethanol with a total incubation volume of 400 μL (pH 7.4). Quinidine (100 μL, 0.96 μM/0.31 μg/mL) was used as a positive inhibitor control. After 25 min incubation, ice cold acetonitrile (200 μL) was used to stop the reaction and the solutions were placed directly on ice. All solutions were centrifuged at 3000 rpm/805 × **g** for 10 min and the supernatants were transferred to HPLC vials for analyses.

HPLC analyses. Aliquots of 40 μL were analysed for the metabolite 6β-OH-testosterone by high pressure liquid

chromatograph (HPLC) (Agilent Technologies, 1200 Series, column; Eclipse XDB-C18, 5 μm, 4.6 × 150 mm) using 40% methanol as the mobile phase. The flow rate was 1 mL/min and the total run time was 25 min. Detection of 6β-OH-testosterone was performed by UV at 240 nm. The retention time was 21 min.

Aliquots of 30 μL were analysed for the metabolite, dextrorphan, by HPLC as above, using 20% acetonitrile and 80% 0.01 M KPO-buffer (pH 3.4) as the mobile phase. The flow rate was 1 mL/min and the total run time 25 min. Detection of dextrorphan was performed using a fluorescence detector with excitation and emission wavelengths of 230 and 330 nm, respectively. The retention time for the metabolite was approximately 4.3 min and for dextromethorphan approximately 21 min.

The peak areas were used for quantitation of metabolites.

Mechanistic inhibition. Time- and NADPH-dependent inhibition assays were performed for AVJ'A' with a method described previously by Hellum and Nilsen (2007). In short, the time-dependent inhibition assay was done by pre-incubating the incubation mixtures at 37 °C for 0, 15, 30 and 45 min. The pre-incubation mixtures contained all the metabolic incubation constituents and IC_{50} concentrations of AVJ'A' for CYP3A4 or CYP2D6, respectively, but without substrates added. After the pre-incubation period, the specific substrates were added: testosterone (50 μL, 0.8 mM) or dextromethorphan (32 μL, 0.1 mM) to initiate the metabolic reaction, and all incubation mixtures were incubated for 10 or 25 min, respectively. The reactions were terminated on ice by adding 200 μL ice-cold methanol or 200 μL acetonitrile, respectively.

The NADPH-dependent assay was performed by pre-incubating as above with and without the presence of the NADPH regenerating system for 0, 30 or 45 min. After pre-incubation, the NADPH regeneration system was added to the tubes without NADPH. Substrate (testosterone or dextromethorphan) was added to all tubes and incubated for another 10 or 25 min. The reaction was terminated on ice by adding 200 μL ice-cold methanol or 200 μL acetonitrile, respectively.

Analytical validation. Calibration curves for 6β-OH-testosterone consisted of seven calibration standards (62.5–10000 nM). The lower limit of quantification (LLOQ) was 62.5 nM (CV 14.4%). The CVs of intra-day assay precision were 1.5%, 2.2% and 1.3% at low [75.0 nM], medium [3.0 μM] and high [6.0 μM] concentrations of 6-β-OH-testosterone, respectively ($n=5$), while inter-day CVs were 5.2%, 4.3% and 3.1%, respectively ($n=15$). The overall inaccuracy was less than 10.0%.

Calibration curves for dextrorphan consisted of seven calibration standards (50–10000 nM). The LLOQ was 50 nM (CV 2.3%). The CVs of intra-day assays precision were 2.3%, 2.6% and 1.9% at low [50.0 nM], medium [800 nM] and high [6000 nM] concentrations of dextrorphan respectively ($n=5$), while inter-day CVs were 2.3%, 3.1% and 3.1%, respectively ($n=15$). The overall inaccuracy was less than 14.0%.

The calibration standards and QCs contained no CYP enzyme as no extractions were necessary and no disturbing peaks appeared from the incubated and centrifuged AVJ samples.

Calculation of CYP activity. The basic (control) activities of CYP3A4 and CYP2D6 were determined on the basis of the amount of metabolite (6β-OH-testosterone or dextrorphan) that was formed after the 10 min or 25 min incubation of substrate (testosterone or dextromethorphan) without the presence of inhibitor. Activity was expressed in general as the amount of metabolite formed (pmol) per pmol CYP enzyme and incubation time (min) ($\text{pmol} \times \text{pmol}^{-1} \times \text{min}^{-1}$) or as a percentage of the basic activity when an inhibitor was added.

Statistics. Data are presented as mean ± SD of three parallels. A two-sample *t*-test was used to test the effect of herbal preparations and inhibitor controls on CYP3A4 or CYP2D6 enzyme activities and regression analyses were used to estimate IC_{50} and IC_{75} inhibition values from inhibition plots. The IC_{75}/IC_{50} ratios were calculated. Confidence intervals (95%) for all IC_{50} values were estimated. A value of $p<0.05$ or non-overlapping confidence intervals were considered to be statistically significant. Statistical analyses were performed in SigmaPlot 2004 version 11.0 (Systat Software, Inc. 225 W Washington Street, Suite 425, Chicago, IL 60606) and Microsoft Excel 2003 (Microsoft Cooperation, Redmond, WA, USA).

RESULTS

The basic (control) activities of CYP3A4 and CYP2D6 were 672.5 ± 46.0 and 15.5 ± 1.8 $\text{pmol} \times \text{pmol}^{-1} \times \text{min}^{-1}$, respectively. Inhibitory plots of the positive inhibitor controls KTZ and quinidine on CYP3A4 and CYP2D6 activities can be seen in Fig. 1 and the respective IC_{50} IC_{75}/IC_{50} inhibition values are summarized in Table 1. Both inhibition plots had significant fits ($p<0.05$) to all experimental points, KTZ concentrations in the range 0.0064–2.0 μM [0.0034–1.06 μg/mL] and quinidine 0.0001–1 μM [3.2×10^{-5}–0.32 μg/mL].The IC_{50} inhibition values for KTZ and quinidine were calculated with 95% confidence intervals of [0.17, 0.40] and [0.025, 0.033] μM, respectively

Figure 2 shows the inhibition of CYP3A4 activity in the presence of AVJ'A' or AVJ'B'. Both preparations inhibited the CYP3A4 activity at concentrations above 1 mg/mL. The IC_{50} value for CYP3A4 inhibition by AVJ'A' was calculated to 8.35 ± 0.72 mg/mL with a 95% confidence interval of [6.94, 9.76] mg/mL. The IC_{75}/IC_{50} ratio was estimated to be 1.8. The IC_{50} value for CYP3A4 inhibition by AVJ'B' was calculated to be 22.4 ± 5.4 mg/mL with a confidence interval of [11.8, 32.9] mg/mL. The IC_{75}/IC_{50} ratio was estimated to be 1.5. The 95% confidence intervals for juice 'A' and 'B' did not overlap and the IC_{50} values were considered significantly different.

