Department of Pharmaceutical Biology[1], Department of Anatomy and Cell Biology[2], Department for Medical Biometry and Epidemiology[3], Philipps University, Marburg and Department of Pharmaceutical Biology[4], Martin-Luther-University, Halle/Saale, Germany

Antiproliferative effect of a polysaccharide fraction of a 20% methanolic extract of stinging nettle roots upon epithelial cells of the human prostate (LNCaP)

J. J. Lichius[1], C. Lenz[2], P. Lindemann[4], H.-H. Müller[3], G. Aumüller[2] and L. Konrad[2]

In Germany, plant extracts are often used in the treatment of early stages of benign prostate hyperplasia (BPH). The effects of different concentrations of the polysaccharide fraction of the 20% methanolic extract of stinging nettle roots (POLY-M) on the cellular proliferation of lymph node carcinoma of the prostate (LNCaP) cells were determined by measurement of the genomic DNA content of the samples. All concentrations of POLY-M showed an inhibitory effect on the growth of the LNCaP cells during 7 days except the two lowest concentrations. The reduced proliferation of POLY-M treated LNCaP cells was significantly ($p < 0.05$) different from the untreated control. The inhibition was time- and concentration-dependent with the maximum suppression (50%) on day 6 and at concentrations of 1.0E-9 and 1.0E-11 mg/ml. No cytotoxic effect of POLY-M on cell proliferation was observed. The *in vitro* results show for the first time an antiproliferative effect of *Urtica* compounds on human prostatic epithelium and confirm our previous *in vivo* findings.

1. Introduction

Benign prostatic hyperplasia (BPH) is the most frequent prostatic disease of elderly men with an incidence of 80% in men above the age of 80. The pathogenesis of BPH, however, is still an unresolved problem. A number of hypotheses have been proposed, e.g. McNeal [1] suggested that the pathological process of BPH is comparable to a "reakwakening of embryonic growth potential". During the last few years, growth factors and their receptors have been discussed as being possibly involved in the development of BPH [2, 3]. Other theories were more concerned with the influence of 5-α-reductase and with different hormone levels [4]. Advanced stages of BPH are usually treated by transurethal resection. In the treatment of early stages of BPH in Germany, plant extracts are often used, whereas in other countries the physicians prefer a "watchful waiting" therapy of BHP.

Extracts prepared from stinging nettle roots (*Urtica dioica* L., Urticaceae) are frequently used in the treatment of BPH grades I and II (symptom score according to Vahlensieck [5]) in Germany. In a study, where Bazoton®, a 20% methanolic extract of stinging nettle roots, was orally applied for six months, the urine flow of the patients was increased and the volume of the resting urine was shown to decrease [6].

Our previous results demonstrated that the 20% methanolic extract of stinging nettle roots (ME-20) had an inhibitory effect (approximately 50%) on cell growth rate of experimentally induced BPH in mice [7]. Based on these observations, the effect of ME-20 on the growth of epithelial and stromal cells of the human prostate was examined. From this experimental data it is evident that ME-20 specifically inhibits epithelial cell growth *in vitro* [8]. Subfractions from ME-20 were prepared and examined in an *in vivo* system. The polysaccharide fraction (POLY-M) was the most effective in inhibiting cell growth [9]. In an attempt to corroborate these results, we proceeded to test POLY-M on the proliferative activity of LNCaP cells in an *in vitro* system. The reasons why this cell line was chosen as a model for the epithelial compartment of the prostate were severalfold: (i) primary non-metastatic epithelial cells obtained from BPH patients rarely proliferate and are therefore unsuitable for proliferation studies, (ii) LNCaP are well characterized prostatic cells which are positive for e.g. PSA (prostate-specific antigen) and PSM (prostate-specific membrane antigen), two highly characteristic markers for human prostatic epithelial cells, (iii) LNCaP cells do not metastasize, and (iv) finasteride (Proscar®), a 5-alpha-reductase inhibitor, one of the standard drugs in the treatment of BPH, also demonstrates antiproliferative effects on LNCaP cells.

2. Investigations and results

According to sugar analysis, about 21% of the methanolic extract (ME-20) is composed of carbohydrates (Table). The POLY-M subfraction of ME-20 is enriched in polysaccharides including about 43% neutral sugars and 7% uronic acids. Thus at least half of the POLY-M fraction is composed of polysaccharides characterized by arabinose, galactose, and glucose as main sugar residues. The high glucose content also indicates the presence of low molecular weight sugar compounds (e.g. sucrose, maltooligosaccharides).

