Estrogenski ili anti-estrogenski učinci ekstrakata tradicionalno korištenih tropskih biljaka na ljudske stanice U2OS

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Estrogenic or anti-estrogenic effects of traditionally used tropic plant extracts on human U2OS cells

Graduation thesis

Zagreb, 2015.

This Graduation thesis was conducted in the Laboratory for Molecular Cell Physiology and Endocrinology at the Department of Biology, Technische Universität Dresden under the leadership of Prof. Dr. Günter Vollmer. This Graduation thesis is submitted to evaluation to the Department of Biology at Faculty of Science, University of Zagreb in order to acquire the Master of Molecular Biology title.

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ESTROGENSKI ILI ANTI-ESTROGENSKI UČINCI EKSTRAKATA TRADIOCIONALNO KORIŠTENIH TROPSKIH BILJAKA NA LJUDSKE STANICE U2OS

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Fitoestrogeni su spojevi biljnog porijekla koji, zahvaljujući strukturnoj sličnosti sa 17β-estradiolom, posjeduju mogućnost vezanja na estrogenske receptore te mogu djelovati kao njihovi agonisti i/ili antagonisti. Fitoestrogenima se pripisuju brojni zdravstveni benefiti povezani sa kardiovaskularnim bolestima, hormonski ovisnim tumorima, osteoporozom i menopauzalnim simptomima. Tropska Afrička biljka, *Mondia whitei* (Hook.f.) Skeels, koristi se u tradicionalnoj medicini za liječenje raznih bolesti, no njezina upotreba najčešće se spominje vezano za spolne probleme muškaraca. To ukazuje na potencijalni mehanizam djelovanja povezan sa hormonima, te su u ovom radu ispitana estrogenska i anti-estrogenska svojstva metanolskog ekstrakta *Mondie whitei* i njegovih frakcija. Istraživanje je uključivalo serije transaktivacijskih testova u U2OS-ERα i U2OS-ERβ stanicama. U ispitanom metanolnskom ekstraktu nije pronađena niti estrogenska niti anti-estrogenska aktivnost. Jedna frakcija ekstrakta pokazala je agonističku aktivnost u obje stanične linije, dok anti-estrogenska aktivnost nije zabilježena niti u jednoj frakciji. Također, ispitana je i toksičnost biljnog ekstrakta i frakcija, no zbog nekih neočekivanih opaženih efekata, savjetuje se ponoviti MTT test. Ovaj rad izrađen je u sklopu većeg "Angola Projekta" čiji je cilj dokazati i znanstveno utemeljiti učinke biljaka korištenih u tradicionalnoj medicini.

(44 stranice, 10 slika, 4 tablice, 50 literaturnih navoda, jezik izvornika: engleski)

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Ključne riječi: tradicionalna afrička medicina, *Mondia whitei*, fitoestrogeni, estrogenski receptor α, estrogenski receptor β, stanična linija osteosarkoma U2OS

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ESTROGENIC OR ANTI-ESTROGENIC EFFECTS OF TRADIOTIONALLY USED TROPICAL PLANT EXTRACTS ON HUMAN U2OS CELLS

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Phytoestrogens are plant-derived compounds that, due to their structural similarity with 17β-estradiol, have an ability to bind to estrogen receptors and act as their agonists or/and antagonists. They have been reported to confer health benefits related to cardiovascular diseases, hormone-dependent cancers, osteoporosis, and menopausal symptoms. A tropical African plant, *Mondia whitei* (Hook.f.) Skeels, is used as a traditional remedy to treat various conditions and diseases, but its use is most commonly reported in the terms of male sexual problems. This indicates potential hormonal-related mechanism of action, so this study evaluated estrogenic and anti-estrogenic properties of methanol extract of *Mondia whitei* and its fractions. Herbal extract and fractions were tested using estrogen receptor subtype specific transactivation assays in U2OS-ERα and U2OS-ERβ cells. In the examined methanol extract, neither estrogenic nor anti-estrogenic properties have been observed. One fraction of the extract showed agonistic activity in both cell lines, whereas none of the fractions showed anti-estrogenic potential. In addition to that, applied herbal treatments were examined for cytotoxicity, but due to some confounding effects observed, MTT assay is advised to be repeated. This study was conducted as part of the bigger project, "Angola Project", which aims to a better, science based understanding of traditionally known medicinal uses of plants.

(44 pages, 10 figures, 4 tables, 50 references, original in: English)

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Key words: traditional African medicine, *Mondia whitei*, phytoestrogens, estrogen receptor α, estrogen receptor β, human U2OS osteosarcoma cell line

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Abbreviations

AF-1 – Activation Function 1

AF-2 – Activation Function 2

ATR – Attractene Transfection Reagent

BCA – Bicinchoninic acid

BSA – bovine serum albumin

DBD – DNA-binding domain

DCC – dextran coated charcoal

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxyde

DNA – deoxyribonucleic acid

Dox – doxycycline

E_1 – estrone

E_2 – 17β-estradiol

E_3 – estriol

ER – estrogen receptor

ERE – estrogen response element

ERα – estrogen receptor α

ERβ – estrogen receptor β

FCS – fetal calf serum

Ful – Fulvestrant

LB – Luria Bertani

LBD – ligand-binding domain

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

MW – Mondia whitei

MWM – methanol extract of Mondia whitei

PBS – phosphate-buffered saline

Tet – tetracycline

U2OS – name of human osteosarcoma cell line

1. Introduction

1.1. Estrogens and phytoestrogens

Estrogens are a group of steroid hormones that control the development and maintenance of an individual's feminine characteristics. They regulate many physiological processes, including normal cell growth, development, and tissue-specific gene regulation in the reproductive tract and in the central nervous and skeletal systems. Estrogens also influence the pathological processes of hormone-dependent diseases, such as breast, endometrial, and ovarian cancers, as well as osteoporosis (Couse & Korach 1999). The naturally occurring estrogens, 17β-estradiol (E_2), estrone (E_1) and estriol (E_3), are C_{18} steroids derived from cholesterol (Gruber et al. 2002). The most potent among them is E_2, while estrone and estriol are, although high-affinity ligands, much weaker agonists on estrogen receptors (ERs) (Heldring et al. 2007). Estrogens are produced primarily in the theca and granulosa cells of the ovaries and, during pregnancy, in the placenta. Some estrogens are produced in smaller amounts by other tissues such as the liver, adrenal glands, breasts and adipose tissue (Gruber et al. 2002).

Phytoestrogens, also called dietary estrogens, are a broad group of plant-derived compounds of nonsteroidal structure that are structurally and/or functionally similar to mammalian estrogens and their active metabolites (Patisaul & Jefferson 2010; Setchell 1998) Phytoestrogens exert their effects primarily through binding to estrogen receptors, wherefore, they can act as estrogen agonists or antagonists. The key structural elements crucial for the estradiol-like effects are: (1) the phenolic ring that is indispensable for binding to estrogen receptors, (2) the ring of isoflavones mimicking a ring of estrogens at the receptors binding site, (3) low molecular weight similar to estrogens (MW=272), (4) distance between two hydroxyl groups at the isoflavones nucleus similar to that occurring in estradiol, (5) optimal hydroxylation pattern (Kariyil 2010). Phytoestrogens were first observed in 1926 but it was unknown if they could have any effect in human or animal metabolism. In the 1940s it was noticed for the first time that red clover (a phytoestrogens-rich plant) pastures had effects on the fertility of grazing sheep (Murkies et al. 1998). Dietary estrogens are weakly estrogenic (10^{-2} to 10^{-3} -fold) when compared with estradiol (Setchell 1998). In vitro assays have found that, although most phytoestrogens, bind both ERα and ERβ, and activate ER-dependent gene transcription through both ER subtypes, they generally have a higher relative binding affinity for ERβ than Erα (Kuiper et al. 1997; Patisaul & Jefferson 2010). The four major classes of phytoestrogens are isoflavones, lignans, stilbenes and coumestans (Mense et al. 2008). They are

found in a wide variety of plants, including soybean and other legumes, flaxseed, alfalfa, pomegranate, wheat, oats, hops. Today, daidzein and genistein are the two most well characterized phytoestrogens and human exposure to these compounds occurs primarily through the consumption of soy-based food and beverage products. Possible effects of phytoestrogens have been implicated in the etiology of hormone-dependent cancers, cardiovascular diseases, osteoporosis, menopausal symptoms, male infertility, obesity and type-2 diabetes (Kariyil 2010). These health benefits have been supported by lower incidence of hormone-dependent diseases in Asian populations than in Western populations. Epidemiological evidence suggest that Asian women have a 3-fold lower breast cancer risk than women in the United States, independent of body weight (Mense et al. 2008). Apart from that, Asian populations have historically had lower rates of cardiovascular diseases, diabetes, obesity, menopausal symptoms, colorectal and prostate cancer (Adlercreutz & Mazur 1997). The explanation for that comes from high soy consumption, which is a cornerstone of the traditional Asian diet (Patisaul & Jefferson 2010). More specifically, in East and Southeast Asia, the average daily intake of phytoestrogens is estimated to be between 20 and 50 mg. In contrast, the typical diet of an adult in the United States contains only 0.15–3 mg phytoestrogens per day, and in Europe the average daily phytoestrogen consumption is estimated to be even lower, falling between 0.49 and 1 mg (Adlercreutz & Mazur 1997; Sirtori et al. 2005). The effect produced by phytoestrogens can depend on the dose of the phytoestrogen. Phytoestrogens acting as estrogen mimics may affect the production and/or the breakdown of estrogen by the body, as well as the levels of estrogen carried in the bloodstream. Phytoestrogens acting differently from estrogen may affect communication pathways between cells, prevent the formation of blood vessels to tumors or alter processes involved in the processing of DNA for cell multiplication (Kariyil 2010).

1.2. Mondia whitei

Mondia whitei (Hook.f.) Skeels is an aromatic plant of Periplocaceae family which occurs throughout tropical Africa, from Senegal east to southern Sudan, and throughout most of Central, East and southern Africa to South Africa (Schmelzer & Gurib-Fakim 2013). It is commonly known as White's ginger, Limte, Nkang Bongo, Yang, La Racine, Gondolosi (Watcho et al. 2006; Lampiao 2008). *Mondia whitei* is a perennial, woody, rather robust and vigorous climber that grows from a large tuberous rootstock. The roots are aromatic and apparently taste like ginger or liquorice and have an aroma reminding of vanilla (Okon et al. 2012) The plant is medicinally used throughout its distribution area. Its roots are used as a traditional remedy to treat various conditions and diseases,

including urinary tract infection, jaundice, headache, gastrointestinal disorders and diarrhea, gonorrhea, postpartum bleeding, pediatric asthma, vomiting, malaria, but the most commonly cited use is as an aphrodisiac (Schmelzer & Gurib-Fakim 2013; Okon et al. 2012; Watcho et al. 2007) Usually the fresh or dried roots or the root bark are chewed for this purpose (Schmelzer & Gurib-Fakim 2013).

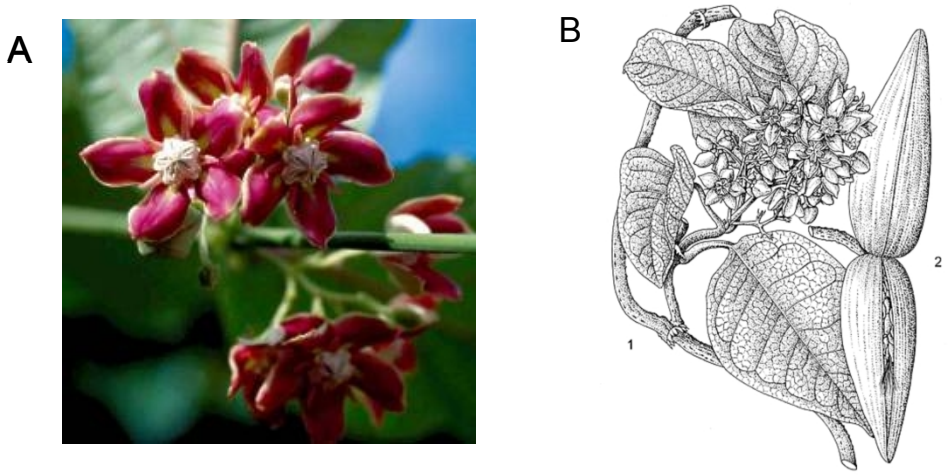


Figure 1. *Mondia whitei*
A) flowers (photography); B) 1-flowering brunch, 2-fruits (draft)
Source: Schmelzer & Gurib-Fakim, 2013.

