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Inhibition of Cytochrome P450 3A4 by Extracts and Kavalactones of *Piper methysticum* (Kava-Kava)

Abstract

Inhibitors of cytochrome P450 3A4 (CYP3A4) were identified in crude extracts from the rhizomes of *Piper methysticum* G. Forst. (Kava-Kava) using bioassay-guided fractionation. After preliminary purification of an ethyl acetate extract with solid phase extraction, the eluate was further fractionated by means of HPLC and fractions were tested for inhibitory potency using cDNA expressed CYP3A4. Positive fractions were analysed with LC/MS using electrospray ionisation and kavapyrones could be identified as the main CYP3A4 inhibitory components of *Piper methysticum*.

Piper methysticum, also called Kava-Kava, is a South Pacific perennial shrub that grows best in warm and humid conditions at altitudes of 150 – 300 m above sea level. A beverage made from the rootstock of the plant has been used for centuries in ceremonies and celebrations because of its calming effect and ability to promote sociability. The German Commission E monograph recommends extracts of the kava rhizome as phytopharmaceuticals against anxiety and restlessness without toxic effects [1].

Recently, kava extracts were withdrawn from the German market due to toxic effects mainly liver damage [2], [3], [4]. The reason for the liver toxicity of *P. methysticum* still remains unknown and the clinical evidence relating to the safety of kava extracts was systematically reviewed [5], [6], [7]. Since cytochrome P450 enzymes play a tremendous role in the hepatic metabolism and elimination of xenobiotics, we investigated the influence of kava extracts on the main drug metabolising enzyme cytochrome P450 3A4 (CYP3A4). This CYP isoform which is expressed in relatively high amounts in the liver but also in the gut [8] is responsible for the metabolism of many therapeutically useful drugs, e.g., midazolam [9], erythromycin [10] and terfenadine [11]. Plant extracts inhibiting CYP3A4 include St. John's wort [12], dong quai [13], milk thistle [14] and - the most prominent example - grapefruit juice, which increases plasma levels of common therapeutics, e.g., cyclosporine [15] and lovastatin [16].

A crude EtOAc extract from kava-kava root powder was fractionated using solid phase extraction on reversed phase silica gel and finally reversed phase HPLC with subsequent testing of fractions for inhibitory potency on CYP3A4. For this purpose the regio- and stereoselective hydroxylation of testosterone to 6-β-hydroxytestosterone was used. Positive fractions were analysed with LC/ESI-MS in order to identify the inhibitory components by their characteristic *m/z* ($[M+H]^+$) signals (for structures and molecular weights see Fig. **1**).

In Fig. **2** the inhibition of CYP3A4 by different kava extracts is shown. Inhibitory activity increased with decreasing polarity of the extraction solvent. The LC/ESI-MS analysis in scan mode revealed the presence of typical lipophilic kava ingredients in all extracts except those made with water and 25% MeOH which contained only small amounts of kavalactones (data not shown). A significant inhibition of CYP3A4 was observed for the methanolic extracts (50/75/100% MeOH) and the acetone and EtOAc

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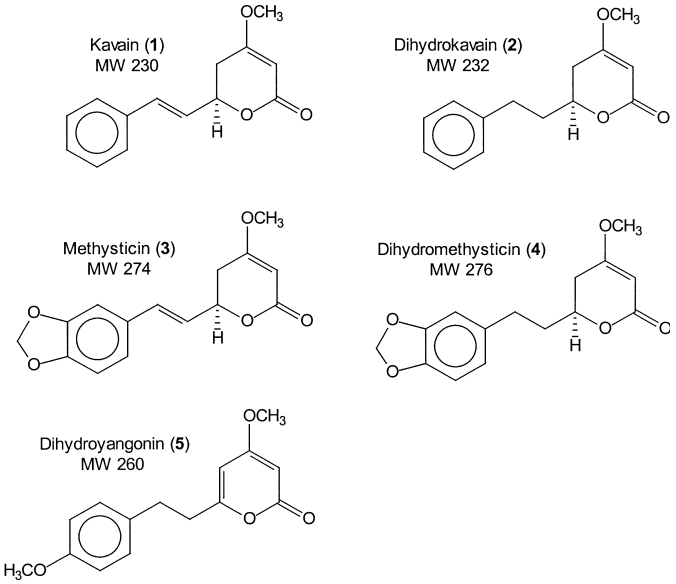

Fig. **1** Structures and molecular weights (MW) of the kavapyrones **1 – 5**.