In addition to an untreated control, LNCaP cells were subjected to different concentrations of POLY-M and then treated in the same manner as the control. Measurements of the genomic DNA-contents were performed during 7 days as an equivalent for proliferation. The growth curves displayed the typical proliferation profile of untreated LNCaP cells, while the POLY-M-treated LNCaP

Table: Sugar composition of the 20% methanolic extract and POLY-M

Extract	ME-20 (mol %)	POLY-M (mol %)
Rhamnose	3.00	8.26
Arabinose	10.56	26.25
Xylose	2.80	1.09
Mannose	4.64	7.98
Galactose	8.80	29.95
Glucose	70.21	26.47
Neutral sugars (in %)	20.9	42.9
Uronic acids (in %)	1.7	7.3

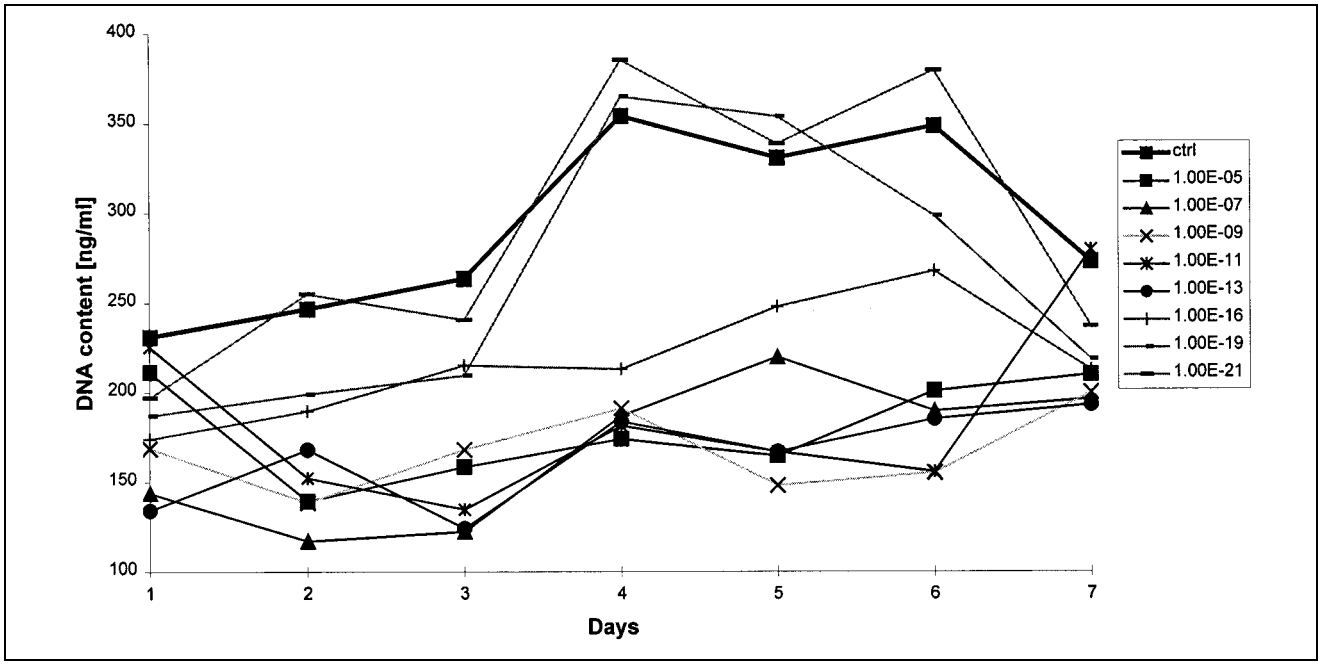

Fig. 1: Growth curves of LNCaP cells showing the untreated control in comparison to the POLY-M-treated cells. POLY-M was used at various concentrations as indicated in Experimental. Proliferation was determined by measuring the genomic DNA content. Each experiment was independently performed three times (n = 3) in duplicate. (SEM are not shown because of better clarity)

cells showed a clear reduction in cell proliferation in comparison to the untreated control (Fig. 1).

