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Research paper

Multiple readout assay for hormonal (androgenic and antiandrogenic) and cytotoxic activity of plant and fungal extracts based on differential prostate cancer cell line behavior

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ABSTRACT

Ethnopharmacological relevance: Prostate cancer is one of the most diagnosed forms of cancer among men in western regions. Many traditional applications or phytotherapeutic concepts propose to inhibit the proliferation of prostate cancer cells. In order to detect influences of plant or fungal extracts and derived fractions on androgen receptor signaling pathways, a differentiating cell proliferation assay was established, which enables the simultaneous detection of hormonal and cytotoxic effects.

Material and methods: The well characterized prostate cancer cell lines LNCaP and PC-3 were used in a multiple readout assay. In all, 186 fractions of 23 traditionally used organisms were screened regarding their effects on proliferation of the two prostate cancer cell lines. The fractions were prepared by accelerated solvent extraction followed by gradient extrography. Extracts of the potential hormonally active plants *Cibotium barometz*, *Heteropterys chrysophylla*, and *Sideroxylon obtusifolium* (= *Bumelia sartorum*) were phytochemically investigated.

Results: Fractions from *Cibotium barometz*, *Cortinarius rubellus*, *Cyrtomium falcatum*, *Heteropterys chrysophylla*, *Nephrolepis exaltata*, *Salvia miltiorrhiza*, *Sideroxylon obtusifolium*, *Trichilia emetica*, and *Trimeria grandifolia* exhibited hormonal influences on prostate cancer cells. Cytotoxic activity towards human cell lines was detected for the first time for fractions from *Aglaia spectabilis (A. gigantea)*, *Nephrolepis exaltata* and *Cortinarius brunneus*.

Conclusions: The differential behavior of the two prostate cancer cell lines allows the discrimination between potential androgenic or antiandrogenic activities and effects on the estrogen or glucocorticoid receptor as well as cytotoxic activities. The combined cell lines assay can help to assess the biological activities of material used in traditional medicine.

1. Introduction

The androgen receptor (AR) and its signaling pathways are known to be involved in the development of a variety of diseases and therefore act as a valuable medicinal targets. One example is represented by the therapy of benign prostate hyperplasia, prostate cancer, and skin disorders (e.g. acne, hirsutism or androgenetic alopecia) with androgen receptor signaling pathway inhibitors. Furthermore, androgenic anabolic compounds are used to cure chronic states of exhaustion caused by AIDS or cancer cachexia due to their appetizing effect. In addition to that, they are applied as male contraceptives, in hormone replacement therapies for elderly males and in case of gender dysmorphia. Based on this knowledge, there has been an increasing interest in the use of phytotherapy to prevent or cure androgen dependent diseases. The development of relevant drugs is thereby mainly based on ethnomedicinal knowledge. In South Africa, for example, the folkloric use of the plant *Bulbine natalensis* (= *Bulbine latifolia*) was reported for the management of male sexual dysfunction. Indeed, an anabolic and androgenic activity of the *Bulbine natalenis*

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stem extract could be demonstrated in male Wistar rats (Yakubu & Afolayan, 2010). Furthermore, extracts of *Serenoa repens* (fruit), *Pygeum africanum* (= *Prunus africana*) (cortex), *Urtica dioica* (radix) (Farag et al. 2013), *Secale cereale* (pollen), *Hypoxis rooperi* (= *Hypoxis hemerocallidea*) (herba), and *Cucurbita pepo* (semen) were studied regarding their potential to cure benign prostate hyperplasia (Oelke et al., 2009). *In vitro* activities could mainly be documented for the inhibition of 5α-reductase and growth factors. In addition, effects on prostate cancer cells were shown for the phytocompounds genistein, lycopin, cucurmin, resveratrol, and epigallocatechin gallate from Green Tea. However, only preventive effects, but no therapeutic effects could be proven so far. Moreover, the ecdysteroid 20-hydroxyecdysone, which is also found in plants, has been suggested as an option for the therapy of muscle atrophy because of its anabolic activity (Tóth et al., 2008). Considering the large number of diseases connected to the androgen receptor and its signaling pathway, this study aimed at the evaluation of the antiandrogenic or androgenic potential of extracts from fungi and medicinal plants. For this purpose, the androgen dependent prostate cancer cell line LNCaP and the androgen independent prostate cancer cell line PC3 were employed. Cell proliferation was evaluated with the help of the colorimetric XTT assay. Screening parameters were chosen to reveal any influence on androgen signaling pathways. Extracts of 23 organisms were prepared and fractionated *via* gradient extrography. In total, 186 fractions were submitted to the cell proliferation assay. Beside plant material with an ethnomedicinal background, basidiomycetes were also included in the study, since secondary fungal metabolites can interfere with androgen receptor function (Zaidman et al., 2008).

2. Materials and methods

2.1. Plant materials

Plant and fungi material was provided by cooperation partners or obtained from botanical gardens, commercial sources or the greenhouse collection of the Leibniz Institute of Plant Biochemistry (Table 1). Plant names have been checked with www.theplantlist.org (accessed at 05/14/2014). The identity of commercially procured material was proved by the providers. Fungal fruiting bodies were collected and determined by Dr. Norbert Arnold, IPB. Reference material is available at the IPB.

2.2. Extraction and fractionation

The dried or lyophilized material of 23 organisms was extracted *via* automated accelerated solvent extraction using Dionex ASE 200®. Unless stated otherwise, extraction was performed in two steps: first using the solvent mixture 2-methoxy-2-methylpropane (MTBE)/ethanol (80/20 v/v) and second by applying pure methanol. Two extraction cycles for each solvent were performed in 22 ml extraction cells. The first solvent system was selected as it was found beneficial to also extract more lipophilic compounds well, e.g. steroids (Seipold et al. 2004; Dumri et al. 2008).

The crude extracts obtained by accelerated solvent extraction or provided by cooperation partners were fractionated *via* gradient extrography. For this purpose, extracts were adsorbed to modified diatomaceous earth (Isolute HMN). Afterwards, the loaded diatomaceous earth was eluted using solvents with increasing polarity [*n*-hexane, ethyl acetate, acetone, methanol, acetone/HCl (0.5 ml 2 N HCl/100 ml acetone), whereby the latter is especially useful for fungal extracts and alkaloids] (Scheme 1). Crude extracts and fractions were tested in the proliferation assay (Scheme 1).

2.3. Cell culture conditions

LNCaP and PC-3 cells were obtained from the DSMZ (Braunschweig, Germany) and were cultured in phenol red free RPMI1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 2 mM L-alanyl-L-glutamine (Biochrom AG, Berlin, Germany), 16 mM HEPES (CC pro, Neustadt, Germany), 100 µg/ml penicillin and streptomycin (both from PAA Laboratories) and 10% FBS (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cell lines were regularly tested for mycoplasm infection.

