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Kavalactones fail to inhibit alcohol dehydrogenase *in vitro*

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Abstract

In recent years, Kava kava (*Piper methysticum*, Forst. f., Piperaceae), a folkloric beverage and popular herbal remedy, has been implicated in a number of liver failure cases. Many hypotheses as to the mechanism of its hepatotoxicity, for example interactions with other co-ingested medication, have been postulated. This present study investigated whether pharmacokinetic interactions between kava constituents and alcohol via alcohol dehydrogenase (ADH) inhibition by individual kavalactones might explain its claimed hepatotoxic effects. Four kavalactones, (±)-kavain, methysticin, yangonin and desmethoxyyangonin, fail to inhibit ADH *in vitro* at 1, 10 or 100 μM concentrations.

Keywords: Kava kava; Kavalactones; *Piper methysticum*; Alcohol dehydrogenase; Inhibition

Introduction

Kava kava, a worldwide popular herbal drug with a long-standing folkloric tradition, is used as a traditional beverage in the Pacific Islands as well as in Western countries due to its anxiolytic and sedative properties (Singh and Singh, 2002). However, in recent years kava has been implicated in several liver failure cases (BfArM, 2002; Schmidt, 2003). This has led to its ban in many countries, which has prompted widespread and robust discussion on its relative benefit and risk both as a social beverage and a herbal remedy (Anke and Ramzan, 2004). A prominent feature of all liver failure case-reports was that almost all patients used other over-the-counter, prescription or herbal medications (BfArM, 2002; Schmidt, 2003). This leads to the hypothesis that herb–drug interactions might have contributed to the observed liver toxicity. As several studies have shown that several kavalactones, the active principles of kava extracts, are potent inhibitors of various CYP enzymes (CYP1A2, CYP2C9, 2C19, 2D6, 3A4 and 4A9/11), that are responsible for the metabolism of the majority of drugs, there is an increased risk of pharmacokinetic herb–drug interactions (Mathews et al., 2002; Unger et al., 2002; Zou et al., 2002). This might then result in elevated and potentially toxic plasma concentrations of co-administered drugs or their metabolites. Influence of kava on ethanol metabolism, for example, a decreased conversion to acetaldehyde due to inhibition of the enzyme alcohol dehydrogenase (ADH), may be a possible reason for the observed liver toxicity with kava.

The purpose of this present study was thus to investigate whether ADH activity is altered by kavalactones as a means of establishing a possible mechanism of

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kavalactone-mediated hepatotoxicity especially if it is consumed with alcohol.

Material and methods

Plant materials

Powdered rhizome of *Piper methysticum* Forst. f. (Kava kava) sourced from Fiji was obtained commercially from MediHerb Pty. Ltd. (Warwick, Australia). This product was not further characterised either qualitatively or quantitatively prior to isolation of the kavalactones.

Plant extraction, isolation and purity

Kava stump root powder was extracted sequentially with dichloromethane and methanol to yield the individual kava extracts. These were then purified by normal phase short column chromatography using stepwise gradient elution with increasing polarity (hexane/ethyl acetate/methanol). Fractions collected were monitored for purity and polarity by thin layer chromatography. Individual kavalactones were crystallised from the purified fractions using hexane and dichloromethane. The structures of the crystalline kavalactones were verified and their purity assessed using ^{1}H-NMR and ^{13}C-NMR spectroscopy and GC-MS. Purity by GS-MS was >99% for all kavalactones isolated for this study.

Reagents

Para-nitrosodimethylaniline (NDMA), β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide, reduced, disodium salt hydrate (NADH) and ADH (from equine liver) were purchased from Sigma (Sydney, Australia). (±)-Kavain (purity 98%) was obtained from ChromaDex (Santa Ana, CA, USA). All other chemicals were of analytical quality.

NDMA solution (40 mM, in 0.1 M potassium-phosphate buffer, pH 8.0) was stored at −4 °C for 1 week. NAD (3.5 mM, in water), NADH (0.7 mM, in 0.01 M NaOH) and ADH (460 μg/50 ml, in 0.1 M potassium-phosphate buffer, pH 8.0) were stored protected from light at −20 °C for 1 week. The four kavalactones (±)-kavain, methysticin, yangonin and desmethoxyyangonin were dissolved in methanol at final concentrations of 5, 0.5, and 0.05 mM and stored at −4 °C for 1 week.