Scientific research conducted so far on this plant involves evaluation of antibacterial and anti-inflammatory activity, antidepressant-like activity, reproductive activity and effects on the hearth (Schmelzer & Gurib-Fakim 2013; Okon et al. 2012; Watcho et al. 2007). Phytochemically, *Mondia whitei* was demonstrated to contain steroids, triterpenes (a mixture of amyrine α- and β-acetate, lupeol, β-sitosterol, and β-sitosterol glucoside) and aromatic compounds (2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, and 4-hydroxy-3-methoxybenzaldehyde), glucose, and polyholosides (Watcho et al. 2006). Other constituents include zinc, iron, calcium, magnesium and vitamins (A, D and K) (Patnam et al. 2005). Interestingly, Watcho et al. (2001 and 2004) report different effects of Mondia whitei aqueous extract in adult male rats depending on the treatment duration: a short-term treatment (8 days) results in the increase in serum and intratesticular testosterone levels, whereas a long-term treatment (55 days) has inhibitory effects on spermatogenesis and reduces fertility. Overall, *Mondia whitei* had been mostly studied in terms of male sexual stimulant, but more research is necessary to elucidate the compounds responsible for the activity and also investigate other pharmacological effects.

1.3. Estrogen receptors

The biological actions of estrogens are mediated by binding to estrogen receptors which belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors (Matthews & Gustafsson 2003). The existence of the receptor molecule that could bind 17β-estradiol was first demonstrated by Jensen and Jacobsen in the late 1950s (Dahlman-Wright et al. 2006). The first ER was cloned in 1986. Until 1995, it was assumed that there was only one ER and that it was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens and anti-estrogens. However, in 1995, a second ER, ERβ, was cloned from a rat prostate cDNA library. The first ER was then renamed to ERα (Nilsson et al. 2001). The two ER subtypes, ERα and ERβ, are products of distinct genes on different chromosomes. ERα is located at chromosomal locus 6q25.1, whereas ERβ is found at position 14q22–24 (Matthews & Gustafsson 2003; Gruber et al. 2002) Several ERα and ERβ splicing variants have been described, but whether all transcripts are expressed as functional proteins and have biological functions remains unclear (Matthews & Gustafsson 2003).

ERα and ERβ can be detected in a broad spectrum of tissues. In some organs, both receptor subtypes are expressed at similar levels, whereas in others, one or the other subtype predominates. In addition, both receptor subtypes may be present in the same tissue but in different cell type (Dahlman-Wright et al. 2006). ERα is expressed primarily in the uterus, liver, kidney, and heart, whereas ERβ is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, hematopoietic and central nervous systems. ERα and ERβ are, however, co-expressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain (Matthews & Gustafsson 2003). There has been suggested a, so-called, "Ying Yang" relationship between ERα and ERβ whereby in the presence of ERα, ERβ inhibits ERα-mediated gene transcription and in the absence of ERα, ERβ can partially replace ERα (Lindberg et al. 2003).

ERα and ERβ contain the evolutionarily conserved structural and functional domains typical of nuclear receptor family members (Matthews & Gustafsson 2003). They are composed of six domains named A-F from N- to C- terminus: (1) the N-terminal A/B domain which modulates transcription in gene- and cell- specific manner through Activation Function 1 (AF-1); (2) highly conserved C region harboring the DNA-binding domain (DBD); (3) D domain which serves as a linker peptide between the DBD and the ligand-binding domain (LBD); (4) E domain (LBD) which

contains Activation Function 2 (AF-2) and (5) F domain that plays role in distinguishing estrogen agonists versus antagonists (Klinge 2001; Ruff et al. 2000). (Fig. 2).

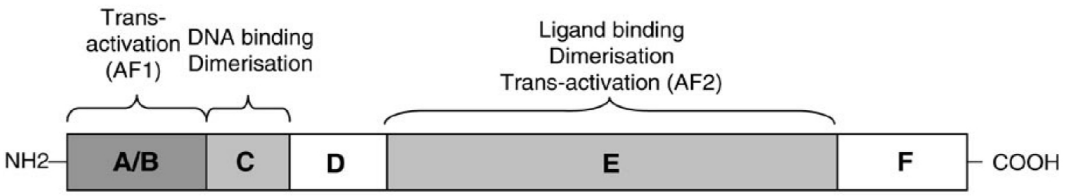


Figure 2. Schematic representation of the functional domain organization of estrogen receptors. Domains from N- to C- terminus are: the A/B domain at the NH2 terminus which contains the AF-1 site, a highly conserved DBD consisting of the C domain, a hinge region D, the LBD (domain E) which contains AF-2 and C-terminal F domain. Presented scheme applies to both ER subtypes. Adapted from: Ruff et al. 2000.

The most conserved region between ERα and ERβ is the DBD featuring two zinc fingers (CI and CII) with which receptor interacts directly with the DNA helix (Kuiper et al. 1997; Klinge 2001). The amino acid sequence identity between ERα and ERβ in this domain is approximately 97%, while the amino acid sequence of the P-box (a motif within DBD critical for receptor-DNA recognition and specificity) is identical between the two receptors (Matthews & Gustafsson 2003; Dahlman-Wright et al. 2006). Thus ERα and ERβ can be expected to bind to various estrogen response elements (EREs) with similar specificity and affinity. The ligand-binding domains are also conserved with approximately 56% homology in amino acid sequence (Dahlman-Wright et al. 2006). Thus both receptor subtypes exhibit similar affinities for E_2 and most other ligands (Barkhem et al. 1998). Nevertheless, there are a number of ligands displaying receptor-selective affinity (Kuiper et al. 1997).

The transactivating functions of ERα and ERβ are mediated by two transcription activation functions that allow the receptors to stimulate the transcription of estrogen-regulated genes: the constitutively active AF-1 located in N-terminal domain and the ligand-dependent AF-2 located in the C-terminal domain of the receptor protein (Klinge 2001; Matthews & Gustafsson 2003). A comparison of the AF-1 domains of the two ERs has revealed that this domain is very active in ERα on a variety of estrogen responsive promoters, but under identical conditions, the activity of AF-1 in ERβ is minimal (Barkhem et al. 1998). Furthermore, these two receptors exhibit distinctive responses to the synthetic anti-estrogens tamoxifen and raloxifene. These ligands are partial ER agonists for ERα but act as pure ER antagonists for ERβ. The differences between the N-terminal

regions of the ERs, in terms of poorly homologous amino acid sequence (18%), have been suggested as a possible explanation for their diversity of responsiveness to several ligands (Matthews & Gustafsson 2003; Dahlman-Wright et al. 2006).
Agonists and antagonists bind at the same site within the core of the LBD, but demonstrate different binding modes (Brzozowski et al. 1997). Crystallographic studies with the LBDs of ERα and ERβ revealed that the AF-2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and that the position of helix 12 is altered by binding of ligands (Nilsson et al. 2001). When the ERα LBD is complexed with agonists such as E_2, helix 12 is positioned over the ligand binding pocket and forms an interaction surface for the recruitment of co-activators. In contrast, when either the ERα- or ERβ-LBDs are complexed with antagonists, helix 12 is displaced from its agonist position and occupies the hydrophobic groove formed by helices 3, 4, and 5, causing helix 12 to disrupt the co-activator interaction surface (Matthews & Gustafsson 2003; Brzozowski et al. 1997). It is evident that different ligands induce different receptor conformations and that the positioning of helix 12 is the key event that permits discrimination between estrogen agonists and antagonists (Nilsson et al. 2001).

For several years it was thought that the only mechanism through which estrogens affected transcription of E_2-sensitive genes was so-called classical ligand-dependent model where activated ER directly bind to EREs, specific DNA sequences found in the regulatory regions of estrogen-responsive genes (Hall et al. 2001; Nilsson et al. 2001). Today, there are known three other ER pathways through which are biological effects of E_2 mediated. The first one is ligand-independent activation of ER where growth factors or cyclic adenosine monophosphate activate intracellular kinase pathways, leading to phosphorylation and activation of ER at ERE-containing promoters in a ligand-independent manner. The second ER signaling mechanism is ERE-independent model where ligand-bound ERs interact with other transcription factor complexes like Fos/Jun (AP-1-responsive elements) or SP-1 (GC-rich SP-1 motifs) and influence transcription of genes whose promoters do not harbor EREs (Heldring et al. 2007). The last known mechanism is cell-surface (non-genomic) signaling in which E_2 activates a putative membrane associated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses (Hall et al 2001).

The classic pathway, where ERs are activated in a ligand-dependent manner, is the relevant signaling pathway for this study and will be, therefore, described in detail. This model states that, in the absence of hormone, ER is sequestered in a multiprotein complex with chaperones that stabilize

the receptor in an unactivated state or mask the DBD of the receptor (Gruber et al. 2002; Hall et al. 2001; Smith & Toft 1993). The exact location of ERs is not entirely clear. They are probably in an equilibrium distribution between the cytoplasm and the nucleus and the equilibrium is then shifted after ligand binding (Gruber et al. 2002). In response to ligand binding, ER undergoes conformational changes, termed "activation", accompanied by dissociation of hsp90, hsp70 and other receptor-associated proteins (Klinge 2001) (Fig. 2). The ligand–receptor complex then moves to the nucleus where two ER dimerize forming homo- (α/α, β/β) or heterodimers (α/β) (Gruber et al. 2002; Matthews & Gustafsson 2003). Liganded ER dimers further bind directly to specific DNA sequences called estrogen response elements (EREs), which are cis-acting enhancers located within the regulatory regions of target genes (Hallet al. 2001). The ERE was first identified by aligning sequences with shared homologies in the 5´flanking regions of the estrogen-regulated vitellogenin genes A1, A2, B1 and B2 from *Xenopus laevis* and chicken and the chicken apo-VLDLII gene (Walker et al. 1984). Many genes that contain EREs have been identified and a 13 bp palindromic inverted repeat 5′-GGTCAnnnTGACC-3′ (n = any nucleotide) was found to be the minimal consensus ERE sequence (Klein-Hitpass et al. 1988). However, only a handful of the most highly estrogen-responsive genes contain perfect consensus EREs. Many genes have been found to contain non-palindromic ERE sequences which vary from the consensus by one or more nucleotides (Driscoll et al. 1998) . ER dimer binding to ERE occurs by interaction of three specific amino acids within the P box of zinc finger CI in the major groove of DNA helix in a sequence-specific manner. Therefore, each ER monomer is bound to DNA in the major groove with the ER dimer located predominantly on one face of DNA helix. The DNA-bound receptors contact the general transcription apparatus either directly or indirectly via cofactor proteins. Examples of ER coactivators include, members of the p160/SRC (Steroid Receptor Coactivator) family: SRC1/NCoA1 (Nuclear Receptor Coactivator-1); NCoA2; NCoA3/AIB1/TRAM1/RAC3; the cointegrators: CBP (CREB-Binding Protein) and p300; and the family of CITED (CBP/P300-Interacting Transactivator, With Glu/Asp-Rich Carboxy-Terminal Domain) proteins (Moggs & Orphanides 2001). It is generally accepted that the ER-coactivator interactions stabilize the formation of a transcription preinitiation complex and facilitate the necessary disruption of chromatin at the ERE (Fig. 3). Depending on the cell and promoter context, the DNA-bound receptor exerts either a positive or negative effect on expression of the downstream target gene.

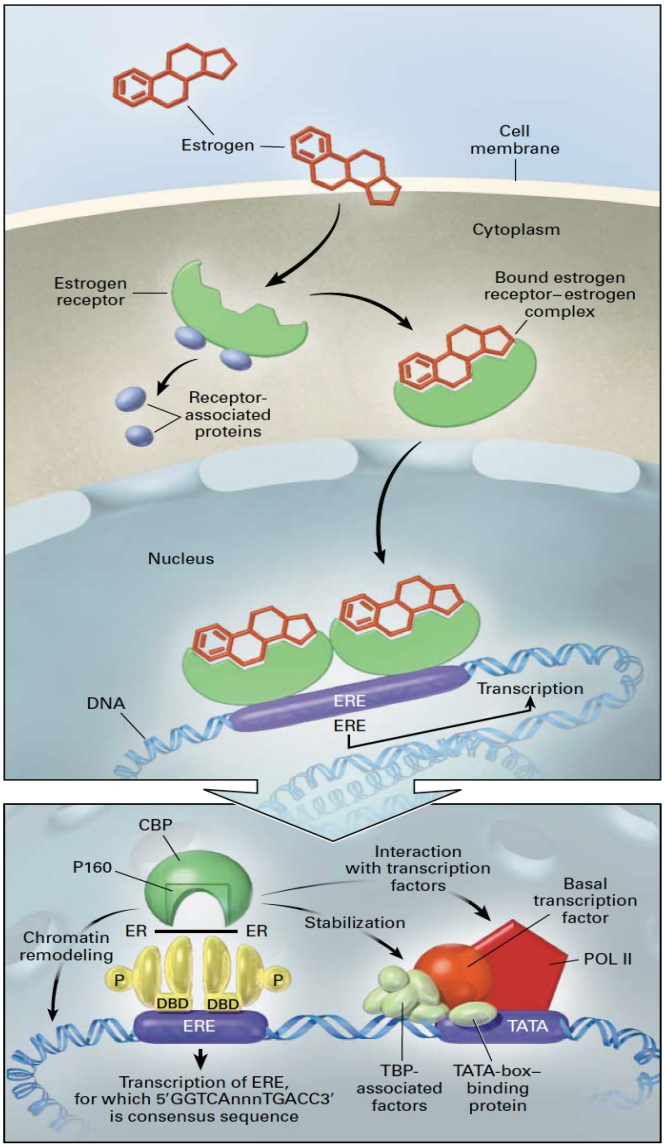


Figure 3. Classic Pathway of Estrogen Signal Transduction.