2.4. Cell proliferation assay

Stock solutions of fractions were prepared in DMSO. For the cell proliferation assay LNCaP and PC-3 cells from passages 5 to 31 were used. The preliminary treatment of the cells included a serum free preincubation period of 7 d after reaching 70% confluency in 25 cm^2 cell culture flasks. Cells were subsequently plated in 100 µl of medium with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) at 8×10^3 LNCaP cells or 5×10^2 PC-3 cells per well as quadruplicates. After 24 h, 100 µl medium with 10% CD-FBS containing 25 µg/ml (exceptions are indicated) of test fraction alone or in addition to 1 nM testosterone (TES) were added. DMSO (0.5%, final v/v) served as negative control. The androgen testosterone (1 nM) was used as positive control for stimulation of LNCaP cell proliferation (group a). The antiandrogenic compounds finasteride and bicalutamide were used in a concentration of 10 µM as positive control for inhibition of testosterone-stimulated LNCaP cell proliferation (group b); the cytotoxic drugs vincristine sulfate and etoposide (10 µM) for inhibition of LNCaP, testosterone-stimulated LNCaP, and PC-3 cell proliferation (group c). The proliferation was determined 5 d later by XTT (Roche Diagnostics Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The absorbance measurements of XTT tests (abs; λ_{max}=490 nm, $\lambda_{reference}$= 600 nm) were compared to the DMSO control to yield percentage values and these values were referred to the proliferation in the DMSO control by subtracting 100%.

$$\text{proliferation in } \% = \frac{\text{abs(test well)}}{\text{abs(DMSO control)}} \times 100 - 100 \ \%$$

Thus, negative proliferation values indicate inhibition and positive values reveal enhancement of cell proliferation. In order to visualize inhibition under testosterone coadministration, the difference to the testosterone positive control was calculated.

$$\text{proliferation in } \% = \frac{\text{abs(test well with TES)}}{\text{abs(DMSO control)}} \times 100 - \frac{\text{abs(TES 1 nM)}}{\text{abs(DMSO control)}} \times 100$$

Plates were also analyzed visually under the microscope to reveal discrepancies between the cell number and XTT values.

2.5. Statistics

The unpaired Lord-test was used to evaluate statistically significant (α=5%) differences.

2.6. Phytochemical investigations

2.6.1. Cibotium barometz (L.) J.Sm

From a fresh rhizome collected in the mountains of Northern Vietnam in March 2008 the hairs were abscised and the remaining rhizome (601 g) extracted exhaustively with methanol. The solvent was removed and the aqueous crude extract was partitioned successively with *n*-heptane (7.65 g) and ethyl acetate

Table 1
Overview of the species (plants and fungi) investigated in this study with corresponding family, extracted part, source, and collection or batch number.

Species (synonyms)	Family	Part	Source	Collection or batch number
Aglaia spectabilis (Miq.) S.S.Jain & *S.Bennet* [a](*Aglaia gigantea* Pellegr.)	Meliaceae	fruit	Vietnam, Academy of Science and Technology, Hanoi	SA 613
Artemisia afra Jacq. ex Willd.	Compositae	aerial part	Kenya, University of Nairobi	QKE019
Carthamus tinctorius L.	Compositae	flower	Yong Quan GmbH	Ch.-B.0406403
Cibotium barometz (L.) J.Sm.	Dicksoniaceae	rhizome	Vietnam, Academy of Science and Technology, Hanoi	SA 685
Cibotium regale Versch. & Lem.	Dicksoniaceae	rhizome	Botan. Garden Halle	Akz.-Nr. 8922
Cibotium schiedei Schltdlt. & Cham.	Dicksoniaceae	rhizome	Botan. Garden Jena	XX-0-JENA-7623578
Cortinarius brunneus (Pers.) Fr.	Cortinariaceae	fruiting body	IPB fungal collection	123/97 Berchtesgadener Land/Schönrauer Filz
Cortinarius rubellus Cooke	Cortinariaceae	fruiting body	IPB fungal collection	89/98 Penzberg
Cyrtomium falcatum (L.f.) C. Presl	Dryopteridaceae	leaves	Green house (IPB)	BOC269
Excoecaria bussei Pax	Euphorbiaceae	bark	Kenya, University of Nairobi	QKE040
Heteropterys chrysophylla (Lam.) Kunth	Malpighiaceae	leaves	Green house (IPB)	BOC267
Microglossa pyrifolia (Lam.) Kuntze	Compositae	aerial part	Kenya, University of Nairobi	QKE045
Monanthotaxis buchananii (Engl.) Verdc.	Annonaceae	leaves	Kenya, University of Nairobi	QKE024
Nephrolepis exaltata (L.) Schott	Nephrolepidaceae	leaves	Green house (IPB)	BOC263
Ozoroa insignis Delile	Anacardiaceae	leaves	Kenya, University of Nairobi	QKE026
Reynoutria multiflora (Thunb.) Moldenke (*Polygonum multiflorum* Thunb.)	Polygonaceae	radix	Yong Quan GmbH	Ch.-B.05038805
Salvia miltiorrhiza Bunge	Lamiaceae	radix	Yong Quan GmbH	Ch.-B.0409405
Sideroxylon obtusifolium (Roem. & *Schult.) T.D. Penn.*[a](*Bumelia sartorum* Mart.)	Sapotaceae	bark	Brazil, Universidade Federal de Rio de Janeiro	RFA-34154
Suregada zanzibariensis Baill.	Euphorbiaceae	leaves	Kenya, University of Nairobi	QKE005
Trichilia emetica Vahl	Meliaceae	leaves	Kenya, University of Nairobi	QKE018
Trimeria grandifolia (Hochst.) Warb.	Salicaceae	leaves	Kenya, University of Nairobi	QKE013
Turraea cornucopia Styles & F.White	Meliaceae	leaves	Kenya, University of Nairobi	QKE030
Vespris nobilis (Delile) Mziray (*Teclea nobilis* Delile)	Rutaceae	leaves	Kenya, University of Nairobi	QKE007

[a] extracts provided by cooperation partners

(6.47 g). The resulting fractions were submitted to the cell proliferation assays. An aliquot of the proliferation enhancing ethyl acetate fraction (1.20 g) was further separated using column chromatography on Sephadex LH20 (solvent: MeOH) and RP18 (methanol/water gradient system) to isolate cibotiumbaroside A (**1**, 50 mg) and a mixture of isophondin (**2**) and two monoglycosides of 3,4-dihydoxy cinnamic acid (25 mg, $[M+Na]^+$ 365.08454, calc. for $C_{15}H_{18}O_9Na^+$ 365.08430). Further fractionation was achieved by preparative HPLC (Merck-Hitachi, RP18, gradient MeOH/water 20:80 to 40:60 in 40 min) and the resulting fractions characterized by NMR and MS.