Figure 3 shows a reduction of CYP2D6 activity in the presence of AVJ'A' or AVJ'B'. The IC_{50} value for AVJ'A' was calculated to be 12.5 ± 2.1 mg/mL. The 95% confidence interval was [8.4, 16.6] mg/mL. The IC_{75}/IC_{50} ratio was calculated to be 2.1. An IC_{50} value for AVJ'B' of 43.0 ± 2.0 mg/mL was found with a 95% confidence interval of [39.1, 46.9] mg/mL. The IC_{75}/IC_{50} ratio was calculated to be 1.2. The 95% confidence intervals for juice 'A' and 'B' did not overlap and the

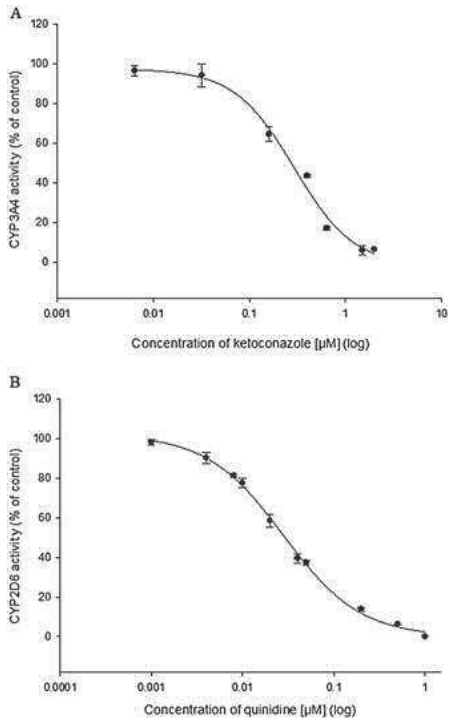

Figure 1. Inhibition of CYP3A4 (A) and CYP2D6 (B) by the positive control inhibitor ketoconazole [0.0064–2.0 μM] or quinidine [0.001–1.0 μM], respectively. All values are presented as mean ± SD of three parallels. Curve fits, $r^2 = 0.995$ or 0.998, respectively.

IC_{50} values were considered significantly different also for CYP2D6.

Figure 4A shows a time-dependent inhibition of both CYP3A4 and CYP2D6 when pre-incubated with AVJ'A'. CYP3A4 showed an exponential decrease ($y=38.507x^{-1.33}$, $r^2=0.953$) in activity with increasing pre-incubation time, while CYP2D6 showed a linear reduction ($y=-0.7878x+35.9$, $r^2=0.989$) in activity. A significant ($p<0.01$) decrease in enzyme activity was seen already after a pre-incubation time of 15 min for both enzymes. This reduction in activity was most profound for CYP3A4 initially, switching to CYP2D6 at 45 min where almost no activity was observed.

Figure 4B shows a decreased CYP3A4 activity when AVJ was pre-incubated both in the absence and presence of NADPH. However, the inhibition was higher in the presence of NADPH than without. The same pattern can be seen in Fig. 4C for the pre-inhibition of CYP2D6.

DISCUSSION

This study investigated the *in vitro* inhibitory potential of crude ethanol extracts from two different commercial types of AVJs towards CYP3A4 or CYP2D6 activities. Also the potential of AVJ to participate in a mechanistic based inhibition of CYP3A4 or CYP2D6 was explored. The basic activities of CYP3A4 and CYP2D6 and the inhibition of the added positive inhibitor controls, KTZ and quinidine, showed a variation during the study within ±15%, which was in agreement with our pre-set limits of variation. All analytical series were accepted by simultaneously run QCs in duplicates at three different concentrations.

Table 1. Inhibition parameters of CYP3A4 and CYP2D6 activities for two different commercially available AVJ preparations

	CYP3A4		CYP2D6	
	IC_{50} mg/mL)	IC_{75}/IC_{50} ratio	IC_{50} (mg/mL)	IC_{75}/IC_{50} ratio
AVJ'A'	8.35±0.72	1.8	12.5±2.1	2.1
AVJ'B'	22.4±5.4	1.5	43.0±2.0	1.2
Ketoconazole[a]	0.15±0.03[c]	2.0		
Quinidine[b]			0.009±0.0006[d]	2.9

Mean values ± SD are given for three parallels.
[a]Positive control inhibitor of CYP3A4.
[b]Positive control inhibitor of CYP2D6.
[c]Corresponding to 0.29 ± 0.06 μM.
[d]Corresponding to 0.029 ± 0.002 μM.

One isolated constituent of AV, rhein, was shown earlier to inhibit both CYP3A4 and CYP2D6 *in vitro* (Tang *et al*., 2009). The reported rhein inhibition constants (K_i) calculated for CYP3A4 and CYP2D6 were 30 and 74 μM, respectively. The levels of rhein in our AVJ'A' or AVJ'B' products, however, are not known. Still, rhein cannot be excluded as a contributing factor to some of the differences found between the two juices in their inhibition potential of the two CYP enzymes.

Both AVJ'A' and AVJ'B' inhibited the CYP3A4 metabolism of testosterone with IC_{50} values of 8.35 ± 0.72 and 22.4 ± 5.4 mg/mL, respectively. The IC_{50} value of AVJ'B' was more than 2-fold higher that of AVJ'A'. The difference was considered significant as no overlap was found in their 95% confidence intervals. These differences might be due to different levels of inhibitory compound(s) in the two AVJ extracts different from rhein. Although the assumed main active constituent in AVJ, acemannan, is present in 1.5 times higher concentration in AVJ'B' compared with AVJ'A', 2.98 and 2.03 mg/mL (Falch and Lorås, 2000), acemannan can not be excluded as an inhibitory compound, because of growth season variation.

Even AVJ'A' must be considered a weak inhibitor of CYP3A4 when compared with similar extracts from other herbs such as horse chestnut (*Aesculus hippocastanum*) and *Gingko biloba* with IC_{50} values of 1.173 and 0.756 mg/mL, respectively (Hellum and Nilsen, 2008; McConn *et al*., 2004). AVJ'A' and AVJ'B' exhibit IC_{50} values in line with other weak CYP3A4 inhibitors as noni juice and mistletoe (Engdal and Nilsen, 2009).

Both AVJ'A' and AVJ'B' inhibited the CYP2D6 metabolism of dextromethorphan with IC_{50} values of 12.5 and 43.0 mg/mL, respectively. When compared with IC_{50} inhibition values from similar extracts of other herbs as St John's wort at 67 μg/mL or A. Grey (*Heliopsis longipes)* at 9.9 μg/mL (Hellum and Nilsen, 2007; Rodeiro *et al*., 2009), the inhibition of AVJ is marginal. Our knowledge on herb–drug interactions with regard to CYP2D6 is limited. However, extracts from Candle

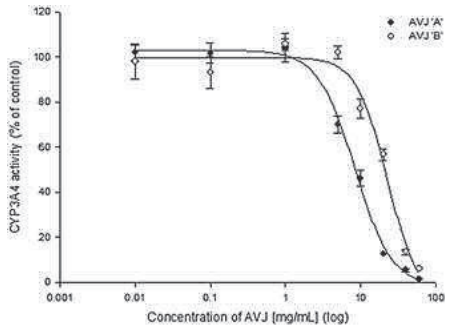

Figure 2. Inhibition of CYP3A4 by AVJ'A' or 'B' [0.01–60mg/mL]. Testosterone [0.10mM] was used as a substrate. All values are presented as mean±SD of three parallels. Curve fits, $r^2 = 0.998$ and 0.991 for AVJ'A' and 'B', respectively.

