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Inhibition of TNFα-induced activation of nuclear factor κB by kava (*Piper methysticum*) derivatives

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Abbreviations:
BSA, bovine serum albumin
DW, dry weight
DYRK1A, dual-specificity tyrosine-phosporylated and regulated kinase 1A
FCS
foetal calf serum
HPLC, high performance liquid chromatography
IκB, inhibitory protein of nuclear factor κB
IKK, IκB kinase
EMSA, electrophoretic mobility shift assay

ABSTRACT

The inducible transcription factor nuclear factor κB (NF-κB) plays a central role in the regulation of immune, inflammatory and carcinogenic responses. While normal activation of NF-κB is required for cell survival and immunity, its deregulated expression is a characteristic of inflammatory and infectious diseases. In this study, we investigated the molecular mechanisms induced by lactones and chalcones isolated from Fijian kava (*Piper methysticum*) used in traditional medicine against urinary tract infections and asthma. In order to understand underlying regulatory mechanisms, inhibition of both NF-κB-driven reporter gene expression and TNFα-induced binding of NF-κB to a consensus response element was achieved at concentrations of 320 μM (flavokavain A), 175 μM (flavokavain B) and 870 μM (kavain and dihydrokavain). Moreover, kavain and flavokavains A and B treatment led to inhibition of both inhibitor of κB (IκB) degradation and subsequent translocation of p50 and p65 NF-κB subunits from the cytoplasm to the nucleus as shown by Western blot analysis. Additionally, kinase selectivity screening demonstrates that flavokavain A, but not kavain, nor flavokavain B, inhibits the IκB kinase (IKK) as well as PRAK (p38-regulated/activated kinase), MAPKAP-K3 (MAPK-activated protein kinase 3), DYRK1A (dual-specificity tyrosine-phosporylated and regulated kinase 1A) and Aurora B. Altogether, these results give a first insight into anti-inflammatory mechanisms triggered by traditionally used chemopreventive kava compounds.

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Luc, luciferase
MAPK, mitogen-activated protein kinase
MAPKAP-K3, MAPK-activated protein kinase 3
NF-κB, nuclear factor κB
NMR, nuclear magnetic resonance
n.s., non-specific binding
PBS, phosphate buffered saline
PRAK, p38-regulated/activated kinase
S.D., standard deviation
TNFα, tumour necrosis factor α

1. Introduction

Nuclear factor κB (NF-κB) is an inducible transcription factor found in virtually all cell types [1,2]. It translocates into the nucleus upon activation, where it regulates the expression of over 200 genes that control the immune system, growth and inflammation [3]. Aberrant regulation of NF-κB leads to the development of many pathological states, especially those involved in carcinogenesis and in inflammation [3,4].

In non-stimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form, bound to the NF-κB inhibitor IκB [5]. Several factors are known to activate NF-κB, including the exposure to cytokines, to proliferation agents, to various carcinogens or to physical stress factors [6]. All NF-κB activation signals converge on the IκB kinase complex IKK, which phosphorylates IκB [1]. Upon phosphorylation, IκB is rapidly degraded and NF-κB is freed to translocate to the nucleus, where it binds to its target DNA sequences to initiate gene transcription [4,7,8].

Because of its implications in numerous diseases, NF-κB has become a major target in drug discovery [9,10]. A large number of natural and synthetic compounds are currently being investigated for NF-κB inhibitory activity. The majority of the compounds known to date as potent NF-κB inhibitors are plant-derived isoprenoids and polyphenolics. Polyphenolics often possess anti-oxidant activity which could account for their NF-κB-inhibitory activity by reducing reactive oxygen species that would otherwise induce NF-κB activation [11].

Kava is extracted from the rhizome of the pepper plant, *Piper methysticum*, which is found in Polynesia, Melanesia, and Micronesia. Kava has been reported as a potent ethnopharmacological remedy for over 3000 years [12–17]. Aqueous extract of roots has been considered as a diuretic and has been used for kidney and bladder ailments. Kava has also been historically used as treatment for gonorrhoea, rheumatism, bronchitis, asthma, as well as stomach aches and headaches. Kava was adopted as a medicinal plant by Europeans soon after its discovery in the Pacific Islands. It was used in Germany for treatments of urinary ailments and gonorrhoea as early as 1850 and the first pharmacological preparations were found in Germany in the 1920s offered as tincture for use as a mild sedative and hypersensitive herb.