2.6.2. *Heteropterys chrysophylla Kunth*

For our experiments this species was grown in the IPB green house. Dried leaves and twigs (20 g) were extracted exhaustively with 80% methanol/20% water. The aqueous residue of the crude extract was successively partitioned with *n*-heptane (0.02 g) and ethyl acetate (0.23 g). The *n*-heptane fraction was separated on a silica gel SPE cartridge using the following eluents: 1) *n*-hexane, 2) chloroform, 3) chloroform-ethyl acetate 1:1, 4) ethyl acetate and 5) methanol. All fractions were submitted to proliferation assays to determine the active sample. The active fraction 3 was further characterized by GC-MS.

Column chromatography of the ethyl acetate fraction (126 mg) on silica gel using chloroform/methanol (8:2) as eluent resulted in the isolation of kaempferol-3-O-α-L-rhamnoside (**5** , 10.3 mg) and kaempferol-3-O-α-L-rhamnose-(2→1)-β-D-xylopyranoside (48.5 mg) as main constituents.

2.6.3. *Sideroxylon obtusifolium T.D.Penn*

661 mg of the ethyl acetate extract of *Sideroxylon obtusifolium* T.D.Penn (synonym *Bumelia sartorum* Mart.) produced by Prof. Kuster in Brazil was submitted to column chromatography on silica gel using a chloroform/methanol gradient system. The fractionation resulted in the isolation of epicatechin (**6**, 28 mg) and in the separation of several fractions containing mixtures of dimeric and oligomeric catechin derivatives, which were further characterized by MS investigations.

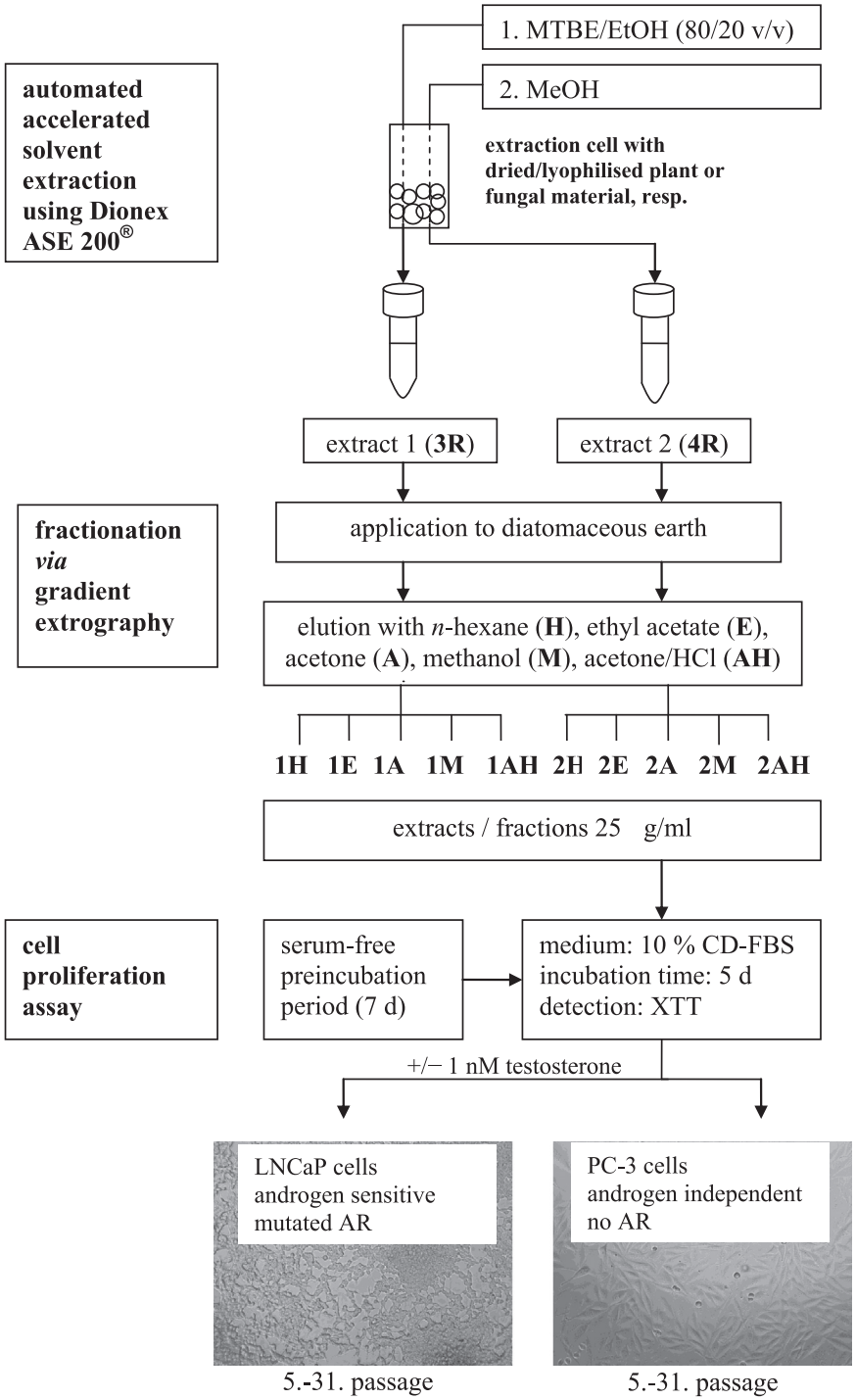
2.7. General conditions

Silica gel (Merck, 63–200 μm) and Sephadex LH-20 (Supelco) were used for column chromatography. Fractions were monitored by TLC using precoated silica gel plates 60 F_{254} (Merck). Spots were observed under UV light and visualized by heating silica gel plates sprayed by vanillin-H_2SO_4 in MeOH.

NMR, 1D: Varian Gemini 300 spectrometer, 300.24 MHz (^{1}H) and 75.5 MHz (^{13}C) in CD_3OD or $CDCl_3$· 2D (HSQC, GHMBC, GCOSY, GROESY): Varian Unity 500 spectrometer, 499.83 MHz. Chemical shifts in ppm were referenced to the internal standard TMS ($\delta=0$) for ^{1}H and to CD_3OD ($\delta=49.0$ ppm) or $CDCl_3$ ($\delta=77.0$ ppm) for ^{13}C, respectively.

MS: The high resolution ESI mass spectra were obtained from a Bruker Apex III 70 eV Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. The sample solutions were introduced continuously *via* a syringe pump with a flow rate of 120 μl/h.