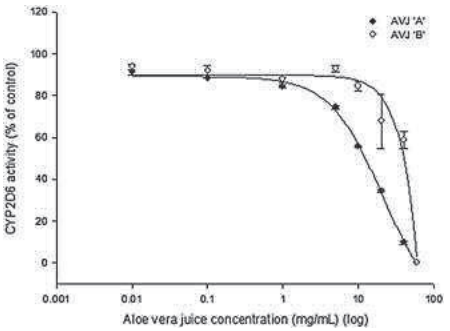

Figure 3. Inhibition of CYP2D6 by AVJ'A' or 'B' [0.01–60mg/mL]. Dextromethorphan [0.008mM] was used as a substrate. All values are presented as mean±SD of three parallels. Curve fits, $r^2 = 0.998$ and 0.987 for AVJ'A' and 'B', respectively.

Bush (*Cassia alata*) leaves, Stonebreaker (*Phyllanthus amarus*) leaves or common valerian (*Valeriana officinalis*) showed IC_{50} values of 165.5±7.50, 182.0±4.81 or 1660±155μg/mL, respectively (Appiah-Opong *et al*., 2008; Hellum and Nilsen, 2007). Thus the *in vitro* inhibition potential of AVJ on CYP2D6 activity seems minor when compared with that reported for other herbs.

The AVJs IC_{75}/IC_{50} ratios were 1.8 or 1.5 for CYP3A4 and lower than that of KTZ with an IC_{75}/IC_{50} ratio of 2.0. The same pattern was seen for the IC_{75}/IC_{50} ratios for CYP2D6. This demonstrates a somewhat steeper decrease in the AVJ inhibition curves with increasing concentrations of AVJ than by KTZ or quinidine. This might indicate a different inhibition mechanism of AVJ on CYP3A4 or CYP2D6 than by KTZ and quinidine, respectively, which both are reversible CYP3A4 or CYP2D6 inhibitors (Bertelsen *et al*., 2003; Zhou *et al*., 2005). In addition, the IC_{75}/IC_{50} ratios of AVJ-A and AVJ-B are also different for CYP2D6, which also might suggest different inhibition profiles.

Several herbs have been identified as mechanism-based inhibitors of CYP3A4 or CYP2D6. To reveal if the AVJ inhibition of CYP3A4 and CYP2D6 is mechanism-based, NADPH- and time-dependent characteristics must be fulfilled (Zhou *et al*., 2005). As AVJ'A' was identified as the most potent inhibitor of both CYP3A4 and CYP2D6 activities this product was used as an AVJ model in the mechanism-based inhibition assays.

As shown in Fig. 4A, AVJ caused a time-dependent decrease of both CYP3A4 and CYP2D6 activities when pre-incubated in the incubation mixture for increasing time intervals before the substrate was added. An exponential time-dependent inhibition pattern of CYP3A4 was also found for the medical plant fennel, *Foeniculum vulgare* (Subehan *et al*., 2006), and for grapefruit juice (Chan *et al*., 1998). On the other hand, CYP2D6 had a linear increase in inhibition as also found for melaleuca (*Melaleuca leucadendron*) (Subehan *et al*., 2006) and common valerian (*Valeriana officinalis*) (Hellum and Nilsen, 2007). This might implicate different affinities of reactive metabolite(s) or constituent(s) from AVJ for CYP3A4 and CYP2D6 (CYP apoprotein and/or haem) or different reactive metabolite(s)/constituent(s) inhibiting either CYP3A4 or CYP2D6. Ethynylestradiol has been documented to modify both the haem and the apoprotein of CYP3A4, showing a linear inhibition curve for CYP3A4 (Lin *et al*., 2002). In addition, ethynylestradiol has demonstrated differences in its binding to haem or apoprotein for different CYP enzymes (Kent *et al*., 2002b). Thus the inhibition pattern for one compound might vary between the CYP enzymes. The exact mechanism for the CYP3A4 and CYP2D6 inhibition by AVJ remains unclear, but it is probably different mechanisms or inhibition patterns for the two CYP enzymes.

As seen in Fig. 4B and C, the pre-incubation of AVJ in the incubation medium with NADPH gave a significant decrease in both CYP3A4 and CYP2D6 activities. The conversion of the inhibitor, AVJ, to a reactive metabolite thus seems to require NADPH as an electron-donor to the CYP enzyme. Hence, the AVJ pattern of inhibition is consistent with a mechanism-based inhibition (irreversible) for both CY3A4 and CYP2D6. However, significant reductions in CYP activities were also seen for both CY3A4 and CYP2D6 when pre-incubated without the NADPH regenerating system (Fig. 4B and C). These inhibitions might come from other components formed from AVJ during the pre-incubation not requiring NADPH, thus a non-CYP mediated inhibition. A mechanistic AVJ inhibition of both CY3A4 and CYP2D6 might thus be of a dual nature.

Grapefruit juice (GFJ) is a well known mechanism-based inhibitor of CYP3A4 (Chan *et al*., 1998). Its furanocoumarin dimers have been found to be extremely potent inhibitors of CYP3A4 activity (Row *et al*., 2006). Bergamotti, another GFJ constituent, inhibits CYP2D6 and is also identified as a weak inhibitor of CYP3A4 (Tassaneeyakul *et al*., 2000). Thus herbal products are complex and made up of multiple chemical compounds that might have interaction potential alone or together. Studies in which pure isolated substances are used do not include the complex nature of herbs and make procedures and interpretations for the whole herb complicated. By including total herb extracts of AVJ and not isolated single components, a higher resemblance to the *in vivo* situation might have been achieved.