When an estrogen molecule binds to ER and the receptor dissociates from its cytoplasmic chaperones. The ligand–receptor complex then moves to the nucleus, where it binds to DNA and initiates transcription. Transcription is catalyzed by RNA polymerase II (POL II) and requires the assembly of various proteins, including the TATA-box–binding protein (TBP) and other associated factors at a TATA box. Other transcription factors join thereafter, completing the preinitiation complex. Activated estrogen receptors interact with several proteins, such as the 160-kD steroidreceptor coactivator protein (P160) and p300–cyclic AMP response-element–binding protein (CBP). This complex binds to the estrogen response element (ERE) through the DNA-binding domain (DBD) of the receptor and stimulates transcription; proposed mechanisms of stimulation include stabilization of the preinitiation complex, chromatin remodeling, and interaction with other transcription factors.

Source: Hall et al. 2001.

1.4. Fulvestrant

Fulvestrant (Faslodex®; Astrazeneca) is an estrogen receptor antagonist with no agonistic effects. It down-regulates cellular levels of the ER and blocks both AF-1 and AF-2 transcription-activating functions of ER (Howell et al. 2005; Wakeling et al. 1991). It is used in the treatment of ER-positive advanced breast cancer in postmenopausal women. The requisite for its development emerged from serious side effects of tamoxifen, which had been previously used in the therapy of breast cancer (Jaiyesimi et al. 1995). Tamoxifen is a selective estrogen receptor modulator (SERM), meaning that it exhibits tissue-specific activity. It acts as an antagonist in breast, but as agonist in

bone and uterus (Deroo & Korach 2006). With long-term use, tamoxifen's estrogen-like properties are associated with two- to three-fold increase in the risk of developing endometrial cancer (Jaiyesimi et al. 1995).

1.5. Cell line

This study was conducted using U2OS-ERα and U2OS-ERβ cell lines, which are characterized by inducible expression of ERα and ERβ, respectively. These two cell lines were obtained from Monroe et al. who developed them from the ER-negative U2OS parental cell line using T-REx™ System by Invitrogen, which will be further described. U2OS cell line is a human osteosarcoma cell line derived in 1964 from a moderately differentiated sarcoma of the tibia of a 15 year old girl. Cells exhibit epithelial adherent morphology (General cell collection: U-2 OS) The U2OS cell line was chosen mainly due to its bone-forming osteoblasts (OB)-like properties, their rapid growth, ease of transfection, and the lack of detectable endogenous ER expression (Monroe et al. 2003).
T-REx™ System by Invitrogen was used to generate cell lines which stably express equivalent levels of either ERα or ERβ, from ER-negative parental cell line. This system is a tetracycline-regulated mammalian expression system that uses regulatory elements from the E. coli Tn10-encoded tetracycline (Tet) resistance operon (Hillen & Berens 1994). The system utilizes two vectors: (1) pcDNA6/TRß vector, a regulatory plasmid which encodes the Tet repressor (TetR) under the control of the human cytomegalovirus immediate-early (CMV) promoter and (2) an inducible expression plasmid, pcDNA4/TOß which contains gene of interest (ERα or ERβ) under the control of the strong human CMV promoter and two tetracycline operator 2 ($TetO_2$) sites. Both plasmids were stably transfected into ER-negative U2OS cells by Monroe et al. In the absence of doxycycline (Dox), which is a Tet analog, the Tet repressor forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence in the promoter of the inducible expression vector and thus silences transcription of the cloned gene (ERα or ERβ gene). Upon addition, Dox binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor-Dox complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest (ERα or ERβ gene) (T-RExTM System - trexsystem_man.pdf)(Figure 4).

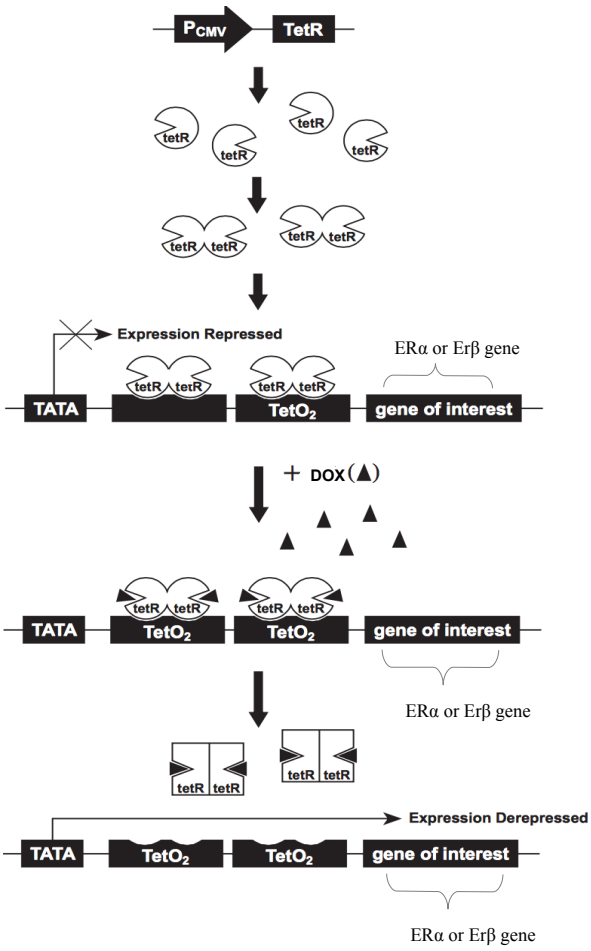


Figure 4. The mechanism and components of the T-REx™ System. TetR protein is expressed from pcDNA6/TR in cultured cells. TetR homodimers bind to $TetO_2$ sequences in the inducible expression vector, repressing transcription of the gene of interest (ERα and ERβ gene, respectively). Upon addition, Dox binds to TetR homodimers. Binding of Dox to TetR causes conformational change in TetR, release from the $TetO_2$ sequences and induction of transcription from the gene of interest (ERα or ERβ gene). Adapted from:T-REx™-System Manual Invitrogen.

1.6. Transfection

Transfection is the process by which nucleic acids are introduced into mammalian cells, stably or transiently. In transient transfection, the introduced nucleic acid exists in the cell only for a limited period of time and is not integrated into the genome. As such, transiently transfected genetic material is not passed from generation to generation during cell division, and it can be lost by

environmental factors or diluted out during cell division. Stable transfection introduces DNA into cells long-term. Stably transfected cells pass the introduced DNA to their progeny, typically because the transfected DNA has been incorporated into the genome, but sometimes via stable inheritance of nongenomic DNA. Common transfection methods are electroporation, calcium phosphate co-precipitation and cationic lipid mediated transfection (https://www.thermofisher.com). In this study, cationic lipid mediated transfection method was applied using Attractene Transfection Reagent by QIAGEN. Attractene Reagent is a nonliposomal lipid that forms a complex with DNA and enters the cell through endocytosis. It enables rapid fast-forward DNA transfection, meaning that cells are seeded and transfected at the same day. This is quicker, saves labor, and increases experimental flexibility compared to protocols where cells are seeded the day before transfection (https://www.qiagen.com).

1.7. Aims and objectives

The aim of this study was to evaluate a tropical African plant, *Mondia whitei*, for estrogenic and anti-estrogenic activity. For this purpose, methanol extract of the plant and 12 different fractions of the extract would be examined in U2OS-ERα and U2OS-ERβ cells. The principal investigation method would be estrogen receptor subtype specific transactivation assays. In addition, cytotoxicity of all herbal treatments would be verified using MTT assay. This study was conducted as a part of the "Angola Project" which aims to better, science based understanding of traditionally known medicinal uses of plants. The presented results were obtained in collaboration with the Instituto Nacional da Biodiversidade e Áreas de Conservação (INBAC) of the Ministério do Ambiente da República de Angola.

2. Materials and methods

2.1. Materials

Table 1. List of used chemicals

Chemical	Manufacturer
0.9% saline solution	TU Dresden
17 β-Estradiol	Sigma
4% CuSO4 x 5H2O solution	TU Dresden
Ampicilin solution (50 mg/ml)	TU Dresden
BC Assay Reagent A	Uptima
Blasticidin S (5 mg/ml)	Invitrogen
BSA (1 mg/ml)	Applichem
DCC	Biowest
DMEM/F12	Biowest
DMSO	Sigma
Doxycycline solution (10 µg/ml)	TU Dresden
Ethanol	TU Dresden
FCS	Biowest
MTT powder	Sigma
PBS	TU Dresden
Trypsin/EDTA (10x)	Biochrom
Zeocin (100 mg/ml)	Invitrogen

Table 2. Overview of used media and solutions

Medium/solution	Composition
1% DCC-supplemented medium	99 ml DMEM/F12 1 ml DCC (100%)
5% DCC-supplemented medium	190 ml DMEM/F12 10 ml DCC (100%)
10% FCS-supplemented medium	180 ml DMEM/F12 20 ml FCS (100%)

DMEM/F12	Provided by manufacturer in 1:1 ratio, already contains penicilin and steptomycin
Freezing medium	14 ml 10% FCS-supplemented medium 4 ml FCS (100%) 2 ml DMSO
Luria Bertani medium supplemented with Ampicilin	50 ml previously prepared Luria Bertani medium 0.05 ml Ampicilin (50 mg/ml)
MTT solubilization solution	100 ml Isopropanol 0.333 ml HCl (12N)
MTT working solution	50 mg MTT formazan powder dissolved in 10 ml PBS
Selective culture medium: 10% FCS-supplemented medium + Blastidicin S (2.5 mg/l) + Zeocin (250 mg/l)	50 ml 10% FCS-supplemented medium 0.025 ml Blasticidin S (5 mg/ml) 0.125 Zeocin (100 mg/ml)

Table 3. List of used kits

Kit	Manufacturer
Attractene Transfection Reagent	QIAGEN
Luciferase Assay System	Promega
Plasmid plus Midi Kit	QIAGEN

Table 4. Overview of used equipment

Equipment	Manufacturer
Spectrophotometer Specord 200	Analytik Jena
Analysis software WinASPECT	Analytik Jena
NanoDrop 1000 spectrophotometer	Thermo Scientific
Analysis software NanoDrop 1000	Thermo Scientific
Microplate Reader Infinite F200	Tecan
Analysis software Magellan	Tecan

Cell culture and *Mondia whitei* extracts and fractions

U2OS-ERα and U2OS-ERβ cell lines were provided by Monroe et al. working group. Methanol extract of *Mondia whitei* and its fractions were obtained from Prof. Stuppner working group (University of Innsbruck, Institute of Pharmay/Pharmacognosy). Powdered plant materials were extracted by methanol after defatting with petroleum ether and the extract was chromatographed using silica gel. HPLC elution profiles were used to identify fractions with similar profiles selected for the in vitro tests. Herbal extract and its fractions were then we diluted in DMSO.

2.2. Methods

2.2.1. Cell culture work

Thawing the cryopreserved U2OS-ERα and U2OS-ERβ cells

The U2OS-ERα and U2OS-ERβ cells used in this work had previously been stored by freezing in liquid nitrogen. Cell aliquots of both cell lines were taken out from cryogenic tank and warmed up at room temperature until they were completely thawed. Such cell aliquots were then transferred into centrifuge tubes with 5 ml selective culture media and centrifuged at 175 x g and room temperature for 5 minutes. Following centrifugation, supernatant was discarded while pellet was resuspended in 1 ml selective media and transferred into 25 cm^2 cell culture flasks with previously added 3 ml selective culture media. Cells were thus incubated for ca. 24 hours in the incubator.

