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# Identification of methysticin as a potent and non-toxic NF-κB inhibitor from kava, potentially responsible for kava's chemopreventive activity

Ahmad Ali Shaik, David Lee Hermanson, Chengguo Xing *

*Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, 308 Harvard St SE, Minneapolis, MN 55455, United States*

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ABSTRACT

Nuclear factor-κB (NF-κB) is a transcription factor that plays an essential role in cancer development. The results of our recent chemopreventive study demonstrate that kava, a beverage in the South Pacific Islands, suppresses NF-κB activation in lung adenoma tissues, potentially a mechanism responsible for kava's chemopreventive activity. Methysticin is identified as a potent NF-κB inhibitor in kava with minimum toxicity. Other kava constituents, including four kavalactones of similar structures to methysticin, demonstrate minimum activities in inhibiting NF-κB.



Nuclear factor-κB (NF-κB) proteins are a family of dimeric transcription factors.[1] Under resting conditions, NF-κB dimers reside in the cytoplasm. Upon activation, such as stimulation from free radicals, inflammation, radiation, or chemical carcinogens, a 65-kDa unit (p65) of the NF-κB dimmers is phosphorylated and translocates to the nucleus. The nuclear p65 activates the transcription of more than 200 genes, which are involved in a variety of cellular processes, such as B cell and T cell development, inflammation, proliferation, and apoptosis.[2,3] The dysregulation of NF-κB, therefore, is associated with many diseases, including cancer, AIDS, asthma, arthritis, diabetes, inflammatory bowel diseases, muscular dystrophy, Alzheimer's disease, and stroke.[4] Appropriate control of the NF-κB signaling pathway, which can be achieved by small-molecule modulators, would provide potential approach for the management of NF-κB related diseases. Thus intensive efforts have been invested to search for NF-κB inhibitors, leading to ~1000 such candidates.[5,6]

Our interest in developing NF-κB inhibitor stems from our recent results of demonstrating kava as a chemopreventive agent against lung cancer development.[7] Kava is the extract of the roots of *Piper methysticum*, a historically long-standing crop in South Pacific islands. Traditionally kava is prepared as a water extract of *P. methysticum* roots and has been consumed safely as a beverage in the South Pacific islands for centuries.[8] Epidemiological data reveal a nice negative correlation between the amount of kava consumed and cancer incidence among the nations in South Pacific, suggesting that kava is potentially chemopreventive.[9] Other than water extraction of *P. methysticum* roots for traditional kava preparation, there is a commercial kava preparation, which is an extract of *P. methysticum* roots using organic solvents (mostly ethanol or acetone) with about 20 compounds isolated and structures determined.[10–16] Commercial kava had been used clinically for anxiety treatment.[17] Due to some controversial idiosyncratic hepatotoxic effects derived from commercial kava,[18–21] it is currently banned in Europe, Australia, and Canada while Food and Drug Administration (FDA) issued a warning in 2002 of commercial kava usage.

We recently demonstrated that commercial kava effectively suppresses 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[*a*]pyrene (B[*a*]P) induced lung adenoma formation in A/J mice.[7] During the eight-month commercial kava treatment, there were no adverse side effects detected in the A/J mice, including food consumption, bodyweight, liver functions, and liver pathology. The absence of signs of toxicity indicates that commercial kava may be safe for long-term usage. It is also possible that the limited number of animals used in this study is unable to detect hepatotoxicity due to its extremely low rate (0.008–0.016 reported hepatotoxic cases/$10^6$ daily commercial kava usage).[22] Mechanistically, NNK- and B[*a*]P-induced lung adenomas have elevated activation of NF-κB,[7] consistent with other reports about the role of NF-κB in tumorigenesis.[23,24] Kava treatment significantly suppresses NF-κB activation in lung adenoma tissues, a potential mechanism responsible for kava's chemopreventive efficacy.