The *in vitro* IC_{50} inhibition values of AVJ'A' and AVJ'B' on CYP2D6 and CYP3A4 metabolism are high and presumably too high to imply *per se* any clinical drug metabolic interactions *in vivo*. However, high intracellular herbal accumulations are shown (Di Marco *et al*., 2003),

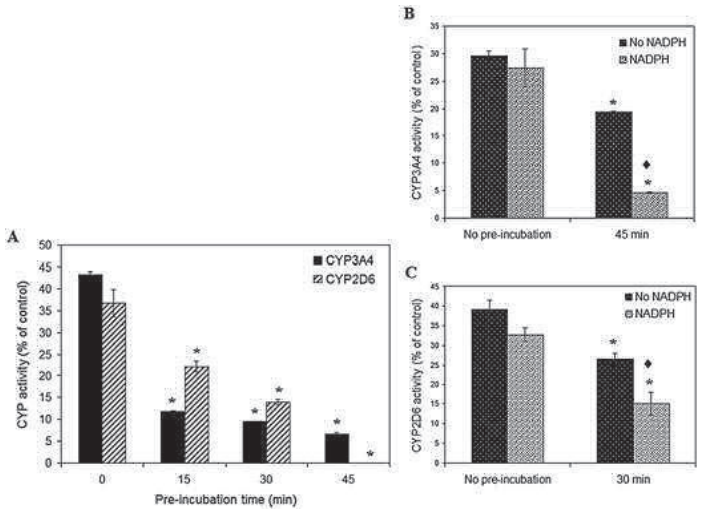

Figure 4. (A) The effect of pre-incubation with IC_{50} concentrations of AVJ'A', 8.4 and 22.1 mg/mL, on CYP3A4 [20 nM] and CYP2D6 [10 nM] activities, respectively. Testosterone [0.10 mM] and dextromethorphan [0.008 mM] were used as substrates. (B) The effect of 45 min pre-incubation with AVJ'A' [8.4 mg/mL] on CYP3A4 [20 nM]-mediated metabolism of testosterone [0.10 mM] in the absence or presence of NADPH. (C) The effect of 30 min pre-incubation with AVJ [22.1 mg/mL] on CYP2D6 [10 nM]-mediated metabolism of dextromethorphan [0.008 mM] in the absence or presence of NADPH. All results are corrected by activities observed without inhibitor present (substrate only). Data are given as mean ± SD of three parallels. * Significantly different from incubation samples with no pre-incubation, time 0 ($p < 0.05$). ♦ Significantly different from 'no NADPH at 45 min' or 'no NADPH at 30 min' values ($p < 0.05$).

making an *in vivo* interpretation somewhat difficult. Since the most potent juice, AVJ'A', had an IC_{25} value of 4.7 mg/mL towards the CYP3A4 activity and the daily recommended intake of AVJ is as high as 50 mL (about 1000 mg) (Organics Australia Online, 2009), this might imply a possible *in vivo* CYP3A4 inhibition potential in the small intestines. Clinical significant AVJ inhibition of CYP2D6 metabolism is not likely to take place *in vivo*.

The dual mechanistic inhibition of both CYP3A4 and CYP2D6 by AVJ might be of clinical importance as severe drug toxicity has been observed as a result of irreversible inactivation of CYP3A4 or CYP2D6. This is found for CYP3A4 inactivators such as erythromycin or clarithromycin when they are co-administered with terfenadine or astemizole (CYP3A4 substrates), causing torsades de pointes (Zhou *et al*., 2005) or when quinidine or paroxetine is co-administered with codeine, causing lack of analgesic effects. One case report is, however, published on similar AVJ interactions between sevoflurane and *Aloe vera* (Lee *et al*., 2004). According to the paper, the interaction was most likely a pharmacological rather than pharmacokinetic interaction. Still, another paper concludes that CYP1E1 is the key metabolizing enzyme of sevoflurane (Restrepo *et al*., 2009). An interaction between CYP and AV can thus not be excluded, and further investigation is needed.

Grapefruit juice is a more potent inhibitor of both CYP3A4 and CYP2D6 than AVJ, both enzymes probably being irreversibly inhibited (Tassaneeyakul *et al*., 2000). Grapefruit juice shows significant clinical interactions in humans with, for instance, cardiovascular medications, e.g. calcium-channel blockers (Ulbricht *et al*., 2008). The combined mechanistic or dual mechanistic inhibition of both CYP3A4 and CYP2D6 by AVJ *in vitro* may thus also have some clinical relevance.

It is concluded that both the investigated AVJ products are capable of inhibiting the metabolic activities of CYP3A4 and CYP2D6, but to a different degree and probably by different inhibition mechanisms. Furthermore, AVJ is involved in a dual mechanistic inhibition of both CYP enzymes. Estimated IC_{50} inhibition values indicate no major interference of AVJ with drug metabolism in man, but the dual mechanistic inhibition of both enzymes might be of clinical significance.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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Paper III

Caco-2 Cell Methodology and Inhibition of the P-glycoprotein Transport of Digoxin by *Aloe vera* Juice.

Ane Djuv and Odd Georg Nilsen

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Caco-2 Cell Methodology and Inhibition of the P-glycoprotein Transport of Digoxin by *Aloe vera* Juice

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The aims of this study were to carry out a thorough quality control setup for essential Caco-2 cell characteristics in P-glycoprotein (P-gp) inhibition studies and to explore if *Aloe vera* juice (AVJ) inhibits the bidirectional transport of the P-gp substrate digoxin (30 nM). Seven AVJ concentrations (0.00001-1.0 mg/mL), anticipated to cover a clinically relevant range, were tested and digoxin apparent permeability coefficients (*Papp*), net *Papp* values ($Papp_{Net}$) and net flux values (J_{Net}) were calculated. Relevant validation parameters for P-gp inhibition studies in Caco-2 cells are suggested to include, as a minimum, an assay linearity test with and without a known P-gp inhibitor, cell cytotoxicity testing (MTT-test) for substrates and inhibitors, and cell integrity testing by TEER and mannitol transport measurements. The question is also raised whether a minimum effect of a reference P-gp inhibitor as verapamil should be demanded. Cell cytotoxicity was seen for digoxin at concentrations ≥3 μM and for AVJ at 10 mg/mL. AVJ did not inhibit the P-gp transport of digoxin in any of the concentrations tested. This indicates that AVJ is no inhibitor of the P-gp mediated transport of digoxin *in vitro* if AVJ is present in clinically relevant concentrations. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Aloe vera; digoxin; inhibition; P-glycoprotein; Caco-2 cells.

INTRODUCTION

Drug–herb interactions are an issue taken more and more seriously. About 30% of the American adult population is reported to use complementary medicine in general and herbal remedies in particular. Herbs are frequently taken together with conventional drugs by patients (28%), and physicians often underestimate their patients' use of herbal remedies (Giveon *et al.*, 2003). This is a challenge as adverse pharmacokinetic interactions between drugs and common herbs are being discovered (Xu *et al.*, 2003; Pal and Mitra, 2006). Fortunately there are several *in vitro* methodologies available for the evaluation of such interactions.

Caco-2 cells derived from human colon carcinoma cells, constitute a promising model for bi-directional transport and interaction studies of P-glycoprotein (P-gp) drug substrates (Artursson, 1991). This model seems suitable for *in vitro* assays and has been shown to correlate with *in vivo* controls (Artursson and Karlsson, 1991; Collett *et al.*, 2004). Despite this, Caco-2 cells form a heterogeneous cell population that is exposed to different growth conditions which cause inter-laboratory variability (Shah *et al.*, 2006). A thorough quality control of the Caco-2 cells will, however, facilitate the possibility of comparing data between laboratories (Hidalgo, 2001). An evaluation of specific cell characteristics should thus be mandatory for the use of Caco-2 cells in general and for P-gp inhibition studies (Artursson, 1991; Hidalgo, 2001; Shah *et al.*, 2006).