ESI mass spectra and collision-induced dissociation (CID) mass spectra were obtained from a TSQ Quantum Ultra AM system equipped with a hot ESI source (HESI), electrospray voltage 3.0 kV, sheath gas: nitrogen; vaporizer temperature: 50 °C; capillary temperature: 250 °C; The MS system was coupled with a Surveyor Plus micro-HPLC (Thermo Electron), equipped with a RP18 column (5 μm, 150 × 1 mm, Hypersil GOLD, Thermo Scientific). For the HPLC a gradient system was used starting from H_2O:CH_3CN 90:10



Scheme 1. Schematic illustration of the extraction and fractionation route employing automated accelerated solvent extraction and subsequent fractionation *via* gradient extrography.

(each of them containing 0.2% HOAc) to 5:95 within 15 min and then hold on 5% for further 30 min; flow rate 70 μl/min.

GC-MS measurements were performed on a Voyager/Trace GC 2000 (Thermo Quest CE Instruments) under the following conditions: 70 eV EI, source temperature 200 °C; column DB-5MS (J&W, 30 m × 0.25 mm, 0.25 μm film thickness); injection temperature 250 °C, interface temperature 350 °C; carrier gas He, flow rate 1.0 ml/min, constant flow mode; splitless injection, column temperature program: 40 °C for 1 min, then raised to 300 °C at a rate of 10 °C/min and then hold on 300 °C for 15 min.

3. Theory

Plant secondary metabolites are well characterized concerning their estrogenic potential, whereas their effects on androgen mediated processes have not been under profound examination up to date. There are several modes by which antiandrogenic or androgenic compounds can affect the androgen receptor signaling pathway. At first, modulatory effects due to binding to the androgen receptor itself and binding to related kinases have to be mentioned. Furthermore, the substances can also affect growth

factor inhibition, expression of the androgen receptor cofactor, and enzymes of the steroid metabolism, such as 5α-reductase. Even cross-reactions with other steroid hormone receptors, e.g. the estrogen receptor (ER), result in an altered expression pattern of androgen dependent genes, such as the prostate-specific antigen (PSA) or the androgen receptor (AR).

In order to account for this complexity, a universal assay employing chemical genomics is preferred rather than a basic AR-binding assay. A cell proliferation assay can reveal several aspects of influences on androgen receptor signaling pathways. Thus, a cell proliferation assay was used, which enables the simultaneous detection of hormonal and cytotoxic effects of plant and fungi extracts and derived fractions based on the differential behavior of the two well-analyzed prostate cancer cell lines LNCaP and PC-3.

LNCaP cells grow androgen-sensitive and express an androgen receptor with a point mutation at the ligand binding domain, low amounts of estrogen receptor β and no estrogen receptor α (Horoszewicz et al., 1983; Lau et al., 2000; Bhattacharyya et al., 2006; Setlur et al., 2008). Regarding the presence of the glucocorticoid receptor (GR) within LNCaP cells, contradictory statements can be found (Kim et al., 2006; Yan et al., 2008).

PC-3 cells do not express an androgen receptor, but estrogen receptor α and β (Lau et al., 2000; Setlur et al., 2008) and are unresponsive towards androgen treatment (Kaighn et al., 1979). Extracts were tested in both cell lines under the same conditions to reveal any influence on androgen receptor signaling pathways. This is necessary because the LNCaP cell line possesses an androgen receptor with a point mutation where alanine is at position 868 instead of threonine. Because of this mutation, the androgen receptor will be unspecifically activated by androgens, estrogens, progesterone and antiandrogens (Veldscholte et al., 1990a, 1990b). Therefore, the extracts were also applied to the PC-3 cell line which does not possess an androgen receptor, but responds to estrogens and progesterone *via* its own receptors.

4. Results and discussion

4.1. Cell proliferation assay

In order to detect influences of plant or fungal extracts and derived fractions on androgen receptor signaling pathways, 186 fractions of 23 organisms were tested in the differentiating cell proliferation assay using the prostate cancer cell lines LNCaP and PC-3.

Automated accelerated solvent extraction was chosen to obtain extracts under standardized conditions. Extraction parameters were optimized regarding yield under mild conditions such as low temperature. The extracts were fractionated due to polarity by gradient extrography with increasing polarity of eluents. In addition to the XTT assay, proliferation was also analyzed by microscopic investigation to detect substances with a reductive potential or with dye properties, which can interfere with the XTT reagent. This is not uncommon in extracts or some fractions thereof. Also unusual phenotypic changes can be observed only by such a visual inspection.

Extract activities can be divided into three main groups (Scheme 2):

a) Stimulation of LNCaP cell proliferation.
b) Inhibition of testosterone-stimulated LNCaP cell proliferation.
c) Inhibition of LNCaP, testosterone-stimulated (TES) LNCaP and PC-3 cell proliferation.

Group a) shows an antiandrogenic or androgenic activity in the LNCaP cell system, whereby it is not possible to distinguish between antiandrogenic and androgenic activity, due to the mutated androgen receptor (Scheme 2, Table 2).

Group b) reveals an antiandrogenic behavior, which might be due to an inhibition of the androgen receptor, inhibition of the 5alpha-reductase, inhibition of the translocation of the activated

a) Antiandrogenic or androgenic effect by stimulation of LNCaP cell proliferation
b) Antiandrogenic effect by inhibition of testosterone-stimulated LNCaP cell proliferation
c) Cytotoxic effect

	LNCaP + TES	LNCaP	PC-3	Effect	Extracts/fractions
a)	↑	↑	-	Effect on the mutated androgen receptor (potential antiandrogenic or androgenic activity)	22[a]
	↑	↑	↑	Unspecific proliferation effects or reducing influence on XTT-reagent or intrinsic dye compounds	1[b]
	↑	↑	↓	Potential effect on the AR with simultaneous activity at the ER or GR	22[b]
b)	↓	-	-	Antiandrogenic effect with potential activity at the androgen receptor	10[a]
	↓	↑	↑	Antiandrogenic effect with overlaying XTT-reducing effect or dye effect	-
	↓	↑	↓	Antiandrogenic effect with simultaneous effect at the ER or GR	2[b]
c)	↓	↓	↓	Cytotoxic effect	14[a]

- no effect
↑ increase of cell proliferation (positive response)
↓ inhibition of cell proliferation (negative response)
[a] relevant effects discussed in this paper and detailed in the tables
[b] detailed in appendix

Scheme 2. Response array and its interpretation in the cell proliferation assay.

androgen receptor or another mechanism not yet revealed (Scheme 2, Table 3).

Group c) exerts a cytotoxic activity (Scheme 2, Table 4).

Extracts which did not influence the PC-3 cell proliferation were of special interest. An increase in PC-3 cell proliferation could be the result of an interference with the reagent of the XTT assay or an unspecific proliferation effect, whereas the inhibition of PC-3 cell proliferation could be due to an interaction with the ER or GR.