This study is the first step of our effort to determine the chemopreventive and potentially hepatotoxic origin of commercial kava, which may lead to a more potent chemopreventive candidate than commercial kava with minimized adverse side effects. Since traditional kava and commercial kava are prepared differently and the

* Corresponding author. Tel.: +1 612 626 5675; fax: +1 612 624 0139.
*E-mail address:* xingx009@umn.edu (C. Xing).

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questionable hepatotoxicity was exclusively detected among commercial kava users,[22,25,26] the two preparations may have different compositions. We therefore prepared traditional kava water extract and compared its composition with that of commercial kava (Gaia Herbs, NC, the same as we have used in our previous in vivo chemopreventive study[7]) by HPLC (HPLC conditions in Supplementary data). As shown in Figure 1B, commercial kava contains non-polar constituents not detectable in traditional kava (Fig. 1A). In order to identify the potential chemopreventive constituent(s), we searched for NF-κB inhibitory chemicals in com-

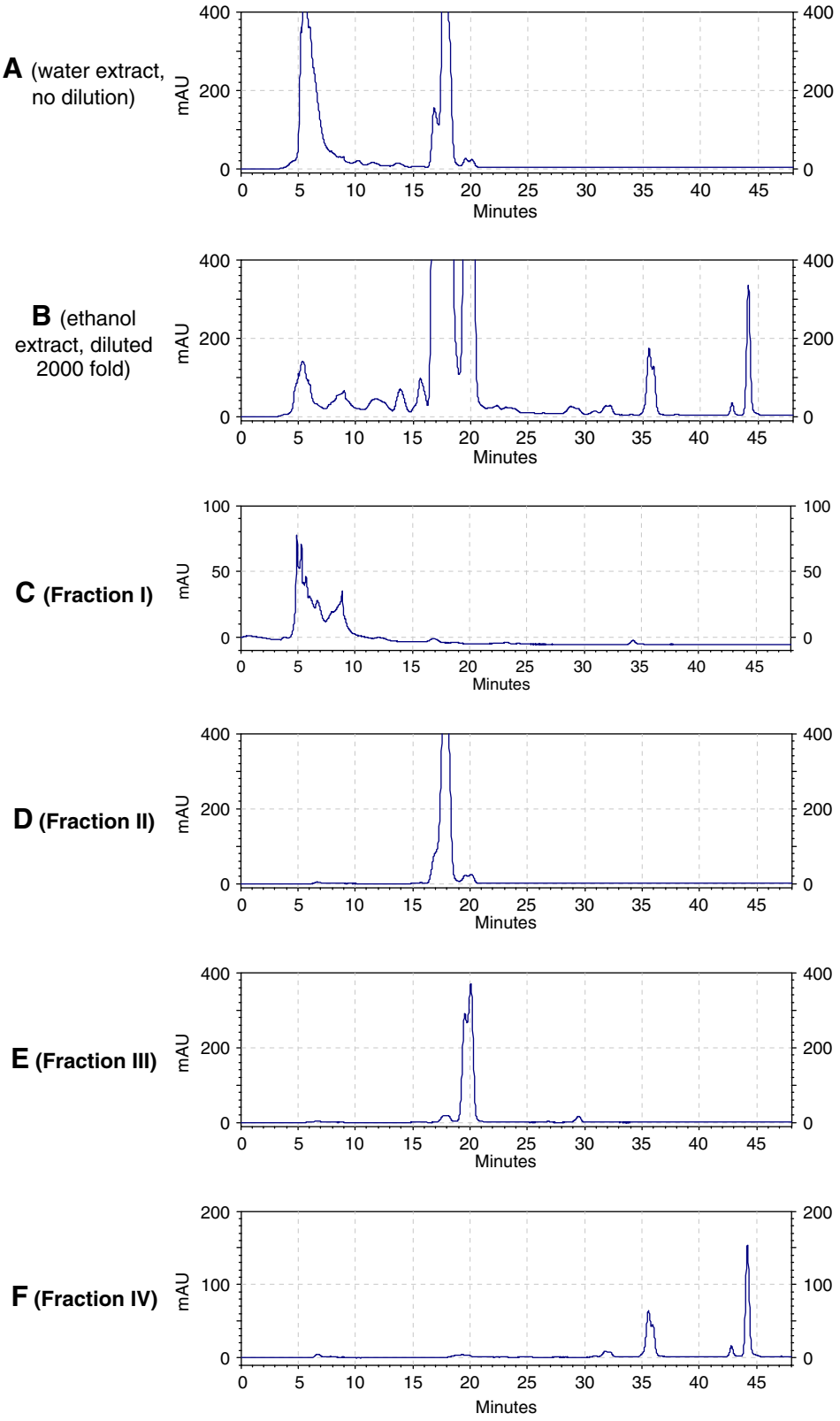
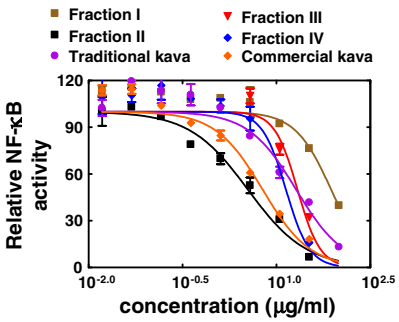