Aloe barbadensis (*Aloe capensis*), *Aloe vera* (AV), is an old medicine plant used as a topical or oral therapeutic agent against different skin diseases and illnesses, and as a constituent in a myriad of cosmetic products (Boudreau and Beland, 2006). Most AV products are made from its fleshy leaves containing aloe latex and aloe gel. They are purchased as tablets (dried aloe gel), juices and purified gels. The National Centre for Toxicological Research (NCTR) in the U.S. Food and Drug Administration (FDA) in their report (FY 2006/2007) called attention to the increasing use of AV in cosmetics, dietary supplements and natural medicine (Slikker, 2007). According to The International Aloe Science Council (IASC), AV products are today converted for over 125 million dollars per year, and the potential market is estimated to be approximately 10 billion dollars (Rodriguez, 2004). Thus, a heavily growing market is assumed for this herb.

Apart from a case report suggesting a possible pharmacodynamic interaction between AV and sevofluorane (Lee *et al.*, 2004) and a very recent publication (Brandin *et al.*, 2007) indicating a minor induction of CYP1A2, CYP3A4 and MDR1 in LS 180 cells, no data are available on the drug interaction potential of AV. As 50% of all marketed drugs are reported to be P-gp substrates (Keogh and Kunta, 2006), and it is assumed that AV will become widespread in the international market, the P-gp interaction potential of AV should be thoroughly evaluated.

The aims of this study were to carry out a thorough quality control setup for essential Caco-2 cell characteristics in P-gp inhibition studies and to explore whether *Aloe vera* juice (AVJ) possesses any inhibitory effect on the bidirectional transport of the P-gp substrate digoxin.

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MATERIALS AND METHODS

Cells and chemicals. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA, cat.nr.HTB-37). L-Glutamine and fetal bovine serum were obtained from Sigma-Aldrich (St Louis, MO, USA). All tissue culture consumables were purchased from Gibco BRL Life Technology (NY, USA) with the exception of the trypsin/EDTA solution (PAA Laboratories GmbH, Linz, Austria). Tetrasodium salt (3-(4.5-dimethylthiazol-3yl)-2.5-difenyltetrazolium bromide) (MTT) (cat. no: M-2128, lot nr: 044K5305), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), non-labelled digoxin (053K1529), phosphate buffered saline (PBS) (lot no. 066K8205), verapamil (cat no. V-4629) and dimethylsulphoxide (DMSO) (cat. no. D-8418) were bought from Sigma-Aldrich (D-8418, St Louis, MO, USA). Isopropanol was purchased from Arcus Norway (L003133). Non-labelled mannitol was obtained from Merck KGaA (Darmstadt, Germany). Hank's balanced salt solution (HBSS) was acquired from Gibco BRL Life Technology (NY, USA). The following radiolabelled compounds were used: ^{3}H-digoxin (23.5 Ci/mmol, 1 mCi/mL) (PerkinElmer cat. no. NET222; lot no. 3559-502) (Wellesley, USA) and ^{14}C-mannitol (54.9 μM; 1.20 μCi/mL) (American Radiolabeled Chemicals, St Louis, MO, USA; cat. no: ARC 127A). The Optiphase Supermix scintillation cocktail (PerkinElmer, Wellesley, USA) was used for counting. *Aloe vera* juice (batch no: 510, 2005) made from *Aloe barbadensis*, Asphodelaceae family, was a gift from Lusitania, Primavida LDA (Portugal).

Cell culture. The human colon carcinoma cell line, Caco-2, was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 25% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, 10 μg/mL insulin and 1% penicillin/streptomycin. The cells were seeded onto 24-well 6.5 mm Transwell plates with 0.4 μm Pore Polycarbonate Membrane Inserts (Corning Inc.) at a density of $0.4–0.5 \times 10^{6}$/cm^{2} and for transport studies. The culture medium was replaced every 2 days. To prevent phenotypic drift only passage 35–40 were used.

Herbal specifications. AV has a high water content of 99–99.5%. The remaining solid material consists mostly of polysaccharides, vitamins and minerals (Boudreau and Boland, 2006). More and more of the components in the AV are known. AV contains 75 potentially active constituents: anthraquinones, saccharides, vitamins, amino acids and enzymes (Vogler and Ernst, 1999). Acemannan is one of the most abundant (>60% of solid matter) and well known of the polysaccharides and is believed to be important for the immunostimulating activity attributed to AV (Zhang and Tizard, 1996). The content of acemannan in our native AVJ was 2.06 mg/mL.

Dissolution of AVJ and digoxin. A 30 mL daily dose of AVJ was centrifuged (3000 rpm for 10 min) and the supernatant was decanted and evaporated to dryness (591 mg). The dried AVJ was then dissolved in a 0.8% DMSO/water solution and sonicated for 10 min at room temperature to make a stock solution of 197.10 mg/mL. A dilution sequence was made of the stock solution with 0.8% DMSO/water and equal volumes were added to the HBSS buffer to achieve seven different AVJ concentrations in the range from 0.97 mg/mL to 0.97×10^{-5} mg/mL or approximately 1.0 mg/mL to 1.0×10^{-5} mg/mL. The concentration of DMSO in all final study solutions was 0.04% (v/v). The addition of AVJ caused the HBSS/HEPES buffer pH to decrease from pH 7.4 to pH < 5.0. To minimize the confounding effects of the low juice pH, the solutions were pH adjusted (Neuhoff *et al.*, 2005).

To achieve a satisfactory dissolution of non-labelled digoxin in the cytotoxicity assay (30 nM–5 μM) and of ^{3}H-labelled digoxin in the transport assays (30 nM), the two final study solutions contained 7.0% and 0.6% (v/v) of ethanol, respectively.

Cell viability. *In vitro* cytotoxicity of AVJ and digoxin were determined by the tetrasodium salt colorimetric assay (MTT). MTT is converted to formazan crystals by mitochondrial lactate dehydrogenases (LDH) in living cells. The measured absorbance of formazan at 570 nm, is thus proportional to the number of living cells (Mosmann, 1983). Caco-2 cells (passage 39) were seeded onto two 24-well plates at a seeding density of 0.06×10^{6}/cm^{2} and incubated with 500 μL of DMEM culture medium in 5% CO_2/95% air at 37 °C for 72 h. The culture medium was exchanged by 500 μL of AVJ or digoxin final study solutions and the cells were incubated for 90 min at 37 °C. The 500 μL final study solutions were replaced by 250 μL MTT (5 mg/mL) and incubated for 40 min at 37 °C. After removal of the MTT solutions, the cells were washed twice with 500 μL cold PBS (4 °C). DMSO (>99.9%) (150 μL) was added to each well for extraction of the formazan product. The 24-well plates were placed on a shaker for 30 min at 37 °C to dissolve the formazan crystals. One hundred microlitres of extract was added to 10 μL isopropanol to avoid bubbles, and the optical density was measured at 570 nm in a Titrek Multiscan Plus MK II spectrophotometer.

