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Is the alkaloid pipermethystine connected with the claimed liver toxicity of Kava products?

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The pyridone alkaloid pipermethystine has been considered to be responsible for alleged hepatoxicity of Kava products. Investigation of a series of retain samples of finished products from the German market and self-produced extracts from root and stem material of *Piper methysticum* clearly showed that pipermethystine (**1**) is absent from all root and retain samples and extracts, with a limit of quantification of 45 ppm. As a positive control, leaves of *P. methysticum* showed an amount of 0.2% of **1**. Thus, if there is any hepatotoxicity, compound **1** should not be the responsible constituent in the case reports with ethanolic extracts produced in Germany.

1. Introduction

Piper methysticum Forst. (Piperaceae) is a tropical shrub cultivated in the South Pacific. Stems and roots (respectively rhizomes) are used for the preparation of the beverage and ceremonial drink known as Kava (Kava-Kava, Kawa, 'awa). Besides the well known tranquilizing and calming properties further pharmacological investigations and clinical studies have shown an anxiolytic activity in humans; the kavalactones are regarded to be responsible for most pharmacological effects (Singh and Singh 2002).
On a quantitative basis, 6 kavalactones are dominating alcoholic root and rhizome extracts; including minor compounds at least 13 of these lactones are known today (He et al. 1997). Compared to root extracts, those of leaves show a different composition. Smith (1979) discovered the pyridone alkaloid pipermethystine (**1**) in leaves of *Piper methysticum*. Though the enantiomeric synthesis of compound **1** has recently been reported (Arrayas et al. 2001), the absolute configuration of **1** has not yet been established. Further studies showed that **1** is one of the major constituents in extracts of leaves (0.3–2.4%; Dragull et al. 2003) and is also present in smaller amounts around 0.023% in stem peelings (Berkulin et al. 2005). In roots, **1** is only a trace component or is even not detectable (Smith 1983; Jhoo et al. 2005).
Recently, a worldwide discussion on potential liver toxicity of extracts obtained from Kava (Piperis methystici rhizoma) was initiated by a series of reports resulting in a ban by the German Federal Institute for Drugs and Medical Devices (BfArM) that was followed by other countries (Anke and Ramzan 2004; Nerurkar et al. 2004). However, most cases were evaluated as doubtful or unsubstantiated on causality assessment; only very few cases seem to be possibly related to the intake of Kava extract products (Schmidt et al. 2002, 2005).
Several theories evolved as to why liver failure, if in fact there is a causal relationship to Kava, may have occurred with European Kava extract preparations (Anke and Ramzan 2004). Dragull et al. (2003) suggested the alkaloid pipermethystine (**1**) being responsible for hepatotoxicity. It was argued that commercial crude drug material may have been adulterated by stem peelings and leaves thereby introducing compound **1** into the commercially used extracts (Dragull et al. 2003; Nerurkar et al. 2004). Compound **1**, isolated from the aerial parts of the cultivar "Isa" (originally from Papua New Guinea), exhibited cytotoxic activity in HepG2 cells at 100 and 50 μM *in vitro*, probably by disrupting mitochondrial function (Nerurkar et al. 2004). The main kavapyrones, however, were not cytotoxic in primary hepatocytes of rats (Singh and Devkota 2003; Schäfer et al. 2005). Likewise, ethanolic Kava extracts corresponding to qualities on the German market at the time of the Kava ban did not show hints on potential liver toxicity in rats (Sorrentino et al. 2006; DiSilvestro et al. 2007). Although Clayton et al. (2007) in fact found liver toxicity in rats with high extract doses, the extract quality used for this study was rather untypical (43% of yangonin) and does neither correspond to crude drug qualities used for Kava drinking in the South Pacific nor to the composition and cultivars of the European extracts alleged to have caused hepatotoxicity. Compound **1** also showed mutagenicity above 2.5 μg/mL in the mouse lymphoma assay (Jhoo et al. 2005). As a result, patents on preparation of

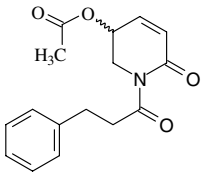

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Table: List of samples investigated