Cultivation of U2OS-ERα and U2OS-ERβ cell lines

All the cell culture experiments were conducted in Class II biosafety cabinets. The cells were maintained in a 37 °C incubator with a humidified atmosphere of 5% CO_2 in air. All the used growth media were stored in refrigerator at 6 °C and prior to use were warmed shortly in a water bath at 37 °C. After thawing, both cell lines were first plated into 25 cm^2 cell culture flasks and following 24 hours incubation, were passaged into 75 cm^2 cell culture flasks in which they were further maintained. Selective medium was first used for cell cultivation following thawing. After few days, medium was changed to 10% FCS-medium, while few days prior to and during transactivation assays, cells were cultured in 5% DCC-medium.

Medium change and subculturing

The medium was changed approximately every second day in order to keep the cells healthy by providing fresh nutrients. The procedure involved removal of old medium using sterile glass pipette and addition of 10 ml fresh medium per 75 cm^2 cell culture flask. In cases when medium was changed from 10% FCS-medium to 5% DCC-medium, cells were rinsed twice with 5 ml PBS prior to addition of new medium.

When ca. 90% confluency had been reached, cells were subcultured (i.e., passaged). For that purpose, old medium was removed and cells were rinsed with 5 ml of PBS. Cells were then enzymatically detached from the surface of culture flask by addition of 3 ml of PBS and 330 µl of 10x Trypsin/EDTA and incubation for 5 minutes in the incubator. Following incubation, cells were observed under the microscope to check if most of the cells had detached. In few cases, only small amount of cells had detached, so the incubation was prolonged for 2 minutes which resulted in larger amount of detached cells. After that, detachment of the cells was stopped by addition of 2 ml of growth medium. The cell suspension was then transferred into 15 ml centrifuge tube and centrifuged at 175 x g and room temperature for 5 minutes. Following centrifugation, supernatant was discarded while pellet was resuspended in 1 ml growth medium. The appropriate amount of thus obtained cell suspension was then transferred into 75 cm^2 cell culture flasks with previously added 10 ml culture medium. Cells were passaged in different amounts, from ¼ to $^1/_{15}$ ratio, depending on the time point and growth rate. When passaged in ¼ ratio it took 2-3 days to reach 90% confluency, while $^1/_{15}$ ratio needed 6-7 days to reach 90% confluency. It has been observed that U2OS-ERβ cells grow slightly faster than U2OS-ERα cells.

Freezing U2OS-ERα and U2OS-ERβ cell lines

Since the highest recommended passage number for both cell lines is ca. 30 before occurrence of more genomic abnormalities, it was necessary to freeze the cells with lower passage number in multiple aliquots in liquid nitrogen to ensure cells for further experiments. The procedure was as same as for cell subculturing until the step following centrifugation, with the exception that cell detachment was stopped by addition of 2 ml freezing medium. After centrifugation, supernatant was discarded and pellet was resuspended in 1 ml freezing medium. The cell suspension was then transferred into cryogenic storage vials that were placed in a freezing container containing isopropyl alcohol. A freezing container was stored at -80°C. Later the cell aliquots were transferred into the fluid nitrogen tank for storage.

2.2.2. Purification of 2X ERE-TK-LUC plasmid from bacteria

Cultivation of bacteria *Escherichia coli*

2X ERE-TK-LUC reporter plasmid, which would be further used in transactivation assays, was purified from previously transformed bacteria *Escherichia coli*. Therefore, the first requirement was to grow an overnight suspension culture of *E. coli* that would be used for plasmid DNA purification. For this purpose, 50 ml of previously prepared Luria Bertani medium supplemented with 0,05 ml of ampicilin was added into 2 Erlenmeyer flasks. *E. coli* glycerol stock was taken out of the –80 °C freezer and thawed at room temperature. When thawed, 20 µl of bacterial aliquot was added into each Erlenmeyer flask. Bacterial culture was grown over the night (ca. 15 hours) in the shaker incubator.

The next morning, optical density of *E.coli* overnight culture was measured in order to estimate growth and metabolic activity of the cells. Optical density was measured at the spectrophotometer at wavelength of 690 nm and pure LB was used as a reference sample. Since measured values of bacterial optical density were above 0.6, bacterial cultures were transferred from incubator into the refrigerator at 6 °C until plasmid purification.

Plasmid DNA Purification using QIAGEN Plasmid Plus Midi Kit

2X ERE-TK-LUC plasmid was purified using QIAGEN Plasmid Plus Midi Kit high-yield protocol which will be further described. Firstly, bacterial suspensions were transferred from Erlenmeyer flasks into Nalgene™ centrifuge tubes and centrifuged in a high speed centrifuge at 6000 x g and 4 °C for 15 minutes. Following centrifugation, supernatant was discarded and pelleted bacteria were resuspended in the first lysis buffer, named Buffer P1. After that, 4 ml of Buffer P2 was added and the suspension was mixed by inversion, which resulted in the appearance of homogeneous blue coloration, and incubated at room temperature for 3 minutes. Buffer S3 was added next and the lysate was mixed by inverting 4-6 times, so the suspension became colorless again with visible white precipitate of genomic DNA, proteins, cell debris, and SDS. Before transferring the lysate to the QIAfilter cartridge, it was centrifuged at 4500 x g and 4 °C for 5 minutes in order to ensure convenient filtration without clogging. Following centrifugation, the clear supernatant was poured into the QIAfilter cartridge and the cell lysate was filtered into a new tube. 2 ml of Buffer BB was then added to the cleared lysate and the suspension was mixed by inverting 6 times. Lysate was further transferred to a QIAGEN Plasmid Plus Midi spin column with a tube extender attached on the QIAvac 24 Plus vacuum manifold. Vacuum source was then switched on, causing the draw of the solution through the QIAGEN Plasmid Plus Midi spin column after which the vacuum source

was switched off again. After that, the tube extender was removed and the bound DNA was washed with 0.7 ml of Buffer ETR using vacuum. Further washing of the DNA was continued with 0.7 ml of Buffer PE, using vacuum again. In order to completely remove the residual wash buffer, the column was then centrifuged at 10,000 x g for 1 min in a microcentrifuge. Following centrifugation, the column was placed into a new 1.5 ml microcentrifuge tube. At the end, the bound plasmid DNA needed to be eluted by adding 100 µl of water and centrifugation at 10,000 x g for 1 min in a microcentrifuge. Thus obtained aqueous solution of plasmid DNA was stored in a -20°C freezer.

Determination of purified plasmid DNA concentration

Concentration of purified plasmid DNA was measured using a NanoDrop™ spectrophotometer. For this purpose, stored plasmid DNA was first taken out of -20°C freezer and thawed at room temperature. To initialize the spectrophotometer, a blank measurement was performed using 1 µl of clean deionized water. After that, concentration of plasmid DNA samples was measured from 1 µl volume of the samples. Following measurement, one sample of plasmid DNA was discarded because of very low concentration value, while the others had satisfactory concentration values and were stored back in a -20°C freezer. The next step would be to verify purified DNA on gel electrophoresis. This was not performed, so in transactivation assays other previously purified and verified plasmid DNA samples were used.

2.2.3. Transactivation assay

Medium change to 5% DCC-medium

The first step was medium change of cultured U2OS-ERα and U2OS-ERβ cells from 10% FCS-medium to 5% DCC-medium in order to reduce estrogen levels in fetal calf serum. This was performed 4 to 5 days prior to transfection.

Doxycycline addition

Doxycycline was added ca. 24 hours prior to transfection to reactivate expression of ERα and ERβ genes, respectively. The final concentration of 100 ng/ml had been previously determined by Monroe et al. as the appropriate (Monroe et al. 2003). For this purpose, 100 µl of doxycycline solution [10 µg/ml] was added per each flask containing 10 ml 5% DCC-medium.

Cell seeding in 24-well plate and transfection with the reporter plasmid

Transfection was performed according to QIAGEN´s Fast-Forward Protocol, which allows cell seeding and transfection on the same day.

The optimal amount of cells for transfection should have been 80,000 cells per well, so for that purpose, cells were counted in Neubauer chamber. Firstly, they were enzymatically detached from the surface of culture flask and resuspended in 5% DCC-medium after centrifugation. 20 µl of the prepared cell suspension was then introduced into Neubauer chamber and the cell count was performed. Cell suspension for seeding was further prepared by diluting appropriate amount of cell suspension in corresponding amount of 5% DCC-medium, so that 500 µl of the cell suspension was dispensed in each well. Doxycycline, in final concentration of 100 ng/ml, was added to cell suspension for seeding as well.

Transfection mix, consisting of serum-free medium (DMEM/F12), ATR and 2X ERE-TK-LUC plasmid was prepared, so that its final volume was 60 µl per well. Total plasmid DNA should have been 0.4 µg per well and the volume of plasmid depended on its concentration. Subsequently, the appropriate volume of ATR [0.75 µl/well] was added. Thus prepared transfection mix was incubated for 10 minutes at room temperature and then added to each well drop by drop. Plate with seeded and transfected cells was incubated for ca. 24 hours in the incubator.

Treatment of the cells with test substances

In this work, a treatment with following substances was undertaken: DMSO, 17β-estradiol, fulvestrant, methanol extract of *Mondia whitei* alone and in combination with fulvestrant and 12 different fractions of *Mondia whitei* extract alone and in combination with fulvestrant. Each treatment was performed in triplicates on the 24-well plate. For this purpose, ca. 24 hours post transfection, old medium was removed and the new medium, in which test substances had already been mixed in the appropriate concentration, was applied on the cells in the volume of 500 µl per well. The new medium consisted of 5% DCC-medium, doxycycline in final concentration of 100 ng/ml and a test substance in the appropriate concentration. It is important to remark that DMSO, as a solvent for test substances, should be used in the final concentration of 0.1%, since it can have cytotoxic effects in higher concentrations. Accordingly, concentration of the stock solutions of test substances was equal to 1000 times their concentration in the well. After applying the treatment, cells were incubated for ca. 24 hours in the incubator.

Luciferase assay

Approximately 22-26 hours post treatment, measurement of the luciferase enzyme luminescent activity was performed. Firstly, cell lysate was prepared by removing the old medium using vacuum

pump, rinsing cells twice with 500 µl unsterile PBS and addition of 100 µl 1X Lysis Buffer in each well. Plates were further frozen at -80 °C for 30 minutes to 2 hours. After thawing plates, cells were scraped from the bottom of wells and cell lysates was transferred into 1.5 ml Eppendorf tubes on ice. When all the lysates had been transferred, they were incubated for 3 minutes at room temperature and then thoroughly vortexed. Each thus prepared sample was then pipetted in duplicates into white 96-well plate, in the volume of 10 µl per well. After that, 50 µl of Luciferase Assay Reagent was added in each well and the produced luminescence was measured at the microplate photometer.

BCA protein assay

Bicinchoninic acid (BCA) protein assay was performed in order to determine total protein concentration in the samples. The assay is based on reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. Bovine serum albumin (BSA) in 0.9% sodium chloride (saline) solution was used as reference standards. Standards covered the range of concentrations from 0 mg/ml to 1 mg/ml and were prepared by diluting BSA stock solution [1 mg/ml] in appropriate amount of 0.9% saline solution. 1X Lysis Buffer was used as blank. All the standards, blank and all the samples were pipetted in triplicates into transparent 96-well plates, in the volume of 10 µl per well. After that, BCA working reagent was prepared by mixing 50 parts of reagent containing bicinchoninic acid with 1 part of 4% $CuSO_4$ x 5 H_2O until the solution was uniform light green color. 250 µl of thus prepared solution was then added into each well and plates were shortly put on a plate shaker to mix each well thoroughly. Plates were further incubated with a lid for 30 minutes in the incubator at 37 °C. Following incubation, absorbance at 560 nm was measured at the microplate photometer.

Calculation of relative luciferase activity and statistical analysis

In order to calculate relative luciferase activity, the measured luminescence per 10 µl was extrapolated to luminescence per 1 ml which was then divided by protein concentration [mg/ml]. Triplicate values [RLU/mg protein] for each treatment group (three wells treated with the same test substance) were then averaged. Value of DMSO treatment was set up as 100% and values of all other treatments were set relative to DMSO in order to obtain relative luciferase activity [% RLU/mg protein]. Described calculation was performed using Open Office.

Statistical analysis, which included one-way ANOVA and Tukey´s test, was performed using Origin software application in order to determine statistical significance of the obtained results. Analysis took in account mean values of three independent experiments and the results were

considered relative to DMSO. An obtained p-value ≤ 0.05 (*) was considered significant, while p-values ≤ 0.01 (**) and p ≤ 0.001 (***) were considered as highly significant.