The major constituents of kava are styryl α-pyrones generally referred to as kavalactones (Fig. 1). The six major kavalactones are the enolides kavain (**1**), methysticin (**2**), and their dihydro derivatives reduced in the cinnamyl side-chain, dihydrokavain (**3**) and dihydromethysticin (**4**) and the dieno-

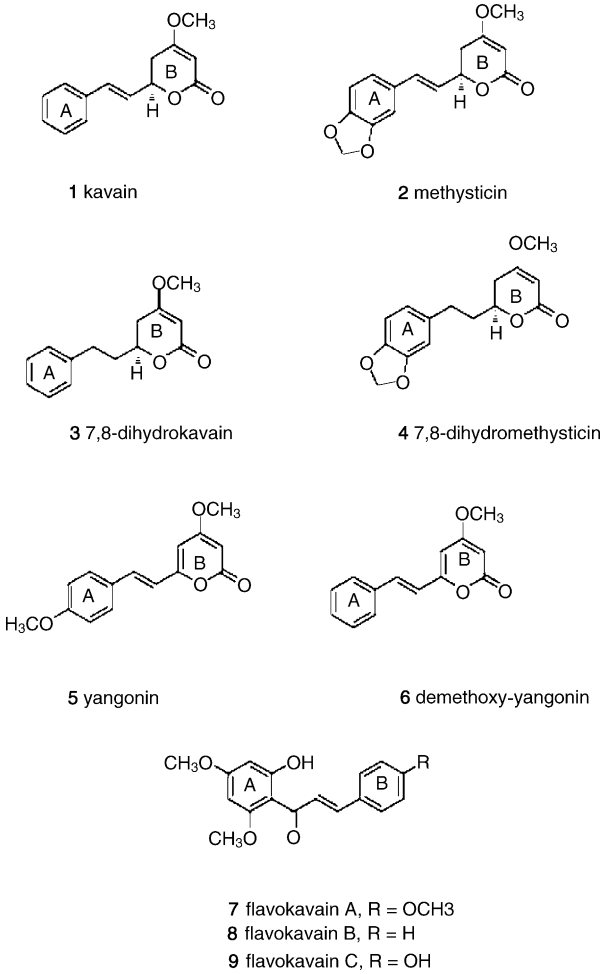


Fig. 1 – The major compounds of kava (*P. methysticum*).

lides yangonin (**5**) and demethoxy-yangonin (**6**) [14–16]. Kava also contains, in smaller amounts, the chalcones flavokavain A (**7**), flavokavain B (**8**), and flavokavain C (**9**), as well as traces of the piperidine alkaloids pipermethysticine and awaine [18,19]. The composition of natural products in kava varies throughout the plant and depends on the age of the plant, as well as on the geographical location of cultivation. The kavalactone content decreases progressively from roots to leaves. Kavain and methysticin are the major components of the roots and rhizomes, whereas their dihydro derivatives constitute the major components of the leaves. The alkaloids, which were recognized as the compounds responsible for rare but severe liver damage observed with chronic consumption of some commercial kava herbal supplements [18] are mainly present in the leaves and stem peelings of kava [14].

In the absence of a comprehensive report about anti-inflammatory effects of kava derivatives leading to inhibition of NF-κB activation, we used gel shift as well as kinase inhibition assays to study the mechanisms of kava-chalcones and -lactones isolated from *P. methysticum* on TNFα-induced NF-κB activation in human leukaemia cells.

2. Materials and methods

2.1. Compounds and purification

TNFα was purchased from Sigma and dissolved at 10 μg/mL in 1× PBS supplemented with 0.5% (w/v) BSA according to the manufacturer's instructions.

Kava (*P. methysticum* G. Forster, Piperaceae) roots were collected, rinsed with water and air-dried in Fiji. The dried roots (750 g dry weight) were then pounded to powdered form and shipped to the University of Aberdeen where they were extracted and partitioned as described previously [20]. Fig. 1 represents the chemical structure of various kava compounds used for this study.