**Figure 1.** HPLC analyses of the compositions of traditional kava, commercial kava, and four fractions from commercial kava.




| | $IC_{50}$ (μg/ml) |
|---|---|
| Traditional kava | 18.3 ± 2.4 |
| Commercial kava | 5.3 ± 0.6 |
| Fraction I | 73 ± 2 |
| Fraction II | 3.6 ± 0.1 |
| Fraction III | 21.0 ± 0.7 |
| Fraction IV | 14.6 ± 0.4 |

**Figure 2.** NF-κB inhibitory activities of traditional kava, commercial kava, and its four fractions. The values ($IC_{50}$) represent the mean ± SE for $n = 2$.

mercial kava through fractionation and monitored the active constituent(s) using a cell-based TNF-α induced NF-κB activation assay as a functional evaluation.

Commercial kava was first fractionated into four fractions by using silica gel chromatography (procedures in Supplementary data). These four fractions were analyzed for their compositions by HPLC following the same HPLC conditions. Fractions I and II mainly contain constituents present in both traditional and commercial kava while Fractions III and IV only contain those constituents not detectable in traditional kava (Fig. 1), including flavokawains A, B, and C in Fraction IV, which have been postulated to be the chemopreventive constituents in kava,[27,28] while our in vivo chemopreventive evaluation indicates that these flavokawains cannot account for the significant chemopreventive activity of kava (manuscript in preparation for Cancer Prevention Research).

Using an in vitro luciferase-based assay with a human lung adenocarcinoma A549 cell line stably transfected with NF-κB-luc,[29] Fraction II (Fig. 2) is demonstrated to have potent NF-κB inhibitory activity ($IC_{50} = 3.6 \pm 0.1$ μg/ml) while Fraction I has minimum inhibitory activity ($IC_{50} = 73 \pm 2$ μg/ml). Fractions III and IV also have much weaker inhibitory activity compared to Fraction II. Fraction II was further purified leading to five kavalactones—kavain, dihydrokavain, dihydromethysticin, methysticin, and desmethoxyyangonin, the identities of which are confirmed by $^{1}H$ NMRs, $^{13}C$ NMRs, optical rotations and mass spectrometry analyses