2.2.4. MTT assay

The purpose of this assay was to evaluate potential cytotoxic effects of the applied *Mondia whitei* methanol extract and fractions. The assay is based on the ability of viable cells to convert a soluble yellow colored tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into an insoluble formazan precipitate. The purple colored formazan crystals may be further dissolved in the variety of organic solvents and the optical density of the resulting solution was measured at a spectrophotometer (Mosmann 1983).

Medium change to 5% DCC-medium

Like transactivation assays, MTT assays started with medium change from 10% FCS-medium to 5% DCC-medium 3 to 4 days prior to treatment.

Doxycycline addition

Doxycycline was added ca. 24 hours prior to treatment. Used volume and concentrations were as same as in transactivation assays.

Cell seeding in 96-well plates and treatment with test substances

Optimal amount of U2OS-ERα and U2OS-ERβ cells per well for MTT assay should have been 7500 cells in 100 µl growth medium. In order to prepare cell suspension with appropriate amount of cells, cultured cells were first counted in Neubauer chamber as described previously for transactivation assays. After counting, cell suspension for seeding in 96-well plate was prepared by diluting appropriate amount of cell suspension in corresponding amount of 1% DCC-medium with addition of doxycycline in the final concentration of 100 ng/ml. Cells were plated into all wells on 96-well plate, except the first row vertically. This row was filled with medium alone to provide the blanks for absorbance readings.

Cells were treated immediately after seeding. Performed treatment included the same substances in the same concentrations as in transactivation assays in order to measure their cytotoxicity. For this purpose, solutions consisting of appropriate amount of 1% DCC-medium, doxycycline and test substances were prepared. Treatments were applied in the volume of 100 µl per well and each treatment group was performed eightfold per experiment. Treatment was not applied to the first row

vertically which, as mentioned previously, contained medium only and the last row vertically which contained untreated control cells. Instead, these two rows received solution of 1% DCC-medium and doxycycline without any test substances in the volume of 100 µl per well. Plated and treated cells were incubated for 3 days (ca. 65 hours) in the incubator at 37°C and 5% CO2.

Measurement of cell viability

After approximately 3 days of incubation, 10 µl of MTT solution was added into each well including controls and cultures were returned to incubator for ca. 90 minutes. To prepare MTT solution, 50 mg of MTT powder was dissolved in 10 ml of PBS and the solution was filtered through 0.22 µm Millipore™ Filter. MTT is light sensitive, so the prepared solution was stored at 6 °C in the dark. Since storage of reconstituted MTT solution at 2-8 °C for more than 2 weeks may cause decomposition and yield erroneous results, the fresh MTT solution was frequently prepared. Incubation of cells with MTT solution was periodically interrupted to check under the microscope whether the formation of formazan crystals had began. Since formazan crystals may damage and destroy cells, incubation with MTT solution should be stopped as soon as they start to form. In this case, incubation period was approximately an hour and a half.

After incubation, cell cultures were removed from the incubator and the media was removed using vacuum. The resulting formazan crystals were dissolved by addition of 100 µl MTT solubilization solution per well. A mixture of isopropanol with 0.04 N HCl was used as the solubilization solution. Dissolution of formazan crystals was enhanced by gentle agitation and the absorbance was measured at microplate photometer at a wavelength of 540 nm, with 690 nm as a reference.

Calculation of relative absorbance and statistical analysis

The results of MTT assay were expressed as relative absorbance that was calculated using Open Office. First it was necessary to determine the average values from eightfold values (one treatment group) and subtract the average value for the blank. Obtained value of DMSO treatment was set up as 100% and values of all other treatment groups were represented relative to DMSO. Statistical analysis was performed as same as previously described in transactivation assays. Data of three independent experiments were took in account and the results were considered relative to DMSO, with p-value ≤ 0.05 (*) as significant and p-values ≤ 0.01 (**) and $p \leq 0.001$ (***) as highly significant.

3. Results

This study aimed to test *Mondia whitei* methanol extract and its fractions for estrogenic and anti-estrogenic activity in human bone-derived U2OS-ERα and U2OS-ERβ cells using estrogen receptor subtype-specific transactivation assays. In addition to that, applied extract and fractions were evaluated for cytotoxicity using MTT assay.

Prior plant extracts transactivation assays, U2OS-ERα (A) and U2OS-ERβ (B) cells were treated with 17β-estradiol in a wide range of concentrations in order to construct a dose-reponse curve of 17β-estradiol (Fig. 5).

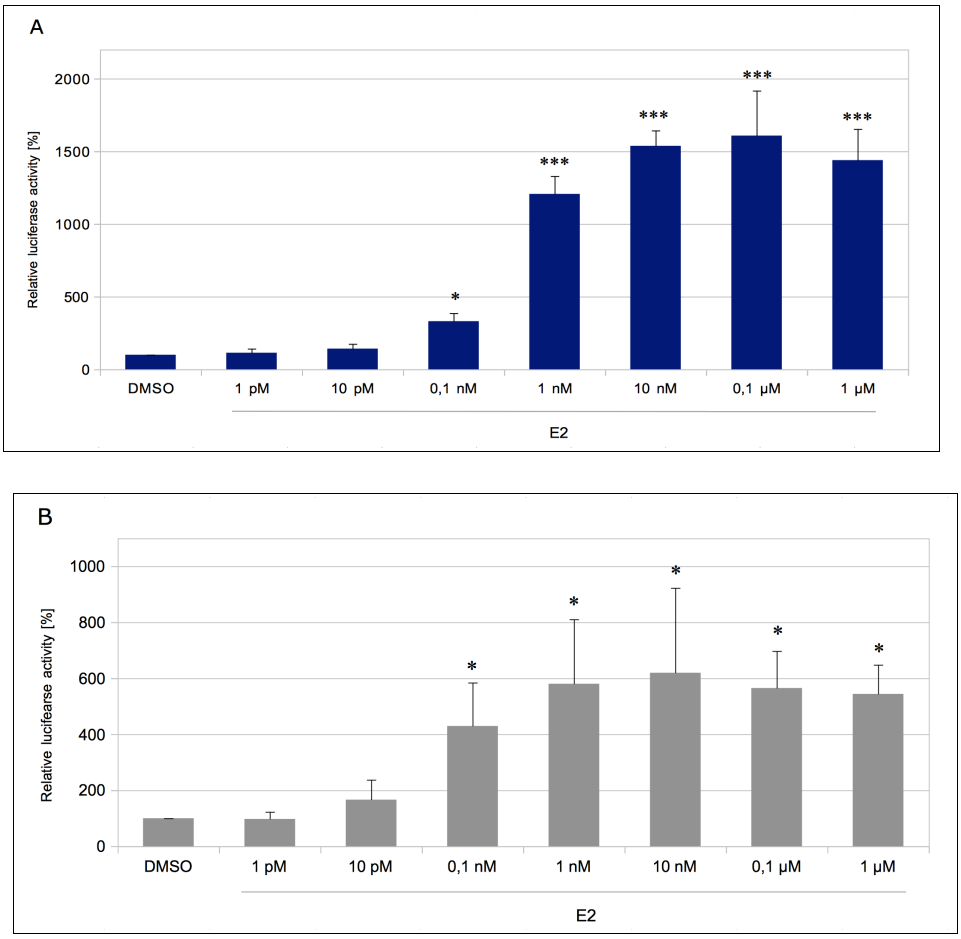
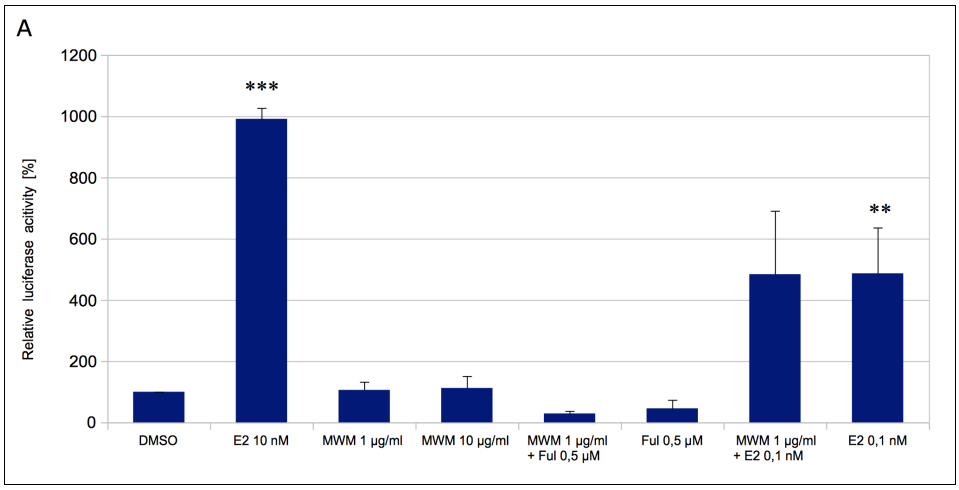


Figure 5. Dose-response curve of 17β-estradiol in U2OS-ERα (A) and U2OS-ERβ (B) cells. Both cell lines were treated with E_2 in the concentration range of 1 pM to 1 µM. DMSO was used as a negative control. Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean relative luciferase activity ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to DMSO as follows: * $p \leq 0{,}05$; ** $p \leq 0{,}01$; *** $p \leq 0{,}001$.

Figure 5 shows relative luciferase activity of U2OS-ERα (A) and U2OS-ERβ (B) cells after treatment with various concentrations of E_2, in the range from 1 pM to 1 µM. DMSO was used as a negative control in all experiments, so the value of that treatment was set as 100% and values of all other treatments were expressed relative to DMSO. It can be observed that estradiol dose dependently induced an increase of the luciferase activity, which is followed by a plateau phase in both cell lines. U2OS-ERα curve exhibits steeper slope than U2OS-ERβ curve. Two lowest applied concentrations just slightly increased luciferase activity compared to the negative control. A significant increase in luciferase activity occurred from concentration 0,1 nM upwards in both U2OS-ERα and U2OS-ERβ cell line. In U2OS-ERα cells a maximal, 16-fold increase was induced by 0.1 µM E_2 concentration, whereas in U2OS-ERβ cells a maximal observed luciferase activity was approximately 600% at 10 nM concentration. Overall, 10 nM concentration of estradiol displayed strong and significant increase of the luciferase activity in both cell lines, and was therefore selected as a positive control in further experiments with plant extracts. Results obtained from this experiment showed dose-response effects of estradiol in both U2OSERα and U2OS-ERβ cell line, suggesting that the selected test system is suitable for the study of plant extracts and fractions.

First, methanol extract of *Mondia whitei* (MWM), in two concentrations, was evaluated for estrogenic and anti-estrogenic properties (Fig. 6).



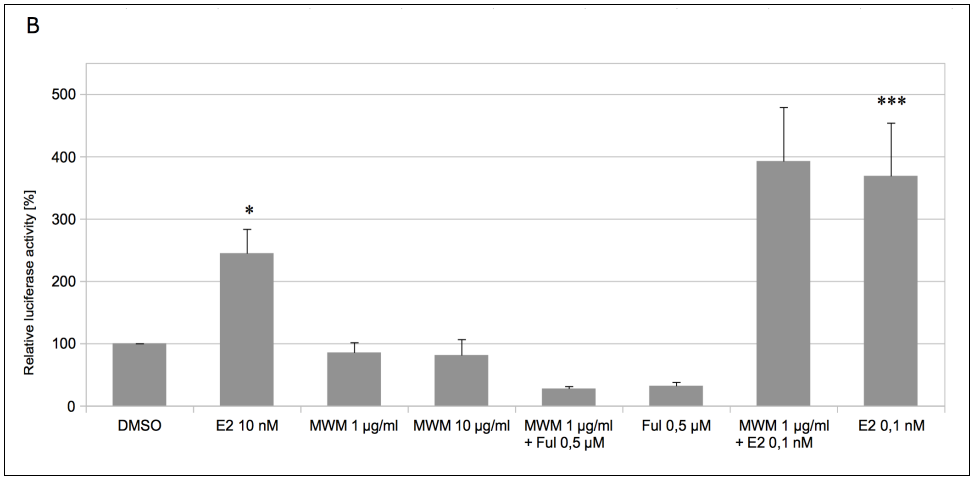


Figure 6. Relative luciferase activity of *Mondia whitei* methanol extract (MWM) in U2OS-ERα (A) and U2OS-ERβ (B) cells.
Performed treatments include negative control (DMSO), positive control (10 nM E_2), methanol extract of Mondia whitei in two concentrations, fulvestrant (ER-antagonist) alone and in combination with MWM, 0,1 nM E_2 alone and in combination with MWM. the concentration range of 1 pM to 1 µM. Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to DMSO as follows: * $p \leq 0{,}05$; **$p \leq 0{,}01$; ***$p \leq 0{,}001$.