In order to demonstrate the growth suppression more clearly, every value obtained for the control was considered to be 100% for each day. The growth suppression in the POLY-M-treated groups was obtained by calculating the reduction in proliferation in comparison to the untreated control (Figs. 2a/b). In both cases the time-dependent reduction in proliferation is detectable. Suppression of the proliferation of LNCaP cells could be observed starting on the first day and reaching a maximum of approximately 50% on days 5 and 6 (Fig. 2a). All concentrations down to 1.0E-16 mg/ml [MED] showed a significant reduction ($p < 0.05$) in proliferation when compared with the control (at day 4–7). This significant reduction in growth of LNCaP cells is concentration-dependent indicating a specific effect of the POLY-M extract and was documented exemplarily for 3 days (Fig. 3). The lowest concentrations of POLY-M did not reveal any antiproliferative effects similar to the control.

The cytotoxic effect of POLY-M on the LNCaP cell was also studied (data not shown). A standardized procedure was used where the influence of different concentrations of POLY-M was compared to the effect of a 0.5 mM hydrogen peroxide solution on the cells. POLY-M showed no cytotoxic effects on the cells.

From this data it was evident that POLY-M inhibited epithelial cell growth *in vitro*. The rate of inhibition observed was even higher than that of earlier experiments obtained with ME-20 [8].

3. Discussion

In the present study the effects of POLY-M on LNCaP cells was investigated. LNCaP cells derived from lymph node carcinoma of the prostate are epithelial but they are still androgen-sensitive cells and exhibit the typical epithelial marker proteins. Therefore this cell line was used to investigate the influence of POLY-M on the epithelial compartment of the prostate *in vitro*.

The results demonstrated a significant reduction in growth rate with a minimal effective dose of 1.0E-16 mg/ml. The growth inhibition was time-dependent during seven days. Furthermore POLY-M treatment revealed also a specific and concentration-dependent effect (Fig. 3). The results concerning the sugar components of POLY-M are most comparable to the polysaccharide RP 5 described by Wagner et al. [10].

Finasteride shows similar effects to those of POLY-M on LNCaP cells [11]. The proliferative activity is also remarkably reduced after finasteride treatment [12]. These effects are mainly due to an inhibition of human 5-alpha-reductase by finasteride [11] thereby reducing the available amount of dihydrotestosterone. For stinging nettle root extracts, neither a 5-alpha-reductase inhibitory activity nor antiandrogen effects could be observed [12]. Therefore, different modes of action of stinging nettle root components have been discussed.

For example, experiments were performed with the sex hormone binding globulin (SHBG), a protein which reversibly binds androgens and estrogens in plasma and therefore regulates the concentration of these hormones in the plasma (and of course also in the prostate). An aqueous extract of stinging nettle roots showed a concentration-dependent inhibition of the binding of SHBG to its receptor [13]. It was also demonstrated that some lignans (from stinging nettle) and their metabolites interfere with the binding of androgens to SHBG [14, 15].

In a clinical study, β-sitosterol, a compound often regarded as "leading substance" for standardization of stinging nettle root extracts, showed a significant improvement of the symptoms and urinary flow of BPH patients [16]. However, it must be mentioned, that humans normally consume considerably larger amounts of sitosterol with their daily assimilation of food compared to therapeutical doses of a plant extract (containing β-sitosterol) [10]. The specific effect of β-sitosterol on BPH is therefore difficult to assess.

Urtica dioica agglutinin (UDA), a lectin from stinging nettle roots, directly inhibited cell proliferation of HeLa