Cell integrity. The cells were cultured for 21–22 days before the medium was replaced with HBSS/HEPES buffer (pH 7.4, 37 °C). TEER (transepithelial electrical resistance) values were measured (Fluke 83 Multimeter) before and after the transport assay. Only Caco-2 monolayers with TEER ≥200 Ω/cm^{2} were included in the results (Artursson, 1991). ^{14}C-mannitol (55 μM) was added to the apical side (250 μL) during the transport assay to measure cell integrity (Artursson *et al.*, 1994). Mannitol *Papp* A–B values $<1.0 \times 10^{-6}$ cm/s were accepted.

Bidirectional transport studies of digoxin. ^{3}H-digoxin dissolved in HBSS/HEPES buffer (30 nM, 23.5 Ci/mmol) was added to the donor side (250 μL on apical side and 500 μL on basolateral side) of the Caco-2 cell monolayer in the presence of test compounds under sink conditions. The digoxin transport was measured up to 180 min in apical to basolateral (A–B) and basolateral to apical (B–A) directions. Verapamil (100 μM) dissolved in HBSS/HEPES buffer was used as a positive inhibitor control (Lin, 2003) also under sink conditions. Seven different AVJ solutions were added bilaterally. Samples of 100 μL were collected from the receiver chambers and analysed by scintillation counting (Beckman LS6500 Multi-Purpose Scintillation Counter). The differences in volumes on

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the apical (250 µL) and basolateral (500 µL) sides were corrected for in the calculations.

Scintillation counting. Analysis of ^{3}H-digoxin was performed by liquid scintillation counting (10 min, Beckman LS6500 Multi-Purpose Scintillation Counter) with a counting efficiency (CE) of 26%. Counts per minute (cpm) were converted to amount (mol) of digoxin by Equation (1), where *Bq* is the background count, *SA* (dpm/mol) is the specific activity of the labelled digoxin, *CE* (cpm/dpm) is the counting efficiency and V_r/V_s is the volume correction for the volume in the receiver (V_r) chamber and the sample volume (V_s):

$$\text{Amount (mol) of digoxin} = \frac{(CPM - Bq)}{CE} \times \frac{1}{SA} \times \frac{V_r}{V_s} \quad (1)$$

Transport parameters. Apparent permeability coefficients (*Papp*) of digoxin were determined according to:

$$Papp = \frac{dQ}{dt} \times \frac{1}{A \cdot C_0}\ (\text{cm/s}), \quad (2)$$

where dQ/dt is the transport rate of digoxin (nmol/s), C_0 the initial concentration of substrate in the donor chamber (nmol/cm^3) and *A* is the surface area of the monolayer (cm^2) (Artursson, 1991).

Papp ratio for digoxin was calculated as:

$$R_{B-A/A-B} = \frac{Papp_{B-A}}{Papp_{A-B}} \quad (3)$$

and the net digoxin *Papp* as:

$$Papp_{Net} = Papp_{B-A} - Papp_{A-B} \quad (4)$$

The net digoxin flux (J_{Net}) was calculated according to:

$$J_{Net} = J_{B-A} - J_{A-B}\ (\text{nmol/h/cm}^2), \quad (5)$$

where the digoxin flux, *J*, is equal to $\frac{dQ}{dt} \times \frac{1}{A}$ (nmol/h/cm^2).

Statistics. Statistical analysis was calculated by Microsoft Office Excel 2003. All data are given as mean ± standard deviation (SD) of 3–4 replicates. Differences between groups were analysed by a two-sample Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. Least square regression lines were considered to fit linearity at *p* values <0.05.

RESULTS

The basic transport characteristics of digoxin (30 nM) in the Caco-2 cell system are shown in Fig. 1, with or without the presence of the P-gp inhibitor verapamil. A linear accumulation ($0.966 < r^2 < 0.998$, $p < 0.05$) of digoxin with time was obtained in the receiver chamber up to 180 min in all cases. Mean experimental *Papp* values of $1.40 \pm 0.06 \times 10^{-6}$ and $5.82 \pm 0.51 \times 10^{-6}$ cm/s were obtained for digoxin alone in the A–B and B–A directions, respectively. Furthermore, a mean *Papp* ratio, $R_{B-A/A-B}$, a mean net *Papp*, $Papp_{Net}$, and a mean net flux, J_{Net} of 4.15 ± 0.24, $4.42 \pm 0.46 \times 10^{-6}$ cm/s and $0.65 \pm 0.06 \times 10^{-3}$ nmol/h/cm^2, respectively, were calculated. The introduction of verapamil (100 µM) increased ($p < 0.001$) the digoxin mean *Papp* (A–B) by 33% and decreased

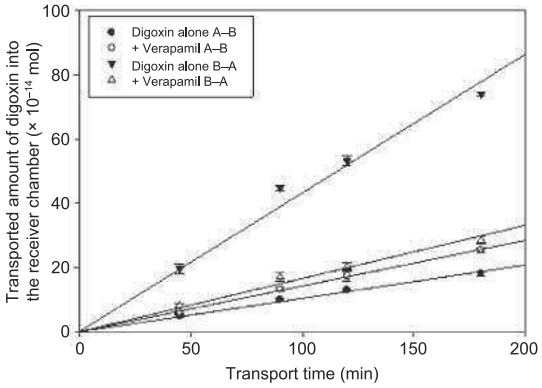

Figure 1. Transepithelial transport of digoxin (30 nM) across the Caco-2 cell monolayer measured in the apical to basolateral (A–B) and basolateral to apical (B–A) directions as a function of time, with or without the presence of the P-gp inhibitor verapamil (100 µM). Data are presented as mean ± SD of three to four replicates.

($p < 0.001$) the digoxin mean *Papp* (B–A), mean $R_{B-A/A-B}$, mean $Papp_{Net}$ and mean J_{Net} by 61%, 70%, 90% and 89%, respectively, as expected from a strong experimental P-gp inhibitor. The inhibiting effect of verapamil on digoxin transport was constant over a range of 50–400 µM (data not shown). Based on these results, a 90 min transport time and a 100 µM concentration of the P-gp inhibitor verapamil were selected for the remaining part of the study.

TEER values were measured for the B–A direction before and after the transport assay. All cells included in the transport assay calculations had TEER values ≥200 Ω/cm^2. About 88% of the wells had TEER values ≥200 Ω/cm^2 after the termination of the transport assay. The mean values for digoxin alone (control, 30 nM), in the presence of the P-gp inhibitor (verapamil, 100 µM) or in the presence of AVJ (0.00001 to 1.0 mg/mL) were 291, 286 and 230 Ω/cm^2, respectively. Mannitol transport (*Papp*) ranged from 2.0×10^{-8} to 3.0×10^{-10} cm/s for cells with TEER values ≥200 Ω/cm^2.