The advantage of the assay is a simultaneous detection of extracts with influence on the androgen receptor signaling pathway and exclusion of extracts with cytotoxic or predominating influence on the ER or GR signaling pathway.

Table 2
Species and fractions with LNCaP cell proliferation stimulating activity (group a): effects on LNCaP and PC-3 cell proliferation (in % referred to the DMSO negative control=0 %).

Species	Fraction[a]	Proliferation (%) ± SD (%)	
		LNCaP	PC-3
Aglaia spectabilis	methanol fraction (M)	20 ± 7	−17 ± 14
Artemisia afra	methanol fraction (M)	58 ± 11	−2 ± 6
Carthamus tinctorius	*n*-hexane fraction (1H)	16 ± 18	1 ± 10
	ethyl acetate fraction (1E)	18 ± 17	−4 ± 12
	acetone fraction (1A)	22 ± 12	−12 ± 15
	acetone fraction (2A)	19 ± 18	9 ± 8
Cibotium barometz	acetone fraction (2A)	100 ± 32	4 ± 11
	methanol crude extract (4R)	105 ± 49	−6 ± 10
Cortinarius brunneus	*n*-hexane fraction (1H)	35 ± 24	9 ± 12
Excoecaria bussei	*n*-hexane fraction (H)	77 ± 36	n.d.
	acetone fraction (A)	47 ± 3	n.d.
	ethanol crude extract	118 ± 24	−32 ± 33
Heteropterys chrysophylla	*n*-hexane fraction (1H)	72 ± 53	−26 ± 34
	ethyl acetate fraction (1E)	10 ± 7	−23 ± 13
	acetone fraction (1A)	29 ± 10	−21 ± 13
Reynoutria multiflora	ethyl acetate fraction (1E)	40 ± 4	−15 ± 12
	acetone fraction (2A)	26 ± 13	−5 ± 10
Vespris nobilis	acetone fraction (A)	45 ± 11	−12 ± 22
Trichilia emetica	ethyl acetate fraction (E)	84 ± 21	7 ± 11
Trimeria grandifolia	*n*-hexane fraction (H)	105 ± 25	17 ± 3
	ethyl acetate fraction (E)	111 ± 14	−11 ± 11
	ethanol crude extract	82 ± 13	−2 ± 2
positive control	1 nM testosterone	86 ± 38	18 ± 7

n.d. not determined

[a] for fraction codes see Scheme 1

4.2. Stimulation of LNCaP cell proliferation (group a)

Fractions were regarded as active when they stimulated the proliferation of LNCaP cells without having a proliferation stimulating activity on PC-3 cells. The androgen receptor in LNCaP cells possesses a point mutation, and therefore can be stimulated not just by androgens, but also by estrogens, progestagens and some antiandrogens such as cyproteronacetate (Veldscholte et al., 1990a). Fractions stimulating the proliferation of LNCaP cells without stimulating the proliferation of PC-3 cells were therefore categorized as modulators of the androgen receptor signaling pathway. They can act potentially as androgens, antiandrogens, progestagens or estrogens. Due to the fact that estrogens and glucocorticoids decrease PC-3 cell proliferation, it is finally possible to identify fractions with androgenic/antiandrogenic activity (Carruba et al., 1994; Nishimura et al., 2001). In order to distinguish, if fractions exert an androgenic or antiandrogenic activity, an assay system containing a wild-type androgen receptor is necessary.

Table 4
Species and corresponding fractions with cytotoxic activities (group c): effects on LNCaP and PC-3 cell proliferation (in % referred to the DMSO negative control=0 %).

Species	Fraction[a] (25 µg/ml)	Proliferation (%) ± SD (%)	
		PC-3	LNCaP
Aglaia spectabilis	ethyl acetate fraction (E)	−101 ± 0,1	−103 ± 1
Carthamus tinctorius	acetone/HCl fraction (2AH)	−12 ± 11	−15 ± 13
Cortinarius brunneus	methanol fraction (2M)	−39 ± 13	−21 ± 9
Cortinarius rubellus	methanol fraction 2M	−25 ± 6	−18 ± 6
Nephrolepis exaltata	ethyl acetate fraction (1E)	−51 ± 8	−18 ± 3
	methanol fraction (1M)	−59 ± 8	−21 ± 10
	acetone/HCl fraction (1AH)	−147 ± 15	−50 ± 23
	n−hexane fraction (2H)	−44 ± 8	−20 ± 4
	ethyl acetate fraction (2E)	−60 ± 8	−16 ± 1
Ozoroa insignis	methanol fraction (M)	−55 ± 4	−37 ± 5
Salvia miltiorrhiza	*n*−hexane fraction (1H)	−98 ± 2	−94 ± 3
	crude extract (3R)	−89 ± 3	−73 ± 5
	n-hexane fraction (2H)	−97 ± 2	−96 ± 5
	methanol crude extract (4R)	−86 ± 3	−63 ± 19
positive control	10 µM vincristine sulfate	−27 ± 3	−90 ± 1
	10 µM etoposide	−27 ± 8	−74 ± 5

[a] for fraction codes see extraction scheme (Scheme 1)

Table 3
Species and corresponding fractions with LNCaP cell proliferation inhibiting activity under testosterone coadministration (group b): effects on LNCaP and PC-3 cell proliferation (in % referred to the DMSO negative control=0 %).

Species	Fraction[a]	Concentration	Proliferation (%) ± SD (%)	
			LNCaP[b]	PC-3
Cibotium barometz	methanol extract (2M)[d]	25 µg/ml	−35 ± 2	−7 ± 9
	fraction of methanol extract (small molecules)[e]	25 µg/ml	−98 ± 22	−12 ± 5
Cortinarius rubellus	crude extract (3R)	25 µg/ml	−32 ± 7	- −11 ± 11
Cyrtomium falcatum	ethyl acetate fraction (2E)	25 µg/ml	−17 ± 4	7 ± 12
Nephrolepis exaltata	acetone fraction (2A)	25 µg/ml	−86 ± 19	−27 ± 19
	methanol fraction (2M)	25 µg/ml	−71 ± 30	−43 ± 16
Salvia miltiorrhiza	acetone/HCl fraction (2AH)	25 µg/ml	−51 ± 34	−2 ± 31
Sideroxylon obtusifolium	aqueous crude extract	1 µg/ml	−25 ± 11	6 ± 5
	fraction of aqueous extract[c]	25 µg/ml	−103 ± 26	30 ± 17
	ethyl acetate crude extract	25 µg/ml	−56 ± 14	8 ± 8
positive control	finasteride	10 µM	−48 ± 3	10 ± 12
	bicalutamide	10 µM	−125 ± 10	5 ± 6