Figure 6. presents results of the first MWM extract study. Again, data of three independent experiments are represented relative to the DMSO control (100%). As mentioned before, 10 nM E_2 was used as a positive control. Luciferase activity induced by both lower (1 µg/ml) and higher (10 µg/ml) concentrations of MWM extract was about as same as luciferase activity of DMSO control in both cell lines, suggesting that *Mondia whitei* methanol extract does not possess neither estrogenic nor anti-estrogenic activity. In order to confirm estrogenic activity, treatment with MWM extract in combination with ER-antagonist Fulvestrant was performed. In both cell lines, luciferase activity of that treatment was not significantly different from Fulvestrant alone treatment, what confirms no estrogenic-like properties of MWM extract. To evaluate anti-estrogenic activity, treatment with MWM extract in combination with 0.1 nM E_2 was performed. 0.1 nM E_2 concentration was chosen based on the dose-response curve and previous conducted studies which have shown that this is the concentration that produced about 50% of maximal effect. Luciferase activity of MWM extract and 0,1 nM E_2 co-reatment reached approximately as same value as 0.1 nM E_2 alone treatment in both U2OS-ERα and U2OS-ERβ cells, confirming no anti-estrogenic-like properties of methanol extract of *Mondia whitei*. However, in U2OS-ERβ cell line luciferase activity of positive control was unexpectedly low comparing to dose-response curve and even lower than 0.1 nM E_2 treatment, so the results for U2OS-ERβ cells should be interpreted with caution.

After testing *Mondia whitei* methanol extract, fractions of *Mondia whitei* extract were evaluated for estrogenic properties (Fig. 7).

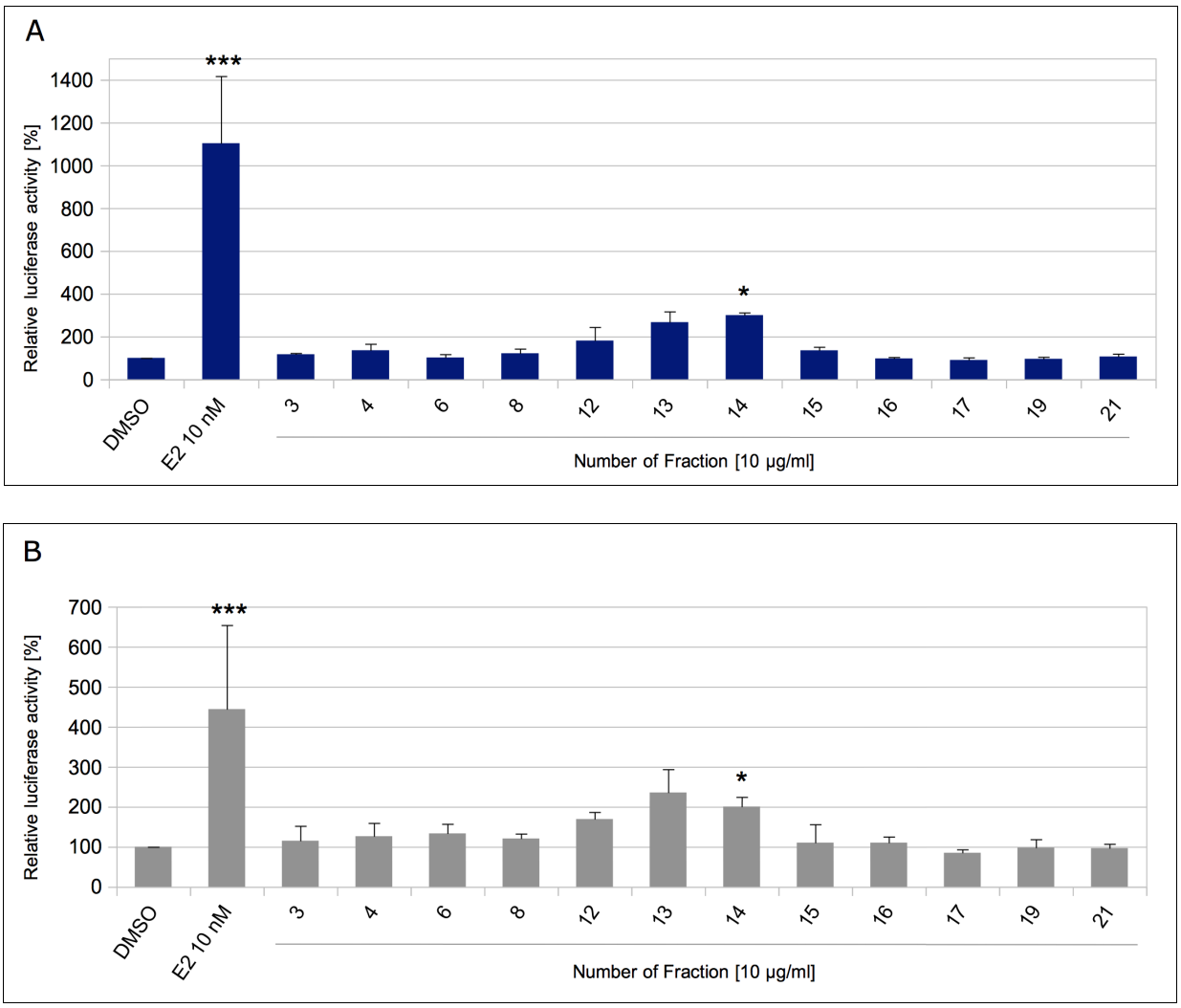


Figure 7. Relative luciferase activity of the fractions of MW extract in U2OS-ERα (A) and U2OS-ERβ (B) cells. U2OS-ERα (A) and U2OS-ERβ (B) cell lines were treated with 12 different fractions of *Mondia whitei* extract in concentration of 10 µg/ml . DMSO was used as a negative control and10 nM E_2 as a positive control. Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to DMSO as follows: * $p \leq 0{,}05$; **$p \leq 0{,}01$; ***$p \leq 0{,}001$.

Results obtained from study on 12 different fractions of MW extract for estrogenic activity are presented in Figure 7. Data are represented as described in previous experiments, with DMSO as a negative control and 10 nM E_2 as a positive control. Fraction number 14 in 10 µg/ml concentration displayed a significant increase in luciferase activity compared to DMSO control in both U2OS-ERα and U2OS-ERβ cell line. More precisely, increased luciferase activity was ca. 350% in U2OS-ERα cells, whereas in U2OS-ERβ cells a 2-fold increase was observed. An increase of luciferase activity was also shown for Fraction number 13, especially in U2OS-ERβ cells, but it was not considered significant because of high standard deviation values. All other tested fractions

displayed approximately as same luciferase activity values as negative control, suggesting they do not possess estrogen agonist activity.

Since obtained results suggested estrogenic-like activity of Fraction 14, it was decided to further investigate this fraction. The next step was, therefore, a dose-response curve of Fraction 14 (Fig. 8).

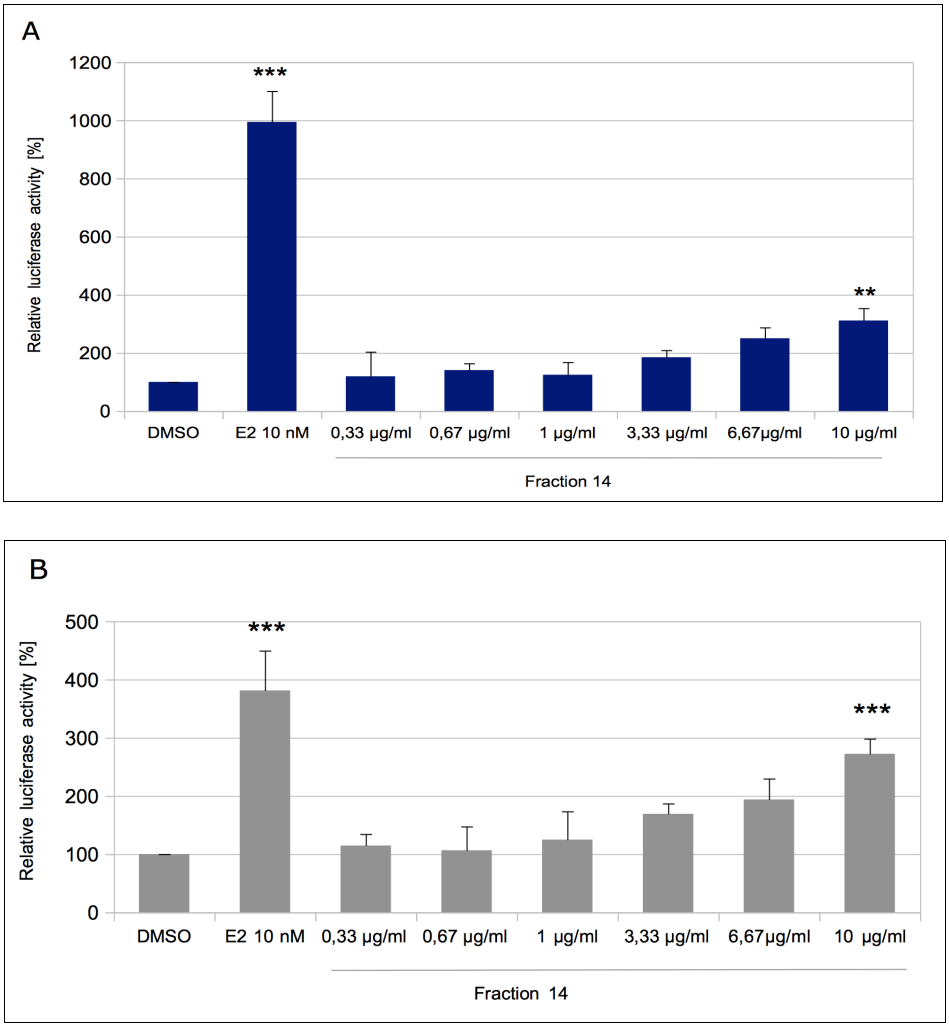


Figure 8. Dose-response curve of Fraction 14 of MW extract in U2OS-ERα (A) and U2OS-ERβ (B) cells.
Both cell lines were treated with Fraction number14 in the concentration range of 0,33 µg/ml to 10 µg/ml. DMSO was used as a negative control and10 nM E_2 as a positive control. Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to DMSO as follows: * $p \leq 0,05$; **$p \leq 0,01$; ***$p \leq 0,001$.

Figure 8. shows relative luciferase activity of U2OS-ERα (A) and U2OS-ERβ (B) cells after treatment with various concentrations of Fraction 14 of MW extract. Applied concentrations were 1 µg/ml and its 2:3 (0,67 µg/ml) and 1:3 (0,33 µg/ml) dilutions and 10 µg/ml which was diluted in ratios 2:3 (6,67 µg/ml) and 1:3 (3,33 µg/ml) as well. Used negative and positive control and principle of data representation were as same as in the previous experiments. It can be observed that

the lowest concentration of Fraction 14 that produced a significant estrogenic effect is 10 µg/ml in both U2OS-ERα and U2OS-ERβ cells. This concentration induced highly significant increase in luciferase activity in both cell lines, but especially in U2O-ERβ cells where the luciferase activity reached ca. 70% of the luciferase activity in E_2 treatment. Concentrations in the range from 0.33 µg/ml to 1 µg/ml induced approximately as same level of luciferase activity as DMSO negative control, whereas, concentrations of 3.33 µg/ml and 6.67 µg/ml induced detectible, but not significant increase in luciferase activity in both cell lines.

The last transactivation assay experiment was investigation of anti-estrogenic properties of fractions of *Mondia whitei* extract (Fig. 9).