In Fig. 2a, the LDH activity showed a statistically significant linear decrease ($y = -0.252x + 1.466$, $r^2 = 0.994$, $p < 0.05$) with increasing concentrations of digoxin. At 5 µM a 61% decrease in LDH activity was found compared with the control. However, no effect on the LDH activity was observed at 30 nM. Figure 2b shows that the LDH activity in the Caco-2 cells was not affected by any of the AVJ concentrations added, with the exception of the highest (10.0 mg/mL). The reduction in LDH activity at this concentration compared with the control was 38%. Solvents present in the incubation medium (7.0% ethanol or 0.04% DMSO) did not influence LDH activity (data not shown).

The effects of different concentrations of AVJ on the bidirectional 90 min transport of digoxin, expressed as *Papp* values, are shown in Table 1 and Fig. 3a. AVJ caused no statistically significant change in the *Papp* values of digoxin in either direction in the investigated range, while the positive inhibitor control confirmed cell functionality. The mean digoxin *Papp* ratio ($R_{B-A/A-B}$) for all AVJ concentrations was calculated as 4.01 ± 0.20 compared with 3.82 ± 0.30 for the digoxin control and 1.33 ± 0.10 for verapamil.

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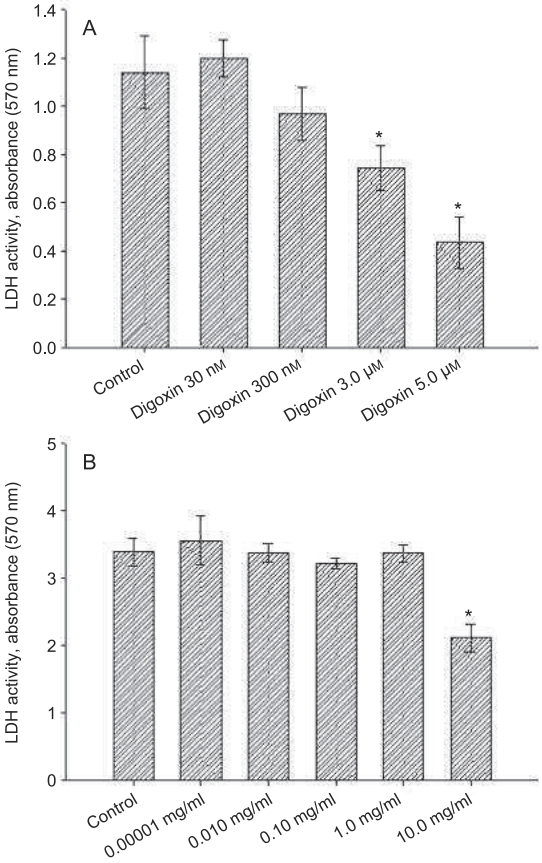

Figure 2. LDH activity in Caco-2 cells after addition of different concentrations of (a) digoxin (30.0 nM–5.0 μM) or (b) AVJ (0.00001–10.0 mg/mL). LDH activity is measured after a 90 min incubation period. Data are presented as mean ± SD of three to four replicates. * $p < 0.05$.

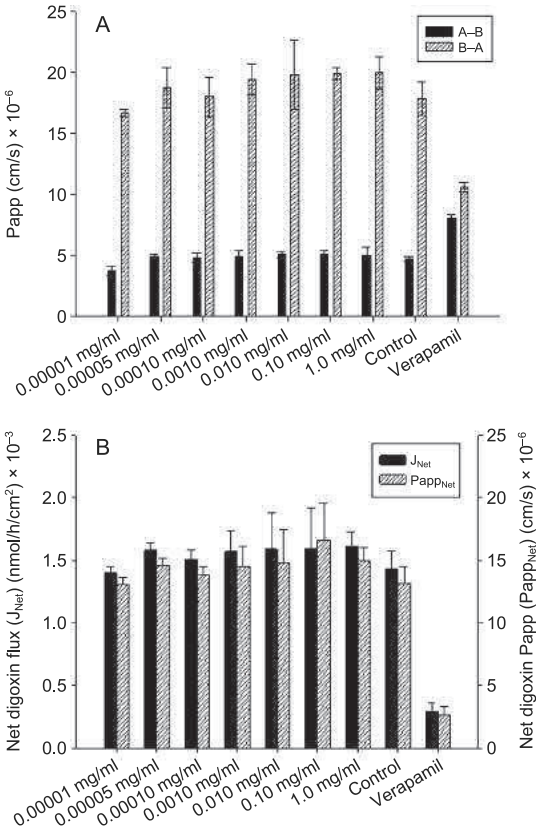

Figure 3. Effects of AVJ (0.00001–1.00 mg/mL) on the transepithelial transport of digoxin (30 nM) in Caco-2 cells expressed as (a) *Papp* values and (b) J_{Net} (left *y*-axis) and $Papp_{Net}$ (right *y*-axis). Control: digoxin alone. Verapamil (100 μM): is added as a positive P-gp inhibitor control. Data are presented as mean ± SD of three to four replicates.

Table 1. Bidirectional transport of digoxin across the Caco-2 monolayer in the presence of AVJ

Test compound	*Papp* (cm/s) × 10^{-6}	
	A–B	B–A
Control[a]	4.69 ± 0.23	17.85 ± 1.39
AVJ 0.00001 mg/mL	3.73 ± 0.38	16.66 ± 0.25
AVJ 0.0005 mg/mL	4.88 ± 0.26	19.50 ± 0.57
AVJ 0.0001 mg/mL	4.79 ± 0.38	18.76 ± 0.57
AVJ 0.001 mg/mL	4.89 ± 0.54	19.42 ± 1.31
AVJ 0.010 mg/mL	5.06 ± 0.23	19.77 ± 2.84
AVJ 0.100 mg/mL	5.14 ± 0.29	19.91 ± 0.46
AVJ 1.000 mg/mL	5.04 ± 0.60	19.98 ± 1.32
Verapamil[b] (0.049 mg/mL)	8.03 ± 0.30	10.57 ± 0.41

[a] Digoxin alone
[b] Verapamil (100 μM) is added as a positive inhibitor control of P-gp. * $p < 0.05$.

Figure 3b shows the corresponding net digoxin flux (J_{Net}) and net digoxin *Papp* ($Papp_{Net}$) values after addition of the different AVJ test solutions. None of these digoxin transport parameters were affected statistically significantly by any of the AVJ concentrations applied, while verapamil reduced ($p < 0.001$) both parameters by 89% and 81%, respectively, when compared with the control. The mean net digoxin flux, J_{Net}, was $1.58 \pm 0.12 \times 10^{-3}$ nmol/h/cm^2 when co-incubated with AVJ, while the control (digoxin alone) was $1.43 \pm 0.14 \times 10^{-3}$ nmol/h/cm^2. The digoxin J_{Net} in the presence of verapamil was $0.29 \pm 0.07 \times 10^{-3}$ nmol/h/cm^2. The mean digoxin *Papp* value, $Papp_{Net}$, was $14.60 \pm 1.09 \times 10^{-6}$ cm/s, when co-incubated with AVJ, while the control and verapamil showed values of $13.18 \pm 1.34 \times 10^{-6}$ cm/s and $2.62 \pm 0.66 \times 10^{-6}$ cm/s, respectively.