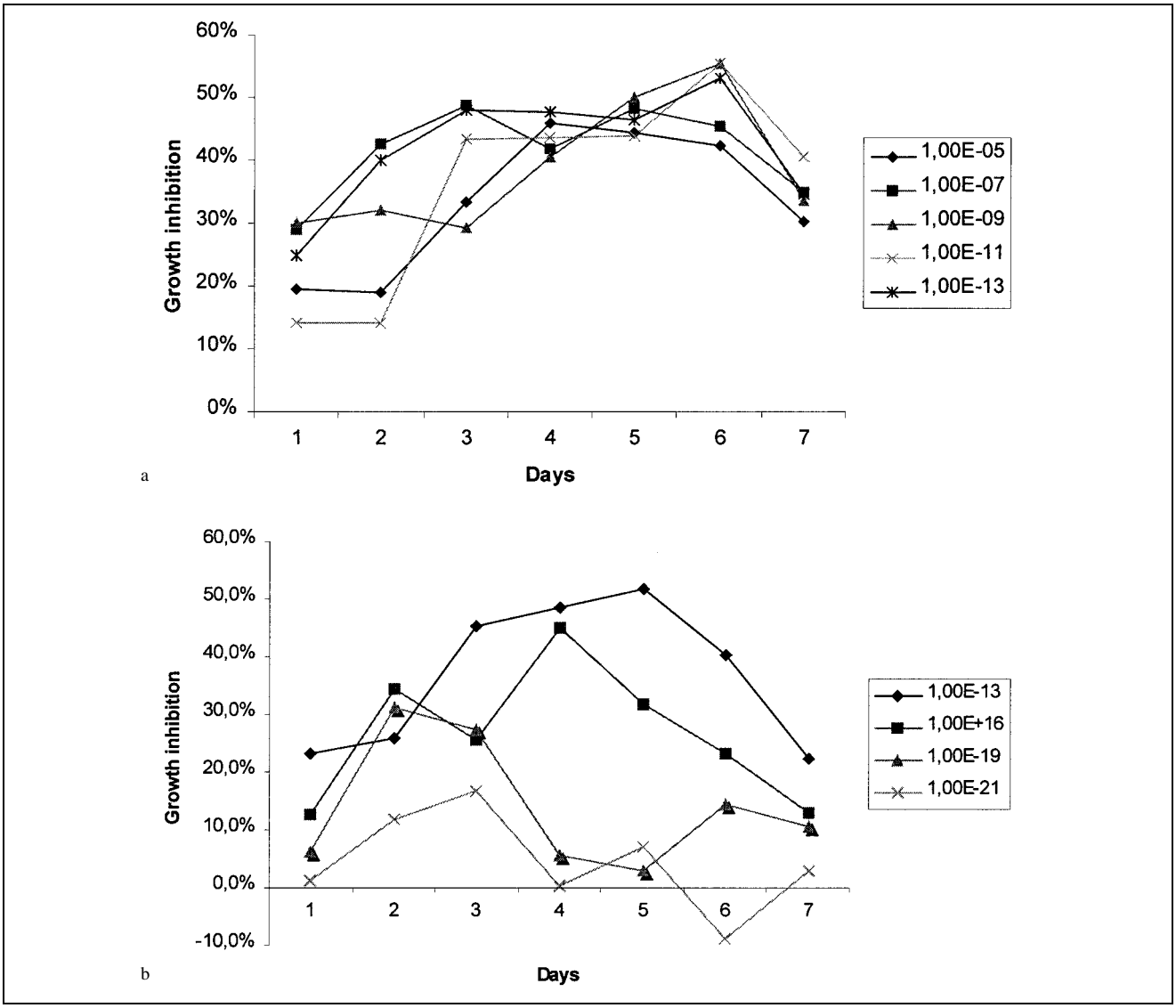

Fig. 2a, b: Effects of the concentration of 1.0E-5 to 1.0E-13 mg/ml (Fig. 2a) and 1.0E-13 to 1.0E-21 mg/ml (Fig. 2b) of POLY-M on the growth of LNCaP cells. Growth suppression in the POLY-M-treated groups of LNCaP cells was obtained by calculating the reduction in proliferation in comparison to the untreated control (= 100% proliferation). The time-dependent reduction in proliferation is detectable reaching a maximum of approximately 50% on days 5 and 6. (SEM are not shown because of better clarity)