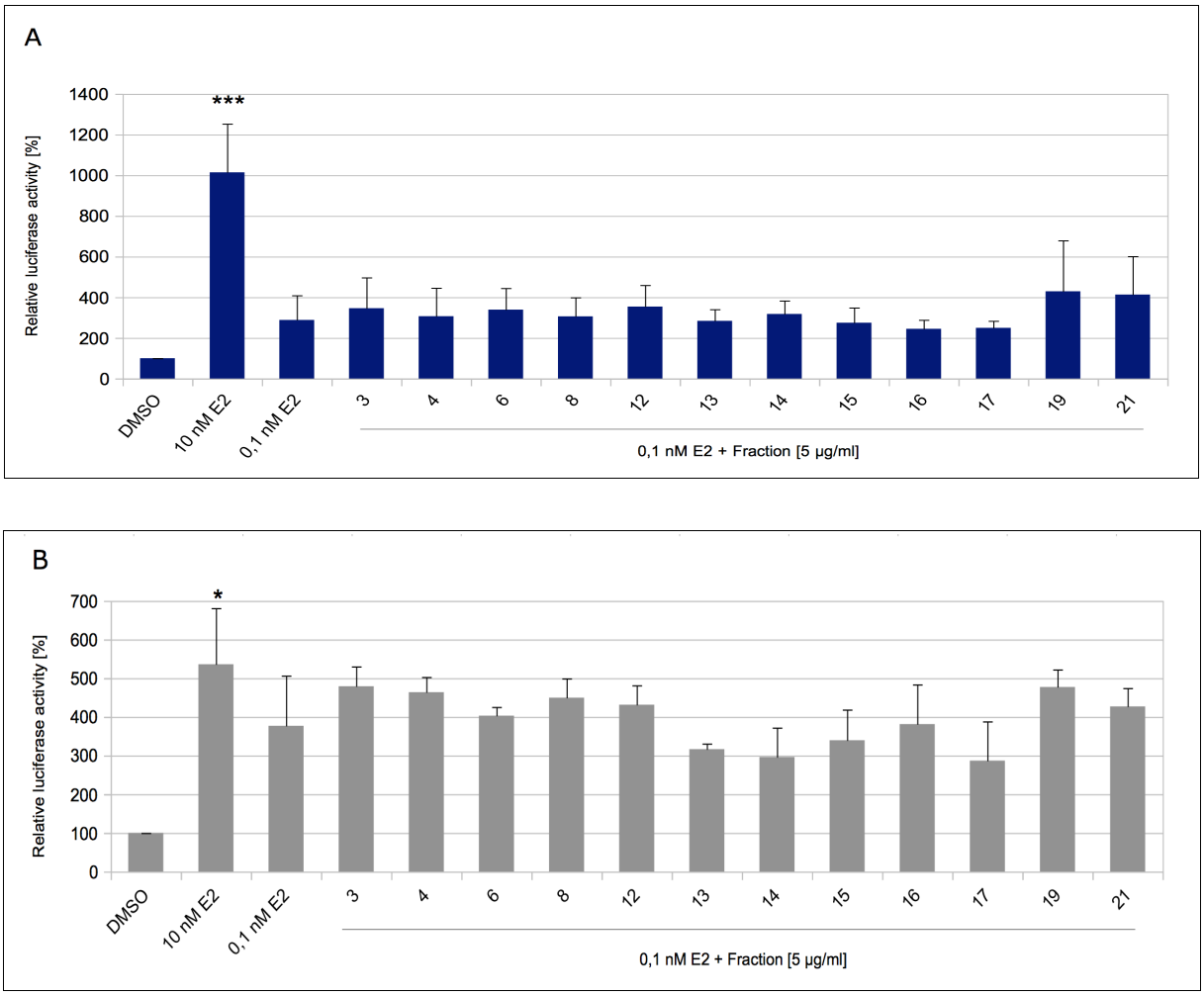


Figure 9. Evaluation of fractions of *Mondia whitei* extract for anti-estrogenic potential in U2OS-ERα (A) and U2OS-ERβ (B) cells.
Treatment for both cell lines included DMSO as a negative control, 10 nM E_2 as a positive control, 01, nM E_2 alone and in combination with each fraction. Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean relative luciferase activity ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to DMSO as follows: * $p \leq 0{,}05$; **$p \leq 0{,}01$; ***$p \leq 0{,}001$.

Results obtained from the experiment on anti-estrogenic activity of fractions of MW extract in U2OS-ERα and U2OS-ERβ cells are presented in Figure 9. Data are represented as described in previous experiments, with DMSO as a negative control and 10 nM E_2 as a positive control. Anti-estrogenic potential of fractions was evaluated by co-treatment with ER-agonist, estradiol in 0.1 nM concentration. Apart from that, treatment with 0.1 nM E_2 alone was performed for comparison. For evaluation of anti-estrogenic potential, relevant comparison is not 0.1 nM E_2 + fractions versus DMSO, but 0.1 nM E_2 + fractions versus 0.1 nM E_2 alone. In both cell lines, luciferase activities induced by all the fractions and 0.1 nM E_2 co-treatments were higher than luciferase activity of DMSO because of estrogenic activity of estradiol. On the other hand, all the fractions in co-treatment with 0.1 nM E_2 induced approximately same levels of luciferase activity as 0.1 nM E_2 alone. These data suggest that none of the fractions possess anti-estrogenic activity in neither U2OS-ERα nor U2OS-ERβ cells.

In addition to evaluation of estrogenic and anti-estrogenic activity, applied *Mondia whitei* extracts and fractions were tested for cytotoxicity (Fig. 10).

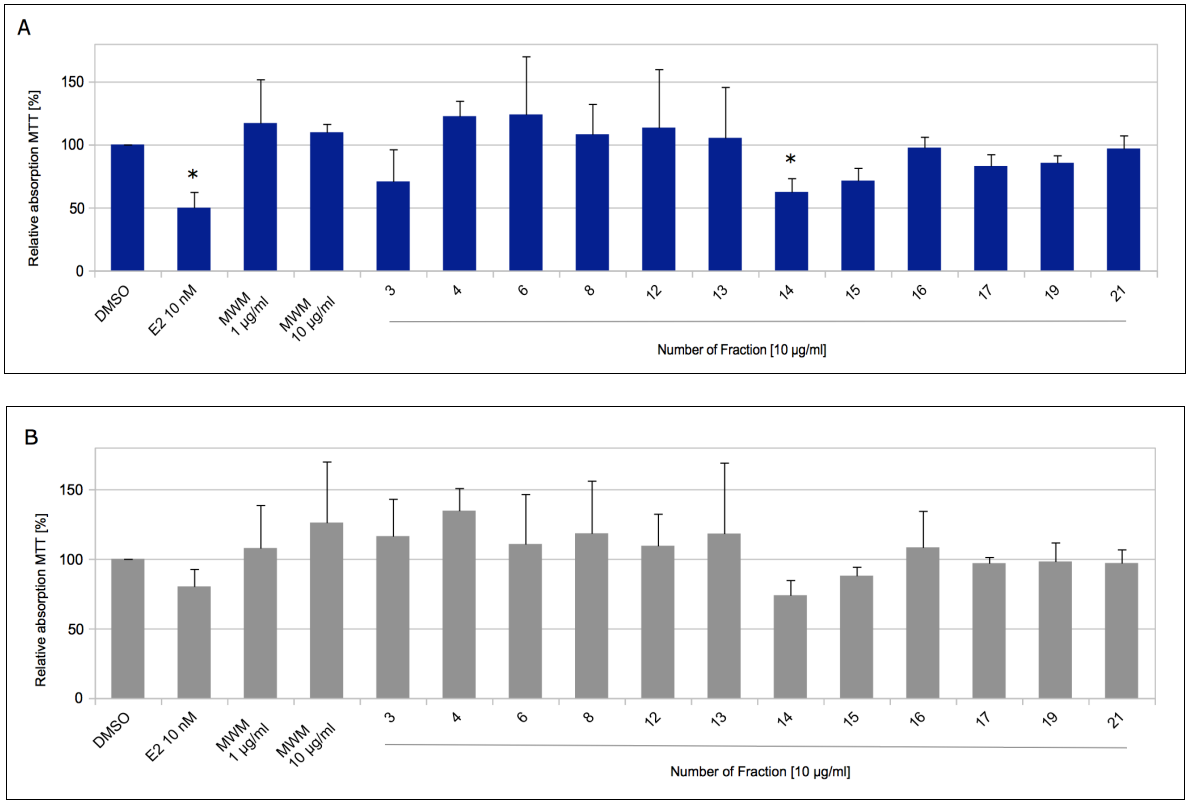


Figure 10. Effect of *Mondia whitei* methanol extract and its fractions on the cell viability determined by MTT assay in U2OS-ERα (A) and U2OS-ERβ (B)cells.
Data of three independent experiments are represented relative to the DMSO control (100%) and expressed as the mean ± standard error. Each treatment group was performed eightfold per experiment. Statistical significance is indicated relative to DMSO as * $p \leq 0{,}05$.

Cytotoxicity of *Mondia whitei* methanol extract and its fractions was measured by MTT assay and the results are presented in Figure 10. Treatment included MWM extract and 12 fractions in the same concentrations which were applied in transactivation assays. Used negative (DMSO) and positive control (10 nM E_2) were as same as in transactivation assays as well. Data of three independent experiments are again represented relative to the DMSO control. In U2OS-ERα cells, none of the applied herbal extracts nor fractions displayed significant cytotoxicity, except the fraction 14. This results, however, should be interpreted with caution because the positive control showed even stronger cytotoxic effect, with only 50% relative absorption value compared to the DMSO control. In U2OS-ERβ cells, none of the applied treatments significantly affected cell viability. Here as well, the strongest cytotoxic effect was detected in 10 nM E_2 and fraction 14 treatment, but it was not statistically significant.

4. Discussion

4.1. Dose-response curve of 17β-estradiol

Prior investigation of plant extract and fractions, a dose-response curve of endogenous ER-agonist, estradiol, was constructed in order to confirm the suitability of the selected test system for the study.

A dose-response relation between the concentration of E_2 and relative luciferase activity was observed in both U2OS-ERα and U2OS-ERβ cell line (Fig. 5) and the results are, in general, comparable with previously conducted studies (Kurras 2013; Moeller et al. 2007). A dose-response effect of estradiol was demonstrated as a sigmoidal curve, where low doses of ligand had no significant effect and high doses plateau at maximal biological response. A plateau phase starts when all the receptor binding sites are occupied, so further increase in ligand concentration will not induce a higher response than the maximum, which had been already achieved. As the E_2 concentration that provokes a maximal response, i.e., increases luciferase activity the most, previous studies report 10 nM (Kurras 2013; Moeller et al. 2007). This concentration has been shown in this experiment as well, to produce a maximal response in U2OS-ERβ cells and strong and significant increase of luciferase activity in U2OS-ERα cells. Therefore, 10 nM concentration of estradiol was selected as a positive control in further experiments. 0.1 nM concentration was reported by Moeller et al. (2007) to provoke a response halfway between the baseline and the maximum, meaning that is the concentration where 50% of receptor binding sites are occupied. Even though that was not confirmed in this experiment, 0.1 nM E_2 concentration was selected in further experiments for validation of potential ER-mediated anti-estrogenic activities of herbal treatments. A co-treatment with estradiol is necessary because antagonists express their activity by blocking agonist-mediated responses rather than provoking a biological response itself upon binding to a receptor.

Some deviations from previous studies could be attributed to the fact that the cells had been thawed only few days before starting the experiment. Cells after thawing may need some time to reach again the full efficiency of their physiological processes.

In addition, DMSO, which was used as a negative control, also induced a certain level of luciferase activity. This proves that, despite using dextran coated charcoal, the serum is not completely steroid-free and there is still low, but detectable background estrogenic activity left. Consequently, weak activities of test substances could overlap with estrogenic potential of the serum.

4.2. Investigation of *Mondia whitei* methanol extract and its fractions in regard to estrogen receptors α and β

In this work, methanol extract of *Mondia whitei* and its fractions were evaluated for estrogenic and anti-estrogenic properties. For methanol extract, no agonistic nor antagonistic activity was detected in neither U2OS-ERα nor U2OS-ERβ cell line (Fig. 6). However, results for U2OS-ERβ cells should be interpreted with caution because of unexpectedly low positive control value. Experiment of MWM extract included treatment with methanol extract alone and in combination with fulvestrant and estradiol. As mentioned above, co-treatment with estradiol is performed to confirm ER-specific anti-estrogenic effect. On the other hand, a co-treatment with fulvestrant is performed in order to verify that potential estrogenic active compounds act through ER-mediated mechanism. Fulvestrant was applied in 0.5 µM concentration, based on some previous studies which have shown that is the concentration which almost completely inhibits 10 nM estradiol-mediated effects (Moeller et al. 2007). There are several possible explanations for deficiency of estrogenic and anti-estrogenic activity of MWM extract. Firstly, extract is a mixture of heterogeneous compounds which can affect each other and that can in turn alter their ability of binding to ERs. It is possible that there are both agonistic and antagonistic compounds present in the extract, which can overlap and annul each other´s effects. In addition to that, compounds may also affect the expression of cofactors what can further influence expression of estrogen-regulated genes. Moreover, Briskin (2000) reported that the medicinal effects of plant materials typically result from the combinations of secondary products present in the plant and secondary metabolite production is influenced by numerous environmental factors. Therefore, a content of specific bioactive compounds may vary between the individual plants within the same species, and also depending on the part of the plant and time of harvest.