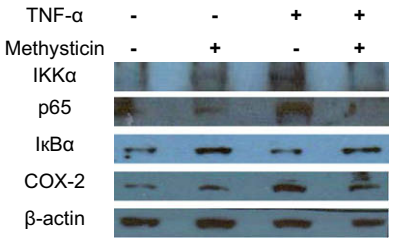



**Figure 5.** Western Blot analyses of methysticin suppressing TNF-α induced NF-κB activation.

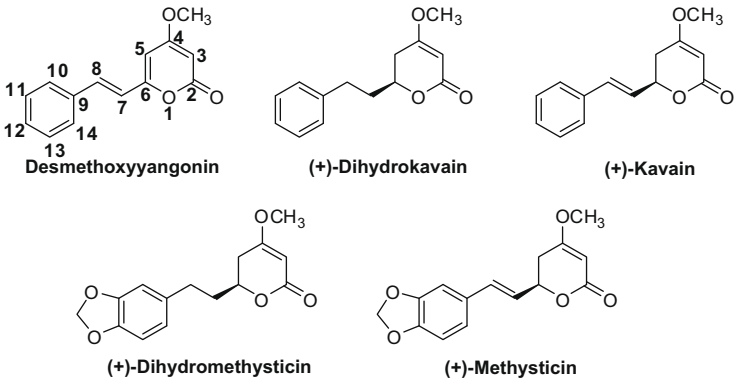
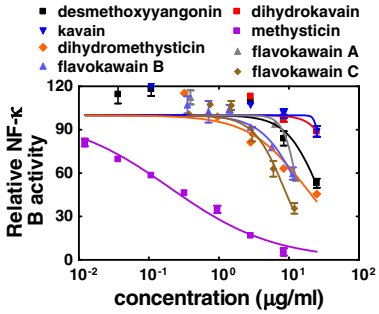
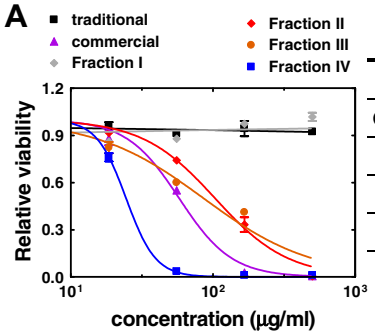



**Figure 3.** Structures and numbering of five kavalactones isolated from Fraction II in kava.

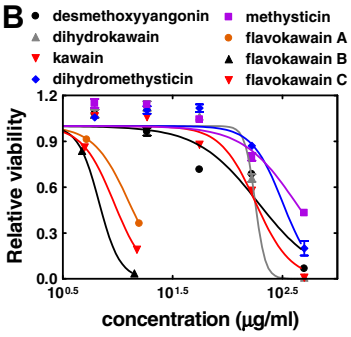



| | $IC_{50}$ (μg/ml) |
|---|---|
| desmethoxyyangonin | 33 ± 7 |
| dihydrokavain | 60 ± 8 |
| kavain | 32 ± 3 |
| dihydromethysticin | 20 ± 3 |
| methysticin | 0.19 ± 0.01 |
| flavokawain A | 11.6 ± 0.6 |
| flavokawain B | 8.0 ± 1.9 |
| flavokawain C | 8.1 ± 1.2 |

**Figure 4.** NF-κB inhibitory activities of kavalactones and flavokawains. The values ($IC_{50}$) represent the mean ± SE for $n = 3$.




| | $IC_{50}$ (μg/ml) |
|---|---|
| Traditional kava | >500 |
| Commercial kava | 61 ± 3 |
| Fraction I | >500 |
| Fraction II | 122 ± 18 |
| Fraction III | 95 ± 9 |
| Fraction IV | 24 ± 0.2 |




| | $IC_{50}$ (μg/ml) |
|---|---|
| desmethoxyyangonin | 173 ± 16 |
| dihydrokavain | 175 ± 3 |
| kavain | 166 ± 14 |
| dihydromethysticin | 313 ± 4 |
| methysticin | 392 ± 24 |
| flavokawain A | 13.8 ± 1.1 |
| flavokawain B | 6.6 ± 0.1 |
| flavokawain C | 9.8 ± 0.6 |

**Figure 6.** Toxicity of various kava, its fractions (A), and pure constituents (B) against Hepa 1c1c7 liver cells. The values ($IC_{50}$) represent the mean ± SE for $n = 3$.