ADH enzyme activity experiments

Spectrophotometric measurement of ADH activity was performed according to Skurskỳ et al. (1979) with minor modifications: the control cuvette (100% enzyme activity) and test cuvettes contained 2 ml reaction mixture with the following ingredients: 25 μM NDMA, 250 μM NAD, 10 μM NADH and 12.5 mM *n*-butanol (to regenerate NADH after reduction) in 0.1 M potassium-phosphate buffer, pH 8.0). The 0% activity reference cuvette had the same composition of other reagents but also contained 10 mM pyrazole, a known ADH inhibitor (Brändén et al., 1975) as a positive control. A 50 μl kavalactone solution was added to each test cuvette to yield final kavalactone concentrations of 1, 10 and 100 μM. Pure methanol was added to the 100% and 0% control cuvettes. After initiation of the reaction by adding 500 μl ADH solution, the cuvettes were incubated for 20 min at 25 °C. The difference in absorbance at 440 nm (ΔA_{440}) between the sample with pyrazole and those without was read after the reaction had been stopped by addition of 50 μl of 0.5 M pyrazole to the samples in which the inhibitor was absent. All spectrophotometric readings were made using a Shimadzu (Shimadzu Oceania, Sydney) UV-1601 spectrophotometer at 25 °C. Absorbance of each sample was read three times over a period of 2 min. Standard deviations (SD) of these three readings were always less than 1.2%.

Statistical analysis

Data are presented as the mean ± SD of % ADH enzyme activity or of ADH enzyme inhibition for each test substance with $n = 4$ replicates. One-way ANOVA with post-hoc Tukey test was performed using GraphPad Prism, version 4.01, for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

The relationship between % ADH inhibition and pyrazole concentration was fitted to an E_{max} model (% inhibition $= E_{max} \times C/IC_{50} + C$) using the non-linear regression program WinNonlin Professional (Pharsight Corp., Palo Alto, CA, USA). Estimates of E_{max} and IC_{50} (and their SD) were generated from the fits.

Results and discussion

In a preliminary experiment, the effect of methanol itself, the solvent used for dissolving the kavalactones on the ADH activity was determined. ADH was inhibited 12.7% ± 3.2% ($p < 0.01$, unpaired *t*-test) compared to control (no methanol). This was judged to be acceptable, as a similar volume of methanol (50 μl) was also added to the control cuvettes to account for this solvent effect. Another experiment with different pyrazole concentrations was carried out to show that the ADH enzyme activity was being appropriately assessed with

the spectrophotometric technique used in this study. Fig. 1 illustrates that the enzyme assay is robust under the present conditions and different degrees of ADH inhibition by pyrazole, a known inhibitor of ADH, were observed. Pyrazole at the highest concentration used (200 μM) inhibited ADH by 92.3 ± 2.5%. The IC_{50} for pyrazole was 5.58 ± 0.26 μM, the K_i value, obtained from the IC_{50} value using the equation ($K_i = IC_{50}/(1 + D/K_D)$), was 0.16 ± 0.076 μM. This K_i for pyrazole compares favourably with the literature value of 0.22 μM (Brändén et al., 1975).

The effects of three different concentrations of the kavalactones, (±)-kavain, methysticin, yangonin and desmethoxyyangonin on ADH activity were then tested at concentrations of 1, 10 and 100 μM. The results show that for methysticin and yangonin ADH activities were not significantly altered from 100% at even the highest concentration. It is noted that the SD for the yangonin experiments were much higher than with the other test compounds. As yangonin is the only coloured/fluorescent substance among the kavalactones, this might have affected the variability in the enzyme assay with yangonin. Nevertheless, the SD observed in the enzyme assays are still relatively low at less than 6%. Tukey test analysis with (±)-kavain and desmethoxyyangonin showed no differences between control and test samples. Minor differences (3% for 1 and 100 μM kavain; 15% for 1 and 100 μM desmethoxyyangonin) were observed when comparing the different concentrations of (±)-kavain and desmethoxyyangonin, respectively. However, ADH activity was never affected by more than 10%, including either lower or higher than 100% enzyme activity (Table 1).

In the present study, the inhibition of ADH by different kavalactones was examined *in vitro* using a simple but widely used robust spectrophotometric technique. None of the four pure kavalactones affected ADH activity by more than 10%. The recommended daily dose for kava is equivalent to 120 mg kavalactones (Kommission, 1990); dosages used in several clinical trials have been equivalent to 240 mg or more kavalactones daily (Schmidt, 2003). Assuming complete oral absorption and a theoretical blood volume of 6 L, an average blood/plasma concentration of 20–40 μg/ml (80–160 μM) kavalactones may be predicted. In humans, a single oral dose of 200 mg (±)-kavain resulted in a maximum plasma concentration of 18 μg/ml (Singh, 2004), thus bioavailability appears to be about 50%. However, other studies have shown that the administration of the complete extract compared to the