Evaluation of *Mondia whitei* for estrogenic and anti-estrogenic activity was further continued with experiments on fractions of the MW extract. Among all the tested fractions, only fraction number 14 displayed significant increase in luciferase activity compared to DMSO control (Fig. 7). These results suggest low, but statistically significant estrogenic activity of fraction 14 in both U2OS-ERα and U2OS-ERβ cells, meaning that fraction 14 contains potential estrogenic active constituents. This fraction was further examined in a wider concentration range. A dose-response curve of fraction 14 shows an increase in the luciferase activity with the increase of concentration (Fig. 9). Concentration of 10 µg/ml was found as the lowest concentration that produced statistically significant agonistic effect. There was no ER-subtype preference detected. Observed estrogenic effect is not as nearly as high as the effect induced by E_2 for several reasons. There are two

important parameters that determine the transcriptional potency and agonist/antagonist character of a ligand: the affinity of a ligand for a particular receptor and the conformational change induced by a ligand after its binding to the receptor. Ligand binding affinity depends, in part, on the three-dimensional structure of the ligand and its hydrophobic/hydrophilic character and, in part, on the volume and shape of the ligand binding cavity and the type of amino acid residues lining the cavity (Barkhem et al. 1998). Upon ligand binding, the receptor undergoes a conformational change, allowing the ER to exist in a spectrum of conformations from active to inactive depending on the nature of the bound ligand. This conformation, in turn, regulates the recruitment of specific transcriptional coregulatory proteins and the resulting transcriptional apparatus (Deroo & Korach 2006). Apparently, compounds contained in fraction 14 possess a structure that enables them to bind to ER, but the binding affinity is not as high as of E_2. That is not unusual for phytoestrogens. Study by Kuiper et al. (1998) showed that binding affinity of many phytoestrogens for ER is generally 1,000- to 10,000-fold lower than E_2 binding affinity. This study included, inter alia, well-known phytoestrogens genistein and daidzein. However, it should be remarked that the concentration of the fraction does not reflect the actual concentration of the compounds contained. The specified concentration only corresponds to the amount of the lyophilisate dissolved in DMSO. Single compounds are present in much smaller quantities and, accordingly, their optimal effect is not exhibited.

12 fractions of *Mondia whitei* extract were also evaluated for anti-estrogenic properties. No fraction displayed a significant difference in luciferase activity when applied in co-treatment with estradiol compared to estradiol alone treatment (Fig. 9). Although none of the fractions showed anti-estrogenic activity in this test system, it cannot be entirely excluded that they do not contain E_2 antagonistic compounds. Even though fractions are more purified than the extract, they still do not contain a single type of bioactive compounds, but a mixture of similar substances which may influence each other. Furthermore, depending on the cell and promoter context, E_2 exerts either a positive or negative effect on expression of the target gene (Hall et al. 2001). An anti-estrogenic effect cannot be equated with the down-regulation of gene transcription. Although this is the case in this test system, regulation *in vivo* is much more complex.

After demonstration of estrogenic activity of one fraction of *Mondia whitei* extract, the next step could be investigation of bioactive compounds that caused this effect. Composition of each fraction depends on the used eluent, so the composition of fraction 14 should be verified with the laboratory that performed the extraction and fractionation. Previously conducted studies on *Mondia whitei* report finding of steroids, triterpenes (a mixture of amyrine α- and β-acetate, lupeol, β-sitosterol,

and β-sitosterol glucoside) and aromatic compounds (2-hydroxy-4methoxybenzaldehyde, 3-hydroxy-4-methoxy benzaldehyde, and 4-hydroxy-3-methoxybenzaldehyde) in various extracts (Watcho et al. 2006). All of these compounds could, due to their chemical structure, present candidates for binding to ER. For example, β-sitosterol has been demonstrated to competitively bind with equivalent affinity to both the α and β-isoforms of the ER (Gutendorf & Westendorf 2001). In addition, Patnam et al. (2005) report isolation of coumarinolignans from the organic fraction of a crude methanol extract of the roots of *Mondia whitei*. Structure determination of this compounds has shown presence of phenolic rings, which are very important parameter for binding to ER (Patnam et al. 2005). Moreover, lignans have been identified as phytoestrogens. It is assumed that fraction number 14 contains compounds which are structurally similar to E_2 and therefore displays estrogenic activity.

It is important to remark that this study was conducted in an artificial system. In addition to that, U2OS is a cancer cell line which, consequently, differs in cell physiology from a healthy tissue. A clearer understanding of the complex mechanism of action through which bioactive compounds in fraction of *Mondia whitei* extract produce their effects, will require further investigation using several different and independent means. Future studies may focus on isolation and characterization of single estrogen active compounds from herbal extracts and identification of their molecular endpoints. To investigate the exact effects on gene expression and protein levels, qPCR or western blot analysis could be performed. Since this study had been conducted in bone-derived cells, future investigations can be made to examine estrogenic and anti-estrogenic activity of herbal extracts and fractions in other tissues. Different cell types may vary in cofactors and promoter sequences of estrogen-responsive genes (Deroo & Korach 2006). In addition to that, in some tissues ERα and ERβ are co-expressed, so formation of heterodimers may also play role in gene regulation by estradiol. It cannot be excluded that bioactive compounds in tested extracts and fractions possess tissue-specific estrogenic activity, i.e., act as selective estrogen receptor modulators (SERMs). Additionally, single bioactive compounds could be tested for their activity in *in vivo* experiments. Only animal experiments can provide an insight in how these potential phytoestrogens could act in overall physiology and homeostasis of the organism. Moreover, such experiments also consider metabolism in the liver and the route of administration.

Another direction would be to evaluate *Mondia whitei* extract and fractions for androgenic and anti-androgenic properties. As mentioned before, Watcho et al. (2001; 2004) observed a short-term androgenic and a long-term anti-spermatogenic and anti-fertility effects of the aqueous extract of

Mondia whitei in adult male rats. Moreover, traditional use of this plant as an aphrodisiac indicates possible androgenic mediated effects.

4.3. Cytotoxicity assay

Cytotoxicity assay on *Mondia whitei* methanol extract and its fractions have yielded unexpected and inconclusive results. Among all the herbal treatments, fraction 14 exhibited the strongest loss of viable cells in both U2OS-ERα and U2OS-ERβ cell lines. However, 10 nM E_2, which was used as a positive control, showed even stronger cytotoxic effect, with significant results in U2OS-ERα cell line (Fig. 10). These results could be attributed to doxycycline, a synthetic tetracycline, which was added to cells 24 hours prior cell seeding. Tetracycline antibiotics have been shown to inhibit matrix metalloproteinases (MMP), retard proliferation, induce apoptosis, and impair mitochondrial function in various experimental settings (Ahler et al. 2013). Human osteosarcoma cells have been studied in these terms as well and (Fife & Sledge 1998) report that doxycycline (10 µg/ml) suppresses cell proliferation and MMP activity and induces apoptosis in U2OS cells *in vitro*. In addition, Ahler et al. (2013) report that even low concentrations of doxycycline, which are commonly employed in inducible gene expression systems (100 ng/ml - 5 µg/ml), can significantly alter the metabolic gene expression profile of the cell and both glycolytic and oxidative metabolism as well as reduce the proliferative rate and increase apoptotic cell death. Moreover, a recent study by Moullan et al. (2015) provide an evidence of interactions between doxycycline and the mitochondria in low µg/ml range, i.e., doses that are typically used in Tet-On/Tet-Off experiments to control gene expression. This study has shown that doxycycline inhibits mitochondrial protein translation and as a consequence, the 13 oxidative phosphorylation subunits encoded by mitochondrial DNA are not properly expressed. This likely leads to unstable oxidative phosphorylation complexes and an adaptive state termed mitonuclear protein imbalance, which disturbs mitochondrial proteostasis (Houtkooper et al. 2013; Moullan et al. 2015). Pathways that were robustly downregulated included mitochondrial transport, mitochondrial protein synthesis, mitochondrial membrane potential, ATP synthesis, and electron transport chain. Concomitant with the induction of mitonuclear protein imbalance, striking adaptive changes in global gene expression profiles of doxycycline-treated cells were observed, with altered expression of almost 10% of genes (Moullan et al. 2015). It should be remarked that MTT assay is based on the reduction of yellow MTT to purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes in the mitochondria of the living cells (Mosmann 1983). MTT assay, therefore, requires functional

mitochondria and, although the results generally correlate with the number of viable cells, the rate of tetrazolium reduction actually reflects the general metabolic activity of cells. Hence, it is possible that viable cells display falsely low MTT readings due to the impaired mitochondrial function and decreased metabolic rate. There is a strong possibility that doxycycline contributes to the confounding effects in this experiment, through two possible mechanisms. First, it is possible that doxycycline masked cell viability by altering mitochondrial dynamics and function. On the other hand, it has been shown that low doxycycline concentrations are sufficient to slow proliferation and increase apoptotic cell death, what could contribute to the loss of viable cells. The observed effects in the MTT assay resulted from synergistic actions of doxycycline and herbal extracts and fractions, rather than the singular action of herbal treatments themselves. Confounding cytotoxic effects of estradiol and fraction 14 showed in this MTT assay, therefore, suggest the repeat of the assay without addition of doxycycline. In addition, this raises the question of doxycycline use in transactivation assays for the induction of ERα and ERβ gene expression. Again the optimization of the experimental system is underscored, emphasizing the importance of rigorous experimental design when using doxycycline in inducible expression systems and design of additional controls.

5. Conclusion

Phytoestrogens are plant-derived compounds that have the ability to cause estrogenic or/and anti-estrogenic effects through interaction with estrogen receptors. There is a growing evidence of health benefits of phytoestrogens, including a lower risk of osteoporosis, heart disease, breast cancer and menopausal symptoms.

Traditional medicine has been using a wide variety of plants for thousands of years, but many of them have not or only insufficiently been studied for their biological activities. Among them is *Mondia whitei*, a tropical African plant, whose usage has been reported mainly in terms of male sexual stimulant. In this study, methanol extract of this plant and its fractions were evaluated for estrogenic and anti-estrogenic properties using estrogen receptor subtype specific transactivation assays in human bone-derived U2OS cells. In addition, their potential cytotoxicity was assessed in MTT assays.

Neither estrogenic nor anti-estrogenic properties were observed for methanol extract of *Mondia whitei*. However, Fraction 14 of methanol extract displayed low, but statistically significant estrogenic activity in both U2OS-ERα and U2OS-ERβ cell line. The lowest concentration that produced agonistic activity was 10 µg/ml. None of the fractions showed anti-estrogenic activity in neither U2OS-ERα nor U2OS-ERβ cells. None of the herbal treatments exhibited significant cytotoxic effects in U2OS-ERβ cells, whereas in U2OS-ERα cells significant cytotoxicity was observed in few treatments, including the positive control. There are strong indications that doxycycline contributed to these confounding results, so it is suggested to repeat the assay without addition of doxycycline.

Evidence of an existing estrogenic potential of *Mondia whitei* forms the basis for further research. These should elucidate how the compounds of this plant interact with hormonal feedback loop and determine whether a use of these phytoestrogens is beneficial and safe.

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7. Curriculum vitae

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WORKING EXPERIENCE

2012-present Occasional part-time job at Croatian Radiotelevison.

EDUCATION AND TRAINING

2013-present	Graduate university study of molecular biology (University of Zagreb, Faculty of Science)
2010-2013	Bachelor of molecular biology (univ.bacc.biol.mol) Undergraduate study of molecular biology (University of Zagreb, Faculty of Science)
2006-2010	II. Gymnasium Zagreb – general education

PROFESSIONAL EXPERIENCE

Internships	Internship within Erasmus exchange program (March – August 2015) Technische Universität Dresden, Department of Biology, Institute of Zoology, Chair of molecular chair physiology and endocrinology Internship within Graduate study program (March – July 2014) University of Zagreb, Faculty of Science, Division of molecular biology, Laboratory for plant tissue culture
Conferences	International Alumni Symposium: Efficacy and Safety of Medical Plants and Dietary Supplements (Dresden, October 2015) The Third Meeting of the Croatian Association for Cancer Research (Zagreb, November, 2014)

Workshops	Scientific publishing and intellectual property protection in life science (Zagreb, November, 2014)
Summer schools	International Summer School: Plant derived polyphenols as drug leads (Dresden, October, 2015)
Other	Undergraduate teaching assistant in Zoology (September 2011 – February 2012)

PROFESSIONAL SKILLS

Other languages	English – level C1 German – level A2

Good communication skills, ability to adapt to multicultural environments, excellent presentation skills, team spirit.
Excellent organisational skills in terms of planning and managing projects.
Proficiency and independence in experimental work, critical analysis and interpretation of results, capacity of working in a timely manner and adhering to deadlines.
Advanced knowledge of Internet browsers and tools as well as MS Office tools and Open Office tools. Good command of R Studio and OriginLab for statistical analysis.