No.	Sample name (German)	Origin	Organ	Company	Type
1	MG1 Extrakt			Mueller Goeppingen	Extract
2	MG2 Extrakt			Mueller Goeppingen	Extract
3	MG3 Extrakt			Mueller Goeppingen	Extract
4	Spissumextrakt Gehrlicher			Gehrlicher	Extract (spissum)
5	EuKavan			Salus	Capsule
6	W508	Vanuatu		Schwabe	Crude drug
7	W504	Vanuatu		Schwabe	Crude drug
8	Kavatonga	Tonga		Phytopharm	Crude drug
9	Kavacur			Biocur	Coated pill
10	Kavatino			Bionorica	Capsule
11	Kavasedon			Harras Pharma	Capsule
12	Kavosporal forte			Mueller Goeppingen	Capsule
13	Kava von ct			ct-Arzneimittel	Capsule
14	Limbao 120			Kanoldt	Capsule
15	Laitan 100			Schwabe	Capsule
16	Laitan (bras.)			Schwabe	Capsule
17	Antares 120			Krewel	Coated tablet
18	Aigin-Kava Hevert Dragees			Hevert Arzneimittel	Coated pill
19	Aigin-Kava Hevert Tropfen			Hevert Arzneimittel	Tincture
20	Kava ratiopharm			Ratiopharm	Capsule
21	Kavacur 120 mg			Biocur	Coated tablet
22	Maoni forte			Lichtwer	Coated tablet
23(I)	"noble Kava" Ava La'au	Samoa	Roots	HERBResearch	Crude drug
23(II)	"noble Kava" Ava La'au	Samoa	Peeling (stem)	HERBResearch	Crude drug
24(I)	"Tudei Kava" Palisi	Vanuatu	Rhizomes & Roots	HERBResearch	Crude drug
24(II)	"Tudei Kava" Palisi	Vanuatu	Peelings (stem)	HERBResearch	Crude drug
24(III)	"Tudei Kava" Palisi	Vanuatu	Roots	HERBResearch	Crude drug (chips)
25Ac75	"noble Kava" Ava La'au	Samoa	Roots	Finzelberg	Extract 75% acetone (spissum)
25Eth	"noble Kava" Ava La'au	Samoa	Roots	Finzelberg	Extract 96% ethanol (spissum)
26Ac75	"Tudei Kava" Palisi	Vanuatu	Roots	Finzelberg	Extract 75% acetone (spissum)
26Eth	"Tudei Kava" Palisi	Vanuatu	Roots	Finzelberg	Extract 96% ethanol (spissum)
27Eth	Produktionsextrakt			Finzelberg	commercial Extract 96% ethanol
28	Peeling Suva	Suva, Fiji	Peelings	Nasigasiga Kava Dealer	Crude drug
29	Kultivar "Matakaro"	Fiji	Leaves	HERBResearch	Crude drug

extracts with low amounts of **1** have been applied (Berkulin et al. 2005; Hauer et al. 2005). However, two week application of 10 mg/kg of **1** to F344-rats did not lead to alterations of liver function tests or apoptosis (Lim et al. 2007).

The aim of the present work was to study the content of potentially toxic pipermethystine (**1**) in extracts from identified crude drug material and in products which were commercially available on the German market at the time of the observation of the liver case reports. Whereas several methods have been presented for the separation and detection of kavalactones (Bilia et al. 2004), methods to determine compound **1** are comparatively rare. We chose a GC/MS system (Duffield and Lidgard 1986; Duffield et al. 1986) originally used to measure kavalactones, but later shown to be likewise suitable for the determination of pipermethystine (Dragull et al. 2003).

2. Investigation, results and discussion

We investigated Kava preparations including a series of retain samples of finished products from the German market, self-produced extracts from root and stem material obtained from two identified Kava cultivars ("noble Kava" Ava La'au from Samoa, "Tudei Kava" Palisi from Vanuatu; extracted with ethanol 96% respectively acetone 75% or 100%), and an extract from the leaves of *Piper methysticum* (Noble Kava cultivar Matakaro from Fiji) as a positive control (Table). The Samoan Kava cultivar Ava La'au was also applied in toxicological testing *in vivo* (DiSilvestro et al. 2007); the Kavalactone composition is indicated in DiSilvestro et al. (2007).