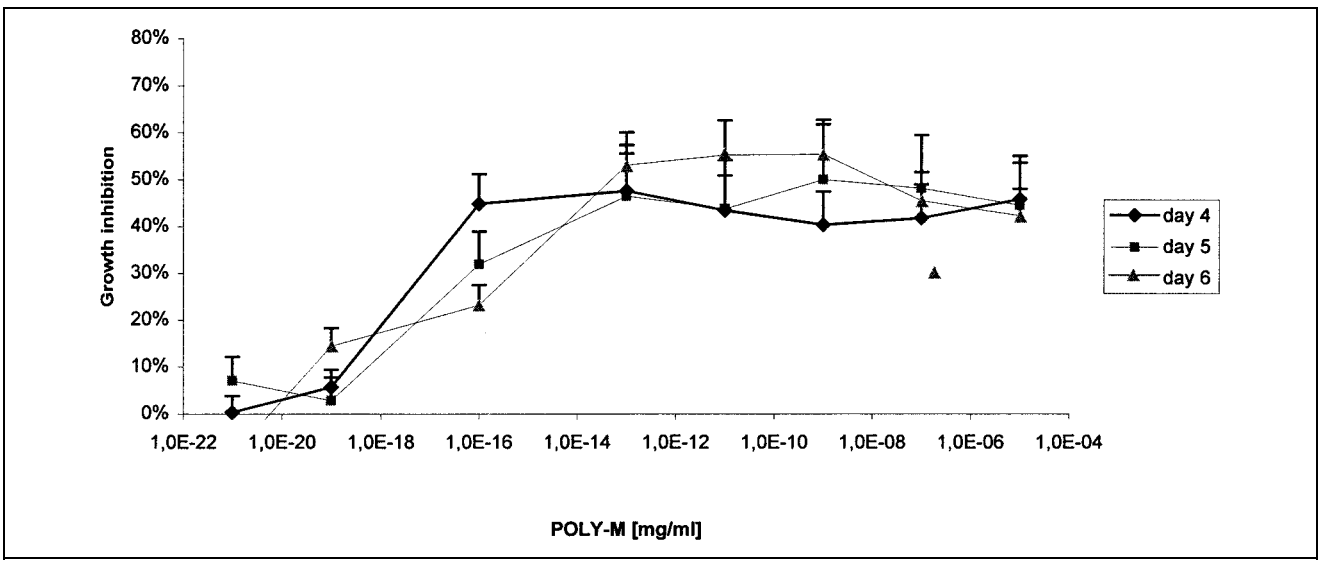

Fig. 3: Concentration response curve of LNCaP cells demonstrating the concentration-dependent reduction [%] in proliferation on day 4, 5, and 6 upon stimulation with varying concentrations of POLY-M. The concentrations of 1.0E-5 to 1.0E-16 are statistically significant ($p < 0.05$) different from the untreated control. Each point represents the means (±SEM) of three separate experiments ($n = 3$) performed in duplicate.

cells and blocked the binding of EGF to its receptor [10]. It was therefore regarded to be responsible for growth inhibiting effects in BPH treatment. In the same study a polysaccharide fraction prepared from an aqueous root extract was demonstrated to elicit an antiinflammatory activity in a rat paw edema test. These results support pharmacological effects of polysaccharides following oral application.

The results obtained with the *in vivo* BPH mouse model [7, 9] and the observations in studies using an *in vitro* approach with prostate epithelial (cancer) and stromal cells clearly showed that epithelial cell growth is significantly reduced by ME-20 [8]. In addition to these results the present study demonstrated growth rate inhibiting effects of POLY-M in *in vitro* cell lines. These results were further substantiated by experimental data indicating a growth-reducing effect of POLY-M in an *in vivo* BPH model [9].

Based on these observations, we postulate that the reduced prostate growth rate observed in an *in vivo* BPH-model and *in vitro* cell lines treated with polar stinging nettle root extracts is at least partly due to a reduced proliferative activity of prostatic epithelial cells.

4. Experimental

4.1. Plant material

Dried roots of *Urtica dioica* L. were purchased from H. Finzelbergs Nachfolger GmbH & Co., Andernach, Germany. Voucher specimens are deposited at the Department of Pharmaceutical Biology in Marburg, Germany.

4.2. POLY-M

20% methanolic-aqueous extracts were obtained from 1005.8 g of dried, milled stinging nettle roots. Maceration was performed with 3.0 l of 20% CH_3OH for 24 h at room temperature. The solution was then transferred to a percolation column and extracted with additional 5.0 l of 20% CH_3OH. After lyophilization, 67.68 g methanolic extract were obtained. The preparation (10.13 g) was resuspended in 100 ml of distilled H_2O and after 8 h centrifuged at 12,000 × g for 15 min. 200 ml C_2H_5OH (200 ml) was then added to the supernatant, the solution placed for 12 h at 4 °C and once again centrifuged at 12,000 × g for 15 min. The resulting pellet was resuspended in 100 ml of distilled H_2O and mixed with 10 g of trichloroacetic acid diluted in 50 ml of distilled H_2O. After 12 h at room temperature the solution was centrifuged at 12,000 × g for 15 min at room temperature and the supernatant dialyzed (3,500 Dalton, Spectra/por 3, Roth, Karlsruhe, Germany) against distilled H_2O. (3 changes). The final amount of polysaccharides (POLY-M) following lyophilization was 373.48 mg.

4.3. Sugar analysis

Analysis of sugar content and composition was performed according to Blakeney et al. [17]. Briefly, freeze dried extracts after addition of myo-inositol as internal standard were hydrolyzed with 2 M trifluoroacetic acid for 60 min at 121 °C. The hydrolyzed material was reduced with $NaBH_4$ and alditols formed were acetylated prior to GLC-analysis on a Permabond OV-225-fused silica column (25 m × 0.25 mm) using a FID. Uronic acid contents were determined by the biphenylol-method [18].