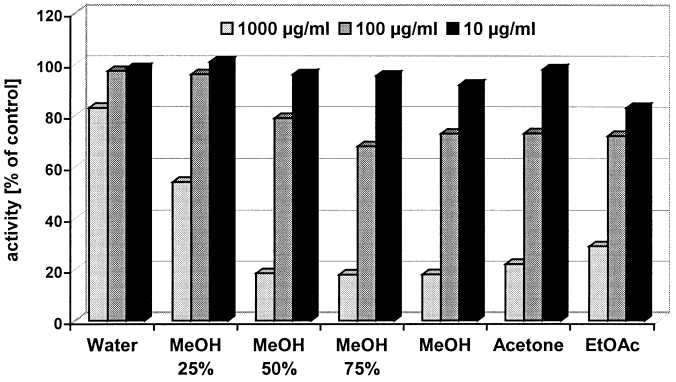

Fig. **2** Influence of various extraction solvents on CYP3A4 activity of kava extracts (results are mean values of triplicate measurements). For experimental details see Materials and Methods.

extracts. Interestingly, the 75 % MeOH extract showed the strongest inhibition at a concentration of 100 µg/ml whereas the EtOAc extract showed strongest inhibitory activity at 10 µg/ml (Fig. **2**). This points to a stronger activity of lipophilic ingredients suggesting a potent inhibition of CYP 3A4 by highly lipophilic kava constituents such as the kavalactones or flavokavines.

When the dried residue of the EtOAc extract (IC_{50}: 300 µg/ml) was dissolved in 50 % MeOH and applied to a solid phase extraction cartridge, a full retention of CYP3A4 inhibitory activity at the reversed phase silica gel was obtained as can be seen in Fig. **3** (fraction A**,** B). Elution of the C18 cartridge with pure MeOH resulted in a total recovery of inhibitory potency (Fig. **3**, fraction C). Fractionation of the MeOH eluate with HPLC revealed three samples with significant inhibition of CYP3A4 (No. 2, 4, 11, Fig. **3**). Fraction No. 11 which showed an inhibitory activity of 30 % (Fig. **3**) was collected between 25 and 27 min suggesting an inhibition of CYP3A4 by highly lipophilic compounds e. g., volatile oils. The other active fractions (No. 2 and 4**,** Fig. **3**) were much more polar and eluted between 7 – 9 min (No. 2) and 11 – 13 min (No. 4), respectively. No signals could be detected by LC/UV and LC/ESI-MS analysis of fraction 11 and 2 (data not shown). This could be the consequence of a low concentration of inhibitory compounds but also a result of weak UV absorption or missing proton affinity for the ESI process resulting in a low sensitivity using MS detection. When fraction No. 4 was analysed with LC/ESI-MS (Fig. **4**), we obtained the characteristic *m/z* signals corresponding to the $[M + H]^+$ ions of the kavapyrones **1 – 5** (Figs. **1** and **4**) [17]. The extracted ion chromatograms shown in Fig. **4** clearly demonstrate the presence of kavain (**1**, *m/z* 231), dihydrokavain (**2**, *m/z* 233), methysticin (**3**, *m/z* 275), dihydromethysticin (**4**, *m/z* 277) and dihydroyangonin (**5**, *m/z* 261). No other signals could be detected using UV and MS detection. Fraction No. 4 was the main CYP3A4 inhibitory sample of the EtOAc extract of *P. methysticum* with 60 % inhibition (Fig. **3**).

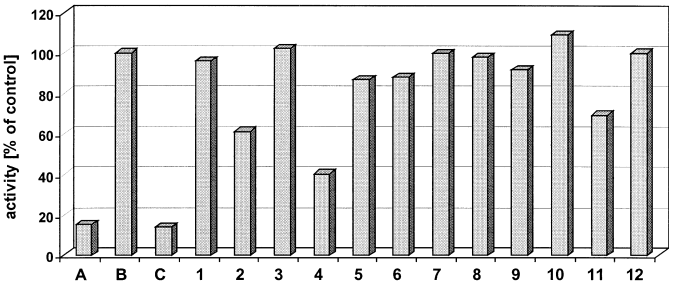

Fig. **3** Inhibition of CYP3A4 by fractions obtained with solid phase extraction (**A-C**) and HPLC fractionation (**1 – 12**). **A:** EtOAc extract dissolved in MeOH 50 %; **B:** extract A passed through the reversed phase extraction column; **C:** MeOH eluate; Results are mean values of triplicate measurements. For experimental details see Materials and Methods.

The aforementioned results clearly demonstrate that extracts of kava-kava contain various inhibitors of CYP3A4 and are likely to cause drug interactions when administered concomitantly with drugs metabolised predominantly by CYP3A4. Bioassay-guided fractionation led to the identification of kavapyrones as the main inhibitory principle. Since one case report described the coma of a woman after simultaneous ingestion of kava and alprazolam, a substance known to be metabolised by CYP3A4, an *in-vitro-in-vivo* correlation is obvious [18], [19]. It has to be clarified whether kavapyrones are inhibitors of CYP3A4 that are metabolised to reactive intermediates causing liver failure or if the inhibition of CYP3A4 by Kava extracts leads to elevated plasma levels