Figure 1a shows the chromatogram (TIC mode) of a typical sample of Piperis methystici rhizoma without sample preparation (as an exception), simply dissolved in MeOH. Besides several signals of kavalactones no signal of **1** (expected at $R_t = 15.90$ min) could be detected. Figure 1b shows the same extract spiked with compound **1**. The chromatogram of pure **1** showed one single peak at $R_t = 15.90$ min (not shown). The recorded mass spectrum of **1** (Fig. 2) matched very well with data from the literature (Smith 1979) and the NIST database entry. We decided to use fragments $m/z = 227$, 131 and 104 for quantification purposes (SIM-mode). As a matter of routine, further GC's were registered after purification of the sample by using a RP-18 cartridge (see sample preparation) in order to protect the column from contamination; **1** was quantitatively eluted from the SPE-cartridge by MeOH–H_2O 50 : 50 as shown by recovery experiments (see Experimental).

Only the reference chromatogram of Kava leaves (sample no. 29) showed a clear signal of **1** at a percentage of 0.2% (Fig. 3). In all other samples no **1** above the LOQ of less than 45 ppm was detected. Only the SIM-chromatogram of samples no. 2 and no. 18 (not shown) showed a poor signal at 15.90 min corresponding to 0.02%; however,

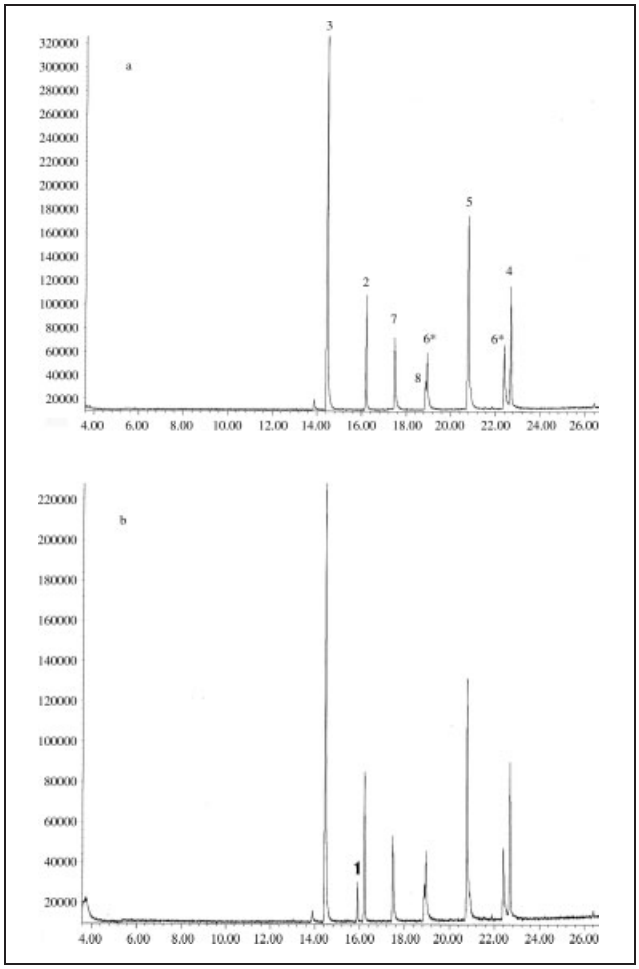

Fig. 1: Chromatogram (TIC mode) of Kava root extract (sample no. 27). **1**: pipermethystine, **2**: kavain, **3**: 7,8-dihydrokavain, **4**: methysticin, **5**: 7,8-dihydroyangonin, **6**: yangonin (* probably cis/trans isomers); **7**: 4′-desmethoxyyangonin, **8**: 5,6,7,8-tetrahydroyangonin. Chromatogram 1a original sample 27 Eth. Chromatogram 1b is spiked with **1**

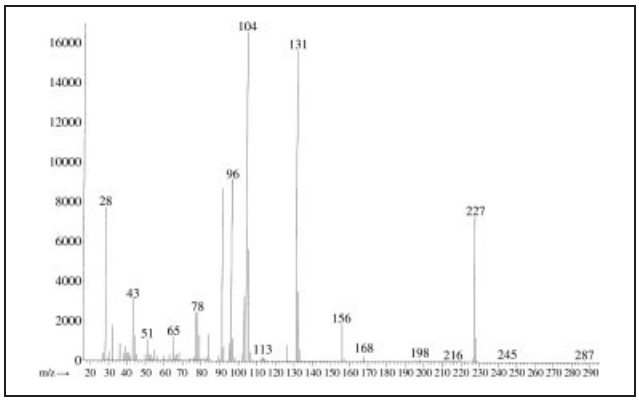

Fig. 2: Mass spectrum of pipermethystine (**1**) (EI)

confirmation of its identity was not possible due to absence of this signal in the "Scan-mode" (TIC). Fig. 4a and 4b show TIC-chromatograms of the "Noble Kava" (No. 25Eth) and "Tudei Kava" (No. 26Eth). Noble Kava from Samoa is obviously characterized by greater amounts of kavain and methysticin in comparison with Tudei Kava from Vanuatu, but compound **1** is not detectable.