4.4 Cell culture

2.0 × 1.0E-5 cells/well of LNCaP (lymph node carcinoma of the prostate; American Type Culture Collection) were seeded in 12-well plates and cultured in 2 ml RPMI-1640 medium supplemented with 10% FCS and 100 ng/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Sigma, Deisenhofen, Germany).

The cells were either cultured without POLY-M (control) or once treated with various concentrations (1.0E-5 mg/ml to 1.0E-21 mg/ml) of POLY-M for 7 days. Each experiment was independently repeated three times (n = 3) in duplicate.

4.5. Proliferation assays

Cell proliferation of LNCaP was quantified by determining the amount of genomic DNA by a fluorometer according to the manufacturer's recommendations (Pharmacia, Freiburg, Germany). In preliminary experiments, we found that the amount of genomic DNA correlates with the number of cells (under the conditions used).

4.6. Cytotoxicity test

1.3 × 1.0E-4 cells were seeded in 24-well plates and incubated in RPMI-1640 supplemented with 10% FCS. After 3 days the medium replaced with one to which POLY-M had been added at various concentrations (1.0E-5, 1.0E-7, 1.0E-9, 1.0E-11, and 1.0E-13 mg/ml). After an 18 h incubation, the medium was discarded and the cells washed with PBS. Viable cells were stained with 0.4% trypan blue and, after an incubation of 5 min, counted in a microscope. As a negative control, cells grown in RPMI-1640 medium without any POLY-M were treated in the same way. As positive control, cells were incubated 10 min at room temperature with 0.5 mM H_2O_2. Each experiment was performed in triplicate.

4.7. Statistical analysis

As a primary endpoint for each concentration the means ± SEM along the time points were calculated. The minimal effective dose (MED) was determined according to Williams [19]. P values of <0.05 were considered to be statistically significant.

Acknowledgements: This study was partly sponsored by Kanoldt, Höchstädt, Germany.

References

1 McNeal, J. E.: Invest. Urol. **15**, 340 (1978)
2 Bonkoff, H.; Remberger, K.: Pathologe **19**, 12 (1998)
3 Storry, M. T.; Hopp, K. A.; Meier, D. A.: Prostate **28**, 219 (1996)
4 Wilson, J. D.; Griffin, J. E.; Russell, D. W.: Endocr. Rev. **14**, 577 (1993)
5 Vahlensieck, W.: Therapiewoche **35**, 176 (1985)
6 Engelmann, U.; Boos, G.; Kreis, H.: Urol. Ausg B **36**, 287 (1996)
7 Lichius, J. J.; Muth, C.: Planta Med. **63**, 307 (1997)
8 Konrad, L.; Müller, H.-H.; Lenz, C.; Laubinger, H.; Aumüller, G.; Lichius, J. J.: Planta Med., submitted
9 Lichius, J. J.; Renneberg, H.; Blaschek, W.; Aumüller, G.; Muth, C.: Planta Med. in press
10 Wagner, H.; Willer, F.; Samtleben, R.; Boos, G.: Phytomedicine **1**, 213 (1994)
11 Bologna, M.; Muzi, P.; Biordi, L.; Festuccia, C.; Vicentini, C.: Urol. **45**, 282 (1995)
12 Rhodes, L.; Primka, R. L.; Berman, C.; Vergult, G.; Gabriel, M.; Pierre-Malice, M.; Gibelin, B.: Prostate **22**, 43 (1993)
13 Hryb, D. J.; Khan, M. S.; Romas, N. A.; Rosner, W.: Planta Med. **61**, 31 (1995)
14 Ganßer, D.; Spiteller, G.: Z. Naturforsch. **50c**, 98 (1995)
15 Schöttner, M.; Ganßer, D.; Spiteller, G.: Planta Med. **63**, 329 (1997)
16 Berges, R. R.; Windeler, J.; Trampisch, H. J.; Senge, T. and the β-sitosterol study group: Lancet **545**, 1529 (1995)
17 Blakeney, A. B.; Harris, P. J.; Stone, B. A.: Carbohydr. Res. **113**, 291 (1983)
18 Blumenkrantz, N.; Asboe-Hansen, G.: Anal. Biochem. **54**, 484 (1973)
19 Williams, D. A.: Biom. **28**, 519 (1972)

Received March 15, 1999
Accepted April 18, 1999

Johannes J. Lichius
Department of Pharmaceutical Biology
Philipps-Universität Marburg
Deutschhausstr. 17A
D-35032 Marburg
lichius@mailer.uni-marburg.de