[a] for fraction codes see extraction scheme (Scheme 1)
[b] negative values (inhibition of TES-stimulated growth) indicate potential antiandrogen activity
[c] fractionated by preparative HPLC from the aqueous crude extract
[d] prepared by fractionating a methanol crude extract of *Cibotium barometz* by gradient extrography according to Scheme 1
[e] methanol extract of *Cibotium barometz* was fractionated *via* gradient extrography and the resulting ethyl acetate and acetone fractions were combined and fractionated with column chromatography (Sephadex LH20; methanol as eluent) to yield a fraction with molecules of the smallest size

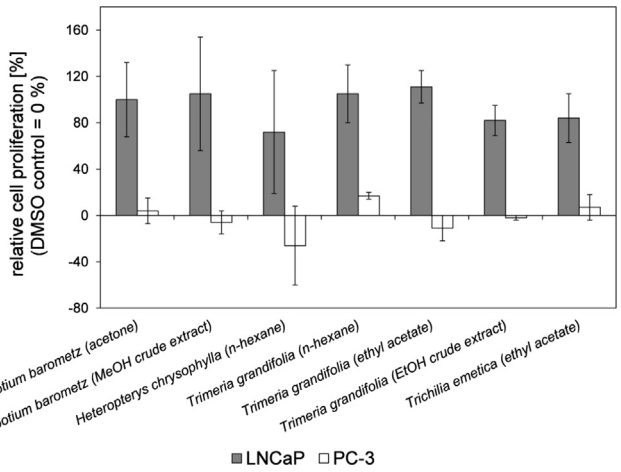


Fig. 1. Species and fractions with the highest stimulating activity on LNCaP cell proliferation.

In total, 47 fractions of 17 organisms reveal a statistically significant stimulating activity on LNCaP cell proliferation (Scheme 2). Out of these 47 active fractions 24 decreased the proliferation of PC-3 cells which was statistically significant. In fact, this is a hint on substances within the fractions which interfere with the estrogen or glucocorticoid receptor in PC-3 cells. Finally, only those fractions were considered as potential modulators of the androgen receptor signaling pathway, which stimulated the LNCaP cell proliferation without affecting the PC-3 cell growth. According to this, 22 out of the 47 fractions were found to exhibit activity on the AR (signaling pathway) (Table 2).

The seven fractions with the strongest activity derived from the plants *Cibotium barometz*, *Heteropterys chrysophylla*, *Trimeria grandifolia*, and *Trichilia emetica* (Fig. 1). Comparison with the related species *Cibotium schiedei* and *Cibotium regale* revealed similar, but higher activities for *Cibotium barometz* (Suppl.).

This is the first time that hormonal activities could be detected for extracts of the species *Aglaia spectabilis*, *Artemisia afra*, *Cortinarius brunneus*, *Excoecaria bussei* and *Vespris nobilis* as well as *Trimeria grandifolia* and *Heteropterys chrysophylla*, whereby extracts of the last two species revealed the highest activity on cell proliferation. Kurubasch aldehyde, a sesquiterpenoid from *Trichilia emetica*, was reported to slightly inhibit the growth of the breast cancer cell line MCF7 (Traore et al., 2007). Substances from the rhizome of *Cibotium barometz* were reported to inhibit the formation of osteoclasts, which might point to an androgenic or estrogenic activity (Cuong et al., 2009). Phytoestrogenic effects, the stimulation of osteoblasts and an anti-estrogenic lignan could be detected for *Carthamus tinctorius* (Yoo et al., 2006; Kim et al., 2008). An inhibitory activity on 5α-reductase was reported for an antraquinone derivative of *Reynoutria multiflora* (Cho et al., 2010) and estrogenic properties were documented for *Reynoutria multiflora* by Kang et al. (2006).

4.3. Inhibition of LNCaP cell proliferation with testosterone coadministration (group b)

The cell proliferation assay was also carried out by applying testosterone (TES) additionally, in order to identify those fractions exhibiting an antiandrogenic potential. In this context fractions were regarded as active when they diminished LNCaP cell proliferation during TES coadministration, but did not affect PC-3 cell proliferation in a statistically significant manner. Finally, 10 fractions from six organisms decreased LNCaP cell proliferation in a statistically significant manner (Table 3). From these six organisms *Sideroxylon obtusifolium, Cyrtomium falcatum,* and *Nephrolepis exaltata* are of special interest, because they show the strongest inhibition effects although no antiandrogenic or androgenic effect has been documented so far. Extracts of these specieshave the potential to be applied as antiandrogenic therapeutics in cosmetic areas like acne, hirsutism and androgenetic alopecia. Further analyses have to be made to prove activity on the wild type receptor (wtAR).

4.4. Cytotoxic activities (group c)

Cytotoxic activities (group c) could be detected for 14 fractions out of seven organisms (Table 4). For fractions or isolated substances from the species *Salvia miltiorrhiza*, *Carthamus tinctorius*, *Ozoroa insignis* and *Cortinarius rubellus* cytotoxic activities towards human cell lines were already reported (Wu et al., 1991; Spoerke & Rumack, 1994; Ryu et al., 1997; Rea et al., 2003; Loo et al., 2004). This effect is so far unreported for the species *Aglaia spectabilis*, *Nephrolepis exaltata* and *Cortinarius brunneus*. Further research concerning these species and context therefore may be of interest in another study.

4.5. Phytochemical investigation

The most promising plant material with a potential hormonal activity (*Cibotium barometz*, *Heteropterys chrysophylla*, and *Sideroxylon obtusifolium*) was phytochemically investigated to obtain information about the potential bioactive metabolites.

4.5.1. Cibotium barometz

Cibotium barometz (L.) J. Sm. (Dicksoniaceae) is a tropical tree fern native to parts of China and SE Asia to the Malay Peninsula. The species is a folk medicinal herb used against rheumatic and menstruation problems, herniated disc, hyperosteogeny and osteoporosis (Tierra & Tierra, 1998).