The data show that pipermethystine (**1**) is absent from all samples above the limit of quantification of 0.0045% (45 ppm) whereas the leaves of Fijian noble Kava (sample

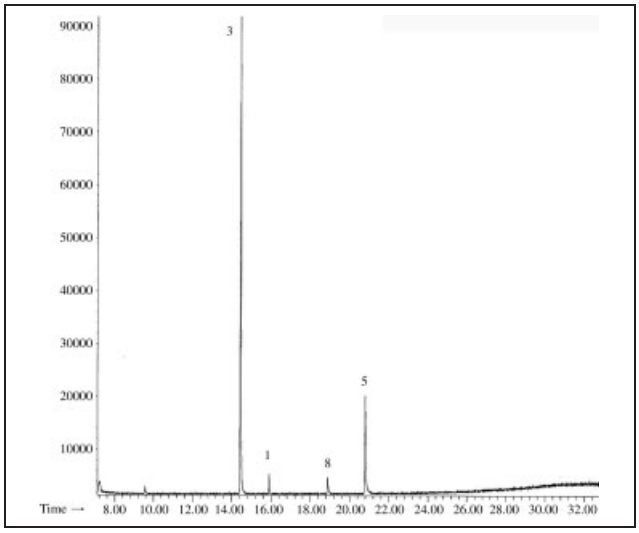

Fig. 3: Chromatogram (TIC-mode) of Kava leaves extract (sample No. 29). Numbering of peaks as in Fig. 1

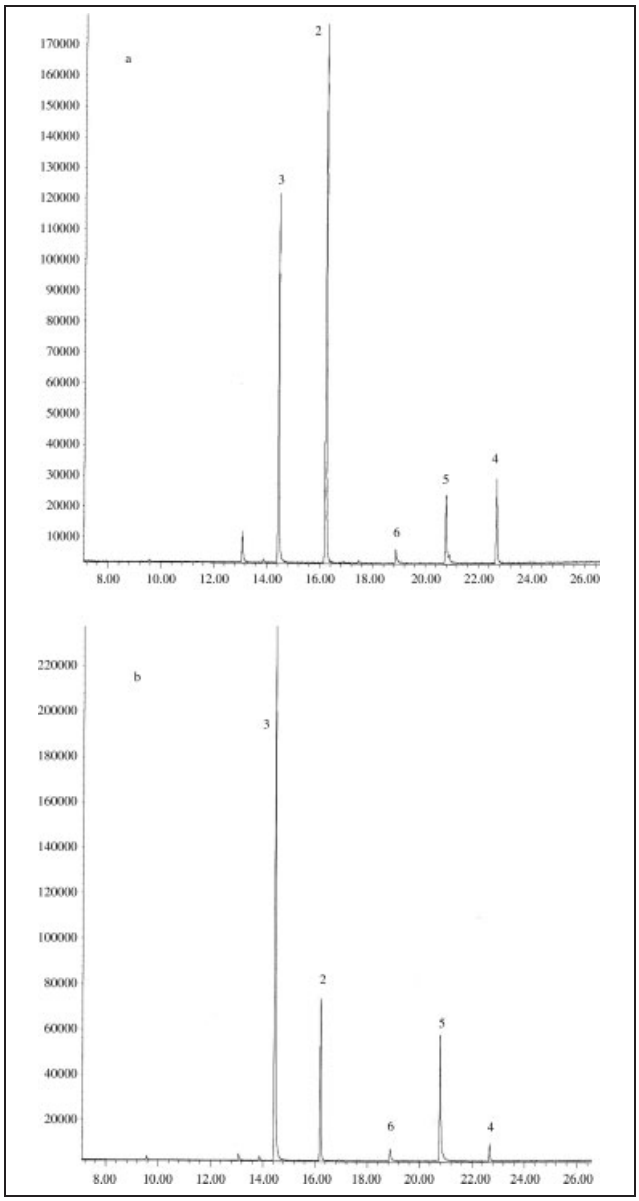

Fig. 4: a) Chromatogram of Kava root extract (sample No. 25Eth, Samoa). b) Chromatogram of Kava root extract (sample No. 26Eth, Vanuatu. Numbering of peaks as in Fig. 1