DISCUSSION

A high inter-laboratory heterogeneity has been reported for Caco-2 cells since the discovery (1975) that these cells are a good P-gp screening model for drug–drug interactions (Hidalgo, 2001; Markowska *et al.*, 2001). According to Hildalgo *et al.* investigations of the substrate transport at regular time intervals, demonstrating linearity, A–B and B–A differences and positive inhibitor effects as shown in Fig. 1, can be one way to facilitate

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comparisons with other laboratories, and to show cell compatibility. The *Papp* values for digoxin were obtained at 90 min for the B–A transport as 6.5×10^{-6} cm/s and A–B transport as 1.5×10^{-6} cm/s. This corresponds to earlier studies (Xu *et al.*, 2003). The positive inhibition control, verapamil, inhibited the digoxin net flux (J_{Net}) by 80–90%. This is in agreement with most of the earlier reported values (Keogh and Kunta, 2006). Our Caco-2 cell system thus fulfils the demands made by Hildalgo (2001) and should hence make inter-laboratory comparisons feasible.

The reported range for verapamil inhibition of P-gp in the literature is, however, quite wide from almost 100% inhibition (Collett *et al.*, 2004) down to 37–41% inhibition (Balimane *et al.*, 2004). These variations between laboratories might indicate that verapamil is not a complete inhibitor of P-gp. Or, that this variation is due to inter-laboratory differences in Caco-2 cell qualities that will influence also the inhibition potency of other P-gp inhibitors. This raises the question whether a lower limit of verapamil inhibition should be introduced for an acceptance of the inhibition studies performed with Caco-2 cells.

Thorough quality screening of the Caco-2 cells as such has been emphasized by several authors (Artursson, 1991; Hidalgo, 2001; Shah *et al.*, 2006). Both TEER and mannitol were included as cell integrity controls, measuring TEER also immediately after the end of the transport assay, and cell cytotoxicity controls (MTT test) for all substrates and inhibitors used, as an integrated part of an overall quality assurance system.

TEER was measured before and immediately after the transport assay. The latter was considered the most relevant. The exclusion of six of 48 monolayers, even after the transport assay, seems satisfactory. All wells included in our calculations showed TEER values $\geq 200\ \Omega/cm^2$. Although there are some inter-laboratory variations in TEER measurements, our measurements are in line with other measurements performed under similar conditions (Markowska *et al.*, 2001). TEER measurements after the transport assay provide a good quality control of the cell integrity and thus experimental results, and should be included in all transport assays on Caco-2 cells.

Mannitol is a large molecule and only crosses the epithelium by paracellular pathways. Mannitol thus demonstrates the existence of paracellular transport pathways and consequently also the integrity of the Caco-2 monolayer. This is considered to be the best indicator for tight junctions (Markowska *et al.*, 2001). *Papp* values less than 1.0×10^{-6} cm/s, as obtained in our study, are in agreement with accepted standards (Artursson *et al.*, 1994). With established tight junctions, no digoxin will leak paracellularly either way.

The MTT test showed that the AVJ decreased significantly the LDH activity at the highest concentration (10.0 mg/mL). This concentration was omitted in our further inhibition studies with AVJ. It corresponds to a daily dose of AVJ of 30 L and is far outside the physiological range.

Digoxin (30 nM), a concentrations used in all our transport assays, did not affect the Caco-2 cells LDH activity. When higher concentrations of digoxin were applied, the LDH activity decreased linearly with the increase in digoxin concentrations (Fig. 3a), which indicates cytotoxicity to the Caco-2 cells. A significant decrease in LDH activity was observed already at 3 μM. This should be noted as digoxin is reported to be used as a P-gp substrate in Caco-2 cell assays in concentrations ranging up to 5 μM (Bhardwaj *et al.*, 2002).

Aloe species are classified both as a natural remedy and as a herbal medicinal product according to the regulations of the Norwegian Medicine Agency (2007). Although several components of AVJ have been identified, their potential pharmaceutical mechanisms and effects are not yet clear (Vogler and Ernst, 1999). An interaction effect of AV on the P-gp drug transporter may, however, greatly influence drug pharmacokinetics and thus drug efficacy (Lin and Yamazaki, 2003; Keogh and Kunta, 2006).

The AVJ concentrations used in this study were selected to cover an anticipated clinically relevant range. The lowest concentrations (0.01– 0.00001 mg/mL) were estimated from a daily dose of 30 mL (19.4 mg/mL dried weight) and AVJ distributed in the total body volume of 60 L with a 100% uptake down to 0.1% uptake from the small intestine. The higher concentrations (1.00–0.10 mg/mL) were considered more relevant for intestinal concentrations. A concentration of 10 mg/mL was omitted due to the observed effects on intracellular LDH activity.

A weak induction of the P-gp gene (MDR1) by AVJ is stated in one report at one concentration (Brandin *et al.*, 2007). Further information should be added to this finding. As P-gp inhibition/induction may occur differently at different concentrations in a biphasic manner (Zhou *et al.*, 2004), an evaluation of the inhibition pattern of AVJ on the P-gp transport of digoxin in Caco-2 cells were considered relevant, especially if a wide range of AVJ concentrations was applied. Our findings from a validated Caco-2 cell system showed, however, no statistically significant inhibitory effects of AVJ on the P-gp mediated transport of digoxin, even though a wide range of anticipated relevant physiological concentrations of AVJ were tested. This might indicate that AVJ in clinically relevant concentrations, do not possess any significant inhibitory potency towards the P-gp mediated efflux transport of digoxin and similar P-gp substrates.

AVJ is a herbal product common in traditional medicine and used against many illnesses such as inflammatory bowel diseases (IBD), cancer, HIV and diabetes (Vogler and Ernst, 1999; Boudreau and Beland, 2006). Prednisone, imatinib (STI-571), docetaxel and indinavir are some drugs used by these patient groups, and are all P-gp substrates. Effects of AVJ on the P-gp drug transport of these substrates thus might be negligible as far as inhibition is concerned. Care should be taken, however, when extrapolating to other P-gp substrates as different efflux sites and binding affinities might be involved.

In conclusion, the Caco-2 cell methodology in our laboratory is in line with other reference laboratories. It is recommended that validation parameters for P-gp inhibition studies should include as a minimum an assay linearity test with and without a known P-gp inhibitor, cell cytotoxicity testing for substrates and inhibitors, and cell integrity testing by TEER and mannitol transport measurements. The question is also raised whether a minimum effect of a reference P-gp inhibitor such as verapamil should be demanded. Special attention should be paid to digoxin as a P-gp substrate in concentrations ≥3 μM and to concentrations of AVJ > 1.0 mg/mL, as cytotoxic effects are indicated at these concentrations.

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No statistically significant inhibitory effects of AVJ was found on the P-gp mediated transport of digoxin, even though a wide range of anticipated relevant physiological concentrations of AVJ were tested. This might indicate that AVJ in clinically relevant concentrations, do not possess any significant inhibitory potency towards the P-gp mediated efflux transport of digoxin and similar P-gp substrates.

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