For our investigations, a fresh rhizome was extracted exhaustively with methanol. The solvent was removed and the aqueous crude extract was partitioned successively with *n*-heptane and ethyl acetate. The ethyl acetate fraction enhanced the proliferation of LNCaP cells independently from testosterone as also shown for the fractions 1E, 1A, 1M, 2H, 2A, 2M, 3R, 4R (Table 1 and Suppl.). Therefore, the ethyl acetate fraction was further separated using column chromatography on Sephadex LH20 and RP18 as well as preparative HPLC to isolate the relevant constituents. However, the proliferation supporting activity could not be attributed to one single compound or a special fraction. MS and NMR investigation revealed the presence of a multitude of structurally related phenols – especially various glycosides of 3,4-dihydroxy benzoic acid (protocatechuic acid) and of 3,4-dihydoxy cinnamic acid (caffeic acid). However, due to the large quantity and similarity of constituents, these compounds could not be separated well. The occurrence of the unsubstituted acids in *Cibotium barometz* was previously reported (Yuan et al., 1996). From the active fraction cibotiumbaroside A (**1**, 3-[(6-*O*-protocatechuoyl-β-D-glucopyranosyloxy)methyl]-2(5H)-furanone, $[M+Na]^+$ 435.08950, calc. for $C_{18}H_{20}O_{11}Na^+$ 435.0897, Fig. 2) could be isolated as a special derivative of protocatechuic acid, which is in good agreement with the findings of Cuong *et al.* in 2009. So far, cibotiumbaroside A was found only in *Cibotium barometz*. Furthermore, 3-(1-hydroxymethyl)-5H-furan-2-one (**2**, isosphondin) was detected. Glycosides of isosphondin were previously shown to possess antifungal, antibacterial and cytotoxic activity (Lorimer et al., 1995). Other substances from the *Cibotium barometz* rhizome (3-[(4-*O*-caffeoyl-β-D-glucopyranosyloxy)methyl]-2,5-dimethoxy-3-hydroxytetrahydrofuran (= cibotiumbaroside B, **3**), corchoinoside C, and two glycoglycerolipids) were reported to inhibit the

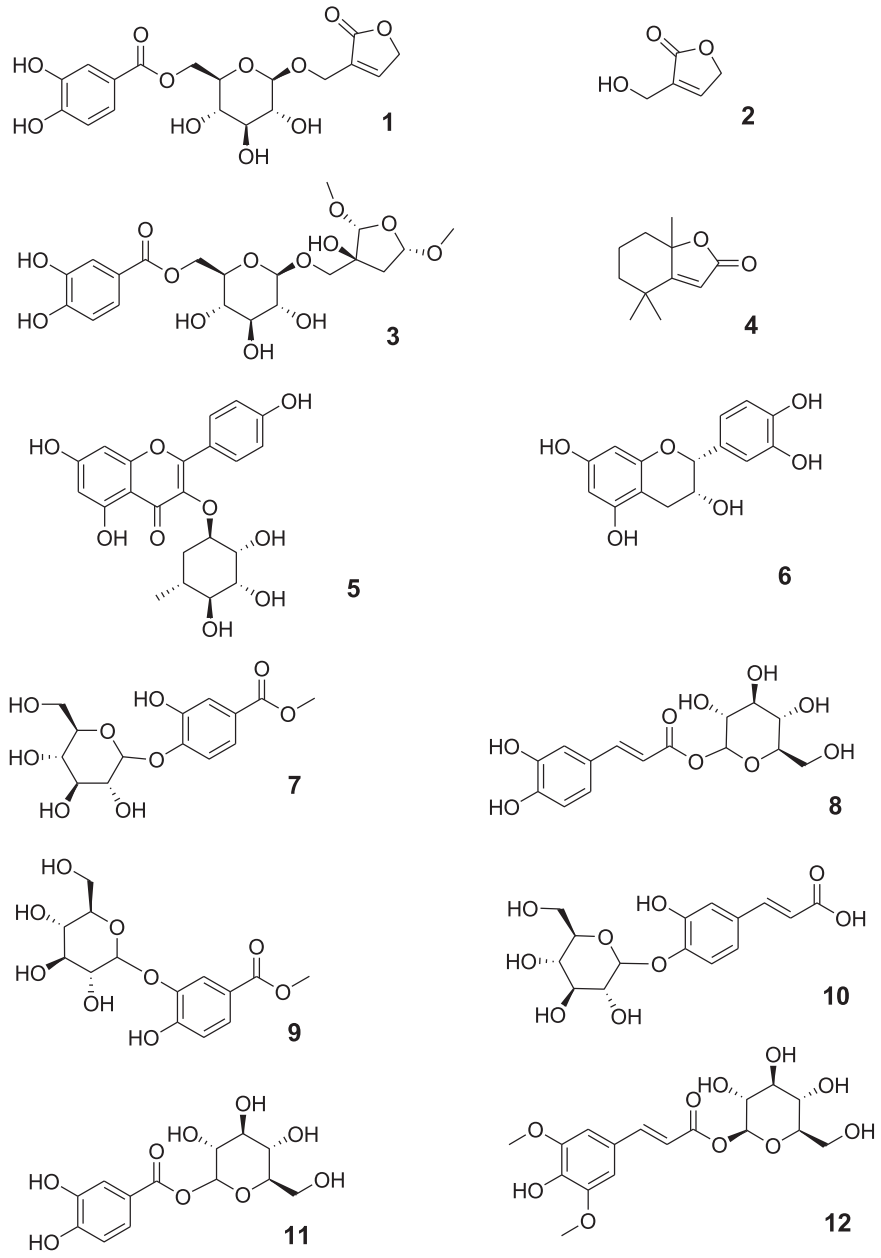


Fig. 2. Compounds detected in *Cibotium barometz* (**1**–**3**), *Heteropterys chrysophylla* (**4**, **5**), or *Bumelia sartorum* (**6**) and substances tested in the cell proliferation assay (**7**–**12**). Compounds **7**–**11** were made synthetically based on structures suggested for *Cibotium barometz*.

formation of osteoclasts, which might point to an androgenic or estrogenic activity (Cuong et al., 2009). In this study, ions which correspond to cibotiumbaroside B (**3**, $[M–H_2O+Na]^+$ *m/z* 507.14867, cal. for $C_{22}H_{28}O_{12}Na^+$ 507.14730) could be determined by MS investigations in fractions of the ethyl acetate extract.

Interestingly, sinapoyl glycosides as well as benzophenone derivatives that exhibit structural similarities to the *Cibotium* constituents were shown to target multiple aspects of androgen receptor signal resulting in an antiandrogen receptor action (Wang et al., 2009). Thus, the effect of simple glucosidic derivatives of protocatechuic, caffeoyl, and sinapoyl residues (**7**–**12**, Fig. 2) was investigated in the differentiating cell proliferation assay. All tested compounds did not significantly influence the proliferation of LNCaP or PC-3 cells and consequently do not possess antiandrogenic/androgenic or cytotoxic properties. Therefore, further investigations regarding the structure and the mode of action of secondary metabolites from *Cibotium barometz* appear necessary.