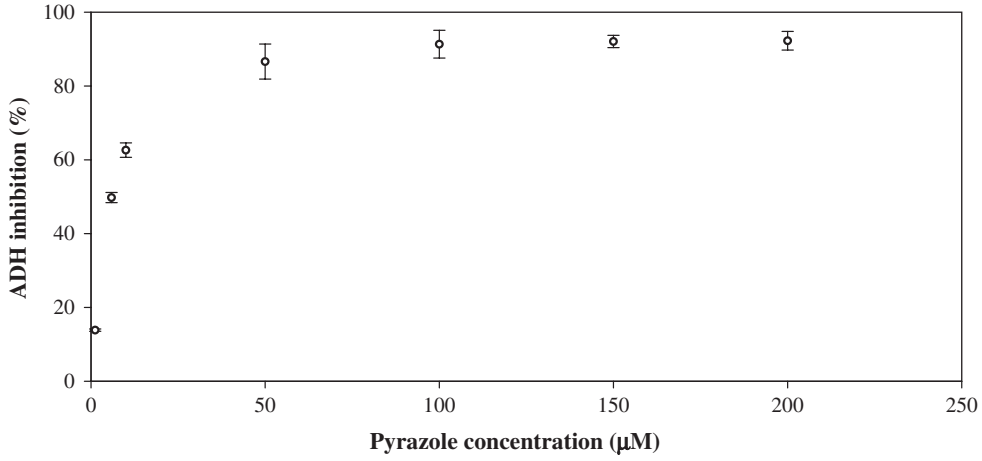

Fig. 1. ADH inhibition by pyrazole, a known ADH inhibitor ($n = 4$ for each concentration).

Table 1. ADH enzyme activity as a function of kavalactone concentration ($n = 4$ each concentration)

Kavalactone	Kavalactone concentration (μM)				ANOVA
	0	1	10	100	*p*
(±)-Kavain	100 ± 1.2%	102.9 ± 0.47%	100.3 ± 0.96%	99.8 ± 0.18%[a]	0.032
Methysticin	100 ± 0.42%	101.0 ± 0.36%	98.5 ± 2.4%	100.5 ± 0.31%	0.61
Yangonin	100 ± 3.3%	97.3 ± 3.2%	94.0 ± 5.6%	96.1 ± 1.2%	0.62
Desmethoxyyangonin	100 ± 0.59%	106.6 ± 1.3%	105.6 ± 0.95%	91.6 ± 2.7%[b]	0.006

[a] $p < 0.05$ compared to (±)-kavain 1 μM (Tukey test).
[b] $p < 0.01$ compared to desmethoxyyangonin 1 μM; $p < 0.05$ compared to desmethoxyyangonin 10 μM (Tukey test).

administration of pure single kavalactones increases the bioavailability by two-fold (for kavain) to more than 20-fold (Keledjian et al., 1988). To account for such variations in systemic kavalactone concentrations, three different kavalactone concentrations were used in this study, reflecting the effect of low (1 μM), therapeutic (10 μM) and higher than therapeutic (100 μM) plasma concentrations. Even at these high concentrations, the kavalactones did not alter ADH activity.

From the observed results it can be postulated that the four kavalactones studied, (±)-kavain, methysticin, yangonin and desmethoxyyangonin fail to inhibit ADH. This implies that at least via this mechanism these kavalactones are unlikely to interact with alcohol. It is expected that similar negative effects on ADH activity would be noted with kava extracts since aqueous, acetonic, ethanolic or organic solvent (toluene) extracts are qualitatively similar (Loew and Franz, 2003) and presumably contain the four kavalactones tested in this study. Thus alcohol metabolism is unlikely to be decreased by kava kava and/or its active constituents, the kavalactones. Potentially toxic plasma alcohol concentrations are therefore unlikely to occur when kava and alcohol are co-ingested. However, this does not completely rule out any risk of pharmacokinetic or pharmacodynamic interactions between kava and alcohol as a decreased conversion of kavalactones to their metabolites may occur *in vivo* due to alcohol intake. In addition, *in vivo* kavalactone metabolites or other minor kava constituents might cause pharmacokinetic interactions (ADH inhibition) between alcohol and kava.

Of course these results also do not rule out any pharmacodynamic interactions between kava and alcohol. Jamieson and Duffield (1990), for example, found increased sleeping times and increased mortality in mice when kava was administered in combination with alcohol compared to kava and alcohol alone. In a clinical study, Foo and Lemon (1997) also observed that kava appeared to potentiate impairment of cognition when combined with alcohol. However, in contrast to this, Herberg (1993) could not detect increased impairment in human test subjects when kava was combined with alcohol. These effects are separate to any anticipated hepatic effects of kava either alone or in combination with alcohol.

In summary it is postulated, based on this *in vitro* study, that the four kavalactones studied do not inhibit ADH and therefore are unlikely to affect alcohol metabolism when kava is taken with alcohol. However, this does not imply that there is no risk for pharmacokinetic interactions between alcohol and kava via other mechanisms. Also, alcohol and kava may interact at a pharmacodynamic level to produce additive or synergistic CNS effects. This however cannot be the explanation for the reported hepatotoxicity with kava.

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