No. 29, used as a positive control) show a content of 0.2% of **1**. Thus application of a daily dose of 300 mg Kava extract corresponding to 210 mg total Kavalactones (highest daily dosage in European extract preparations) contains less than 13.5 μg of pipermethystine. Considering the average weight of 70 kg of an adult person, the approximate amount of compound **1** is less than 0.2 μg/kg (less than 0.2 ppb), a value far from the dose of 10 mg/kg which did not show toxicity on two weeks application to rats (Lim et al. 2007). So far no pharmacokinetic data of compound **1** exist. Absorption from the GI-tract and/or metabolism in enterocytes may further reduce the available quantity of **1** in the human body.

In this study, pipermethystine contents were measured in roots from Kava material favoured for daily Kava drinking (Ava La'au from Samoa), and in a "no drink" quality (Palisi from Vanuatu). Material from both cultivars was used in the preparation of European acetone extracts (Lebot 2006; Schmidt 2007). In both cultivars, no pipermethystine was evidenced in the roots.

The quality of the extracts prepared with ethanol, but not of those prepared with acetone, was shown to have been rather uniform (Schmidt 2007). GMP conditions in production of herbal medicinal products, combined with the definition of the quality of the drug material in the German Drug Codex (DAC) would ensure that no aerial parts were used for the preparation of the ethanolic extracts. With pipermethystine (**1**) not relevantly present in the tested European Kava preparations this compound appears unlikely to have contributed to hepatotoxicity in the European case reports.

3. Experimental

3.1 Sample preparation

Material (compare Table): Dried and ground plant material, extracts, total contents of capsules, aliquots of ground pills and coated tablets, aliquots of tinctures (amount: 0.1–1g). Vouchers of all samples are stored under MS-PB-223.

Extraction: Exhaustive extraction with methanol (Ultra Turrax® T8, 25000 rpm), filled up to 25 ml, an aliquot of 1 mL was evaporated to dryness and re-dissolved in 1.0 ml MeOH-Water 10 : 90.

Solid-phase-extraction: The solution was loaded on a RP18-SPE-cartridge conditioned with MeOH-Water 10 : 90. Elution protocol: (1) 10 mL MeOH-water 10 : 90, (2) 10 mL MeOH-water 50 : 50, (3) 10 mL MeOH 100%, (4) 10 mL MeOH-water 10 : 90; evaporation of eluate 2 to dryness, re-dissolved in 1.0 mL MeOH, GC/MS-analysis.

Recovery: Kava leaves (500 mg, sample 29) were treated as described above. Solutions 1–3 were analyzed by GC/MS (SIM-mode). 97.8% of **1** was found in eluate 2, whereas only 1.3% and 0.9% could be detected in eluates 1 and 3, respectively.

3.2 GC/MS (EI)

Samples were analyzed for their content of pipermethystine (**1**) by GC-MS using total ion currency (TIC) and selective ion monitoring (SIM) detection. Limit of quantification (LOQ) was below 45 ppm (SIM). Agilent Technologies: 5973 Mass Selective Detector, 6890N GC-System, 7683B Injector, Temp. 150 °C → 5 °C/min → 300 °C (30 min isotherm.), Column: HP5MS 0.25 mm × 30 m × 0.25 μm, Vol.: 1 μL; Assignment of peaks by comparison of retention order and mass-spectra with data from the literature (Duffield and Lidgard 1986; Duffield et al. 1986) and NIST database.

Calibration, Limit of quantification (LOQ): Pipermethystine (**1**, R_t = 15.90 min); 0.18–75.0 μg/mL (in MeOH), Modus: SIM: m/z: 227, 131, 104. Linear equation: $y = 1321.2x - 1307.6$, $R^2 = 0.992$; LOQ (SIM): 1.76 μg/mL; weighted sample = 1.0 g, dissolution factor = 25; from this follows: LOQ = 0.044 g/kg (below 45 ppm).

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