4.5.2. *Heteropterys chrysophylla*

Heteropterys chrysophylla (Lam.) Kunth (Malphigiaceae) occurs from Brazil to Bolivia. There are no detailed reports about the chemical composition of this species. The flowers are known to secrete an acylglycerol containing oil (cf. Seipold et al. 2004; Dumri et al. 2008). The extract of *Heteropterys chrysophylla* was shown to exhibit antifungal activity against several *Cladosporium* species (Agripino et al., 2004). For phytochemical investigations, the aqueous residue of the crude extract was successively partitioned with *n*-heptane and ethyl acetate. Since the non-polar *n*-hexane fraction 1H exhibited the strongest LNCaP cell proliferation stimulating activity, the *n*-heptane fraction was further separated on a silica gel SPE cartridge using eluents of different polarity. The fraction eluted with a mixture of chloroform-ethyl acetate (1:1) stimulated the proliferation of LNCaP cells, and reduced the cell number of the androgen insensitive PC3 cells, which might hint at an influence on the estrogen- or glucocorticoid receptor. GC-MS

investigations of this fraction indicated the presence of palmitic acid and dihydroactinolide (**4**) as main constituents. These compounds are accompanied by traces of several saturated fatty acids with chain lengths of 12, 14, and 18 carbons and of 6,10,14-trimethyl-2-pentadecanone. To our knowledge none of these compounds were reported to possess a hormonal or cytotoxic activity on human cells. However, dihydroactinolide was shown to exert phytoregulatory activities, e.g. seed germination inhibition (Kato et al., 2002).

The ethyl acetate fraction of *Heteropterys chrysophylla* is dominated by the two flavonol glycosides kaempferol-3-O-α-L-rhamnoside (**5**) and kaempferol-3-O-α-L-rhamnose-(2→1)-β-D-xylopyranoside. The compounds were isolated by column chromatography on silica gel and identified by comparison of spectral data with the literature data (Bilia et al., 1996). In addition, the structure of kaempferol-3-O-α-L-rhamnose-(2→1)-β-D-xylopyranoside was verified by 2D NMR measurements. Kaempferol-3-O-α-L-rhamnopyranoside (**5**) was previously found to be a weak ligand for estrogen receptors α and β *in vitro* with a greater affinity for ER β (Mallavadhani et al. 2006), and might therefore be responsible for the observed effects of the ethyl acetate and acetone fractions 1E and 1A.

4.5.3. *Sideroxylon obtusifolium*

Sideroxylon obtusifolium (Roem. & Shult.) T.D.Penn (Sapotaceae) is a tree native to Brazil and other parts of Southern America. In the Brazilian folklore the bark is widely used for traditional medicines. The plant is known to contain saponins. One of the main bioactive constituents is the triterpenoid bassic acid which exhibits hypoglycemic and anti-leishmaniosis activity (Almeida et al., 1985). Further triterpene derivatives can function as anticancer agents, immunostimulants and cytokinin formation promoters (Fujimoto et al., 2004).

In this study the ethyl acetate extract of *Sideroxylon obtusifolium* exhibited antiandrogenic activity shown by the inhibition of the proliferation of LNCaP cells under the influence of testosterone. TLC investigation of this extract indicated the presence of polyphenolic compounds solely. Column chromatography on silica gel using chloroform-methanol mixtures as eluent resulted in the isolation of epicatechin (**6**) as the main constituent. The compound was identified by comparison of MS and NMR data with reference data (Foo et al., 2000). Epicatechin was accompanied by small amounts of catechin. In addition, MS investigation revealed the occurrence of heptahydroxyflavan and of several dimeric, trimeric and tetrameric proanthocyanidins. The same extract was shown to exhibit antibacterial activity against *Staphylococcus aureus* strains, high antioxidant activity as well as a low acute toxicity (Ruela et al., 2011). Since epicatechin and related polyphenolic compounds interact with many proteins and therefore possess many biological activities, a potential use as androgen receptor antagonist seems doubtful.

5. Conclusions

The employed cell proliferation assay enables the simultaneous detection of hormonal and cytotoxic effects of plant extracts or compound mixtures, which cannot be analyzed by receptor binding studies. The differential behavior of the two prostate cancer cell lines LNCaP and PC-3 allows the discrimination between potential androgenic and antiandrogenic activities and influences the estrogen or glucocorticoid receptor as well as cytotoxic activities.

The observed effects of the investigated plant and fungal material correlate partly with the traditional use of these materials and with already described pharmacologically active constituents thereof. In other cases the hormonal or cytotoxic activities were determined for the first time in this study.

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Table A.1
Species and fractions with LNCaP cell proliferation stimulating activity while inhibiting PC-3 cell proliferation (DMSO control=0 %).

Species	Fraction	Proliferation (%) ± SD (%)	
		LNCaP	PC-3
Aglaia spectabilis	*n*-hexane fraction (H)	8 ± 1	−80 ± 3
Artemisia afra	*n*-hexane fraction (H)	58 ± 40	−99 ± 0,1
	acetone fraction (A)	125 ± 13	−84 ± 1
Cibotium barometz	ethyl acetate fraction (1E)	35 ± 17	−13 ± 10
	acetone fraction (1A)	38 ± 21	−20 ± 21
	methanol fraction (1M)	45 ± 28	−25 ± 15
	n − hexane fraction (2H)	16 ± 14	−21 ± 26
	methanol fraction (2M)	44 ± 15	−8 ± 9
	crude extract (3R)	49 ± 21	−18 ± 12
Cyrtomium falcatum	methanol fraction (1M)	13 ± 3	−62 ± 2
Monanthotaxis buchananii	acetone fraction (A)	46 ± 17	−10 ± 4
Reynoutria multiflorum	acetone fraction (1A)	41 ± 6	−32 ± 3
Salvia miltiorrhiza	ethyl acetate fraction (1E)	49 ± 29	−84 ± 5
	acetone fraction (1A)	30 ± 23	−49 ± 12
	ethyl acetate fraction (2E)	33 ± 61	−76 ± 4
	acetone fraction (2A)	13 ± 14	−13 ± 17
Suregada zanzibariensis	ethyl acetate fraction (E)	175 ± 19	−81 ± 4
	acetone fraction (A)	121 ± 36	−58 ± 7
Teclea nobilis	ethyl acetate fraction (E)	16 ± 3	−35 ± 3
Turrea cornucopia	*n*-hexane fraction (H)	37 ± 18	−99 ± 1
	acetone fraction (A)	24 ± 11	−12 ± 3
	methanol fraction (M)	17 ± 5	−9 ± 1

Table A.2
Species and fractions of other categories according to Scheme 2.

Species	Fraction	Proliferation (%) ± SD (%)		
		LNCaP	LNCaP + 1 nM TES	PC-3
Sideroxylon obtusifolium	aqueous crude extract 100 μg/ml[a]	18 ± 6	−102 ± 7	−73 ± 2
Reynoutria multiflorum	crude extract (3R)[a]	14 ± 5	−58 ± 31	−62 ± 9
Trimeria grandifolia	acetone fraction (A)[b]	112 ± 18	106 ± 33	9 ± 5

[a] antiandrogenic effect with simultaneous effect at the ER or GR
[b] unspecific proliferation effects or reducing influence on XTT-reagent or intrinsic dye compounds

Appendix A

See Tables A1 and A2.

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