as reported before (Fig. 3).[16,30–32] Very surprisingly, methysticin is the only kavalactone in Fraction II that have potent NF-κB inhibitory activity ($IC_{50}$ = 0.19 ± 0.01 μg/ml), which is ~18 times more potent than Fraction II and ~100 times more potent than traditional kava (Fig. 4). Commercial methysticin (LKT Laboratories, MN) demonstrates the same NF-κB inhibitory activity (data not shown). Kavain ($IC_{50}$ = 32 ± 3 μg/ml), dihydrokavain ($IC_{50}$ = 60 ± 8 μg/ml), dihydromethysticin ($IC_{50}$ = 20 ± 3 μg/ml), and desmethoxyyangonin ($IC_{50}$ = 33 ± 7 μg/ml) are about 100–300 times less active than methysticin. Based on the in vitro NF-κB inhibitory activities among the five kavalactones, it is clear that the 7,8-alkene functional group is essential since methysticin is 100 times more potent that dihydromethysticin and kavain is about two times more potent than dihydrokavain. The 11,12-dioxymethylene functional group is also essential since kavain is ~170 times less active than methysticin. The NF-κB inhibitory activity of methysticin was further evaluated by Western Blot analyses of several proteins involved in NF-κB signaling pathway, including IKKα, IκBα, p65, and COX-2, which have been widely used as indication of NF-κB activation/inhibition.[33–36] As shown in Figure 5, TNF-α treatment leads to the elevated levels of IKKα, p65, and COX-2 and the decrease of IκBα, demonstrating the activation of NF-κB. Methysticin treatment suppressed the elevation of IKKα, p65, and COX-2 and prevented the degradation of IκBα. These Western immunoblotting results, in combination with the luciferase-based assay, establish that methysticin inhibits NF-κB activation. Further structure–activity relationship studies are undergoing. For comparison, flavokawains A, B, and C demonstrate minimum NF-κB inhibitory activities relative to methysticin (Fig. 4).

Next we evaluated the relative toxicity of these chemicals against a liver cell line, Hepa 1c1c7, as a preliminary study to explore the potential toxicity of these compounds to liver.[37] Traditional kava and Fraction I of commercial kava demonstrated no toxicity ($IC_{50}$ >500 μg/ml, Fig. 6A). Fractions II also demonstrated minimum toxicity to liver cells ($IC_{50}$ = 122 ± 18 μg/ml). Fraction III demonstrates a bit stronger toxicity ($IC_{50}$ = 95 ± 9 μg/ml). Commercial kava ($IC_{50}$ = 61 ± 3 μg/ml) and Fraction IV ($IC_{50}$ = 24 ± 0.2 μg/ml) demonstrate significantly higher toxicity (Fig. 6A). These are consistent with the results from Jhoo et al. and Li et al.[38,39] Since Fraction IV is only detectable in commercial kava and both of them demonstrate high toxicity toward liver cells, the hepatotoxicity associated exclusively with commercial kava users may derive from the chemicals in Fraction IV in commercial kava. Among the five kavalactones in Fraction II, methysticin demonstrate no toxicity towards liver cells ($IC_{50}$ ~400 μg/ml) while the other four kavalactones demonstrate weak toxicity (Fig. 6B). Methysticin also demonstrated minimum toxicity against a panel of human cell lines, including A549, HL-60, and CCRF (Supplementary data). Flavokawains A, B, and C, three components detected in Fraction IV, demonstrated much higher toxicity against liver cells ($IC_{50}$ <15 μg/ml), consistent with the high toxicity of Fraction IV against liver cells.

In summary, methysticin has been identified from kava to have potent NF-κB inhibitory activity and minimum toxicity. The NF-κB inhibitory activities of kavalactones are highly structure-dependent in that the 7,8-alkene and 11,12-dioxymethylene functional groups are indispensible. Methysticin also demonstrated no toxicity while the non-polar constituents in commercial kava, which contains flavokawains A, B, and C, are much more toxic. In combination with the results of our previous chemoprevention studies of kava against lung tumorigenesis, methysticin may be responsible for kava's chemopreventive efficacy and potentially be void of adverse liver side effects. Methysticin, therefore, represents a new promising candidate for lung cancer chemoprevention, which is currently under evaluation.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.003.

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