Piper cubeba Targets Multiple Aspects of the Androgen-Signalling Pathway. A Potential Phytotherapy against Prostate Cancer Growth?

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Text 1: HPLC analysis

An aliquot of the extract was analyzed by a Waters HPLC system with UV-VIS detection (280 – 600 nm) using a Nucleosil 120 – 3, C18 (250 × 4.6 mm) column with a pre-column of the same material (both Macherey Nagel; Oensingen, Switzerland) as stationary phase. The mobile phase consisted of two solvent systems [A: 0.1% trifluoroacetic acid in water (v/v) and B: 100% acetonitrile in a gradient (0 – 30 min 90% A, 30 – 45 min 50% A, 45 – 46 min 10% A, 46 – 50 min 10% A)]. The column temperature was 40 °C and the flow rate was set at 1.0 mL/min. The detection was carried out at 280 nm and quantification of the lignan in the extract was performed by the external standard method using cubebin ( Extrasynthese; Genay Cedex, France) as reference substance (Fig. 1S).

![HPLC chromatogram of P9605. Peaks 1, 2 and 4 indicate the presence of lignans based on their typical UV spectra. Peak 3 is identified to be cubebin.](image)

Text 2: 5α-reductase activity

This assay was performed using homogenates of HEK293 cells overexpressing 5α-reductase type II. They were purchased from Dr. Hartmann (Department of Pharmaceutical and Medical Chemistry, University of Saarbrücken, Germany). cDNA encoding 5α-reductase type II were inserted into a pRcCMV vector and expressed in these cells. The samples were pre-incubated with the cell homogenate (20 µg/mL per assay) for 5 min at 37 °C. After which 80 nM 3H-
testosterone (substrate) was added to each well and incubated for 20 min at 37 °C. The remaining $^3$H-testosterone and new steroids produced were extracted by ethyl acetate. Thin layer chromatography (TLC) (equal volumes of cyclohexane and ethyl acetate were used as elution solvent) was preformed to separate the steroids. The TLC plate was then developed using a detection spray containing anisaldehyde, concentrated sulphuric acid, acetic acid glacial and methanol. To quantify the amount of $^3$H-testosterone remaining and $^3$H-DHT produced, bands on the TLC corresponding to the respective steroids were cut out and counted via the scintillation counter. The activity of the enzyme was determined by calculating the conversion of $^3$H-testosterone to $^3$H-DHT.

**Text 3: Western blot analysis**

LNCaP cells were cultured in 25 cm$^2$ cell culture flasks until near confluent (80%) before the addition of P9605 and cubebin (Sigma; St. Louis, MO, USA) at concentrations 7.5, 15, 30 µg/mL and incubated for 48 hours. Cells were then washed once with PBS and lysed in RIPA buffer containing phosphatase and protease inhibitors. Protein concentrations were determined by Bicinchoinic acid (BCA) protein determination test (Pierce; Rockford, Ill, USA). Equal amounts of protein were mixed with 1× loading buffer (Laemmli Buffer, 5% 2-mercaptoethanol) and fractionated by electrophoresis on 8% SDS polyacrylamide gels. Proteins were electro transferred to nitrocellulose membranes (Millipore; Bedford, MA, USA) and then blocked 1 hour at 4 °C with 5% w/v non-fat dry milk dissolved in Tween-Tris buffered saline (TTBS). Blots were then incubated for overnight at 4 °C with primary mouse anti-human androgen receptor (Progen; Heidelberg, Germany) and anti-β-actin (Sigma; St. Louis, MO, USA). The membranes were washed 3 times with TTBS before incubating for 1 hour with secondary goat anti-mouse IgGs conjugated with alkaline phosphatase (BioRad; Hercules, CA, USA). The blots were then developed with chemiluminescent substrate and enhancer (Bio-Rad), followed by exposure to X-ray film. The images were scanned and analysed by Quantity One software programme (Bio-Rad).