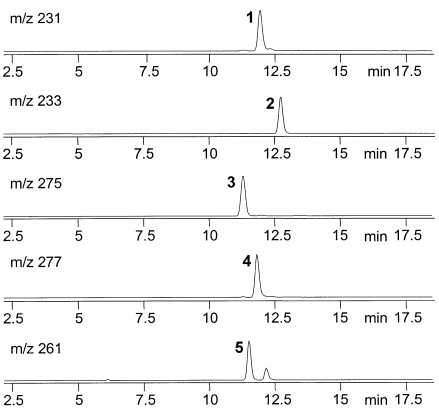

Fig. **4** Extracted ion chromatograms obtained after LC/ESI-MS analysis of fraction 4 in full scan mode. Peak numbers and *m/z* values of the obtained signals correspond to the structures and molecular weights given in Fig. **1**. For experimental details see Materials and Methods.

of simultaneously ingested drugs with potential liver toxicity. Further studies are necessary to elucidate a possible correlation between cytochrome P450 enzymes and liver damage caused by extracts of *Piper methysticum*.

Materials and Methods

Kava-Kava capsules filled with root powder (2.5% kavalactones, batch No. 921552) were from Nature's Way Products Inc. (Springville, Utah, USA). A voucher specimen is deposited at the Institute of Pharmacy (Würzburg, Germany). Testosterone, prednisone, ketoconazole, NADPH and formic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Kava-Kava extract was made by extracting 1 g root powder with 10 ml of water, 25% MeOH, 50% MeOH, 75% MeOH, MeOH, acetone and EtOAc for 30 min in an ultrasonic bath at 40 °C. The resulting yellow coloured extract was transferred to glass vials, centrifuged (3000 rpm) and stored in the dark at room temperature.

One ml of the EtOAc extract was evaporated to dryness and redissolved in 5.0 ml 50% MeOH. This solution was passed through an RP18 endcapped solid phase extraction cartridge (900 mg, Macherey & Nagel, Düren, Germany) preconditioned with MeOH, water and finally 50% MeOH (5 ml each). After the cartridge was dried by applying vacuum for 3 min, elution was performed with MeOH (5 ml). The eluate was diluted with water to give a final MeOH concentration of 50% and after centrifugation, 100 µl were injected onto a 150 × 4.6 mm C_8, 3.5 µm analytical column (Zorbax XDB C8, HP, Palo Alto, USA). After five minutes, 12 fractions (2 min each) were collected and 50 µl of each sample was evaporated to dryness at 50 °C in an eppendorf tube for determination of inhibitory activity. The solvents used for HPLC fractionation were water (**A**) and MeOH (**B**) and the gradient was as follows: 55% **A** (0 min), 5% **A** (22 min), 5% **A** (29 min). Flow rate was 0.5 ml/min and column temperature was set to 40 °C. For LC/ESI-MS analysis of kava extracts and HPLC fractions the injection volume was reduced to 25 µl and the same HPLC conditions were applied except that MS detection was used for identification of substances. All ESI-MS parameters used are the same as described below for the CYP3A4 assay.

The crude kava extracts (100/10/1 mg/ml) or samples from HPLC fractionation were incubated with substrate (testosterone, 50 µM), NADPH (0.4 mM) and CYP3A4 (0.1 mg/ml) from baculovirus infected insect cells (Gentest, Woburn, USA; protein content 5 mg/ml) in 100 mM K/Na phosphate buffer pH 7.4 for 30 min at 37 °C. Substrate solution (5 µl), kava extract (5 µl), phosphate buffer (240 µl) and enzyme (50 µl, 1.0 mg/ml protein) were preincubated for 5 min at 37 °C and the reaction was started through the addition of 200 µl NADPH solution (1 mM in phosphate buffer). Control incubations were carried out with 5 µl solvent instead of kava extract. Ketoconazole, a potent inhibitor of CYP3A4, was used as a positive control. After 30 min the reaction was stopped by addition of 500 µl ice-cold MeOH containing 10 µg/ml prednisone and after centrifugation at 10,900 g and 4 °C for 15 min the supernatant was transferred to HPLC vials and 25 µl were injected onto a Zorbax XDB 3.5 µm C8 column (150 × 4.6 mm) for LC/ESI-MS. Samples were analysed on a Hewlett-Packard (HP, Palo Alto, CA, USA) LC/MS system consisting of the following series 1100 HPLC components: G1312A binary pump, G1322A degasser, G1316A column thermostat and G1946A mass selective detector. The mobile phase for LC/ESI-MS quantification of 6-β-hydroxytestosterone consisted of 0.02% formic acid (**A**) and methanol (**B**) and the flow rate was set to 0.5 ml/min. The following gradient was run: 0 min: 55% (**A**), 12 min: 50% (**A**). After 12 min the column was flushed with pure MeOH for 3 min and re-equilibrated with 55% (**A**) for 5 min. Positive ions ($[M + H]^+$) of 6-β-hydroxytestosterone and the internal standard prednisone were recorded in SIM mode at m/z = 305 and m/z = 359, respectively.

Capillary voltage was 4000 V and drying gas flow (nitrogen) was set to 10 L/min (300 °C). Nebuliser gas flow (nitrogen) was 50 psig.

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