The lipophilic fraction was further purified by normal phase HPLC on a silica column, using a 9:1 (v/v) hexane/ethyl acetate solvent system to yield 92.5 mg of kavain (**1**), 32.4 mg of flavokavain A (**7**), and 25.8 mg of flavokavain B (**8**). The hydrophilic fraction was further purified by liquid–liquid fractionation using a 1:1 (v/v) methanol/water solvent system, followed by reverse phase HPLC on a C18 column, using a 70:30:0.1 (v/v) methanol/water/acetic acid solvent system. The aqueous fraction yielded a mixture of kavain (**1**) and dihydromethysticin (**4**), which were separated by normal phase semi-preparative HPLC on a silica column, using a 3:1 (v/v) hexane/ethyl acetate solvent system, to yield 18.7 mg of dihydromethysticin (**4**) and trace amounts of kavain (**1**). The organic fraction yielded 24.9 mg of yangonin (**5**), as well as a mixture of kavain (**1**), methysticin (**2**), dihydrokavain (**3**), dihydromethysticin (**4**), and demethoxy-yangonin (**6**), which were separated by normal phase HPLC on a silica column, using a 3:1 (v/v) hexane/ethyl acetate solvent system to yield 142.5 mg of methysticin (**2**), 26.4 mg of dihydrokavain (**3**), and trace amounts of kavain (**1**) and of demethoxy-yangonin (**6**). The chemical structure of the compounds was elucidated by comparison of the obtained NMR data (^{1}H, ^{13}C, COSY, HMBC, HSQC; 400 MHz; $CDCl_3$) to previously reported information [21,22]. The compounds were stored as dry powder or oil at 4 °C and prepared in DMSO at a concentration of 20 mg/mL for the biological assays. Control cells were treated with equivalent amounts of DMSO.

2.2. Cell culture

K562 (human chronic myelogenous leukaemia) and Jurkat (T cell leukaemia) cells (DSMZ) were cultured in RPMI medium (Bio-Whittaker) supplemented with 10% (v/v) foetal calf serum (FCS) (Perbio) and 1% (v/v) antibiotic-antimycotic (Bio-Whittaker) at 37 °C and 5% of CO_2.

2.3. Cytotoxicity assay

Percentages of cell survival were determined using a CellTiterGlo Assay kit (Promega).

2.4. Transient transfection and luciferase reporter gene assay

Transient transfections of K562 cells were performed as described previously [23]. A 5 μg of luciferase reporter gene construct containing five repeats of a consensus NF-κB site (Stratagene) and 5 μg *Renilla* luciferase plasmid (Promega) were used for each pulse. Following electroporation, the cells were resuspended in growth medium (RPMI/FCS 10%) and incubated at 37 °C and 5% of CO_2. 20 h after transfection, the cells were harvested and resuspended in growth medium (RPMI/FCS 10%) to a final concentration of 10^6 cells/mL and treated for 2 h with or without kava derivatives. The cells were then challenged with 20 ng/mL TNFα for 2 h. 75 μl Dual–Glo™ Luciferase Reagent (Promega) were added to the cells for a 10 min incubation at 22 °C before luciferase activity was measured. Then, 75 μL Dual–Glo™ Stop & Glo® Reagent (Promega) were added for 10 min at 22 °C in order to assay *Renilla* activity. Luciferase and *Renilla* activities were measured using an Orion microplate luminometer (Berthold) by integrating light emission for 10 s. The results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to *Renilla* luciferase activity.

2.5. Electrophoretic mobility shift assay (EMSA)

K562 or Jurkat cells were resuspended in growth medium (RPMI/FCS 10%) to a final concentration of 10^6 cells/mL and treated for 2 h with or without kava derivatives. The cells were then challenged with 20 ng/mL TNFα for 2 h. Nuclear extracts were prepared as described [23] and stored at −80 °C. The oligonucleotide NF-κBc (consensus NF-κB site) (Eurogentec) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and its complementary sequence were used as probe. The probe was hybridised and labelled with [γ-^{32}P]ATP (MP-Biomedicals) and the EMSA was performed as published before [23]. Briefly, 10 μg of nuclear extract were incubated in binding buffer with the [γ-^{32}P] ATP labelled probe for 20 min. The DNA–protein complexes were analyzed by electrophoresis on a 5% native polyacrylamide gel

Table 1 – Inhibition of protein kinase activities by various kava compounds

		Kavain (1) (10 μM)	±S.D.	Kavain (1) (50 μM)	±S.D.	Flavokavain A (7) (10 μM)	±S.D.	Flavokavain A (7) (50 μM)	±S.D.	Flavokavain B (8) (10 μM)	±S.D.	Flavokavain B (8) (50 μM)	±S.D.
AMPK	AMP-activated protein kinase	106.23	2.00	103.91	3.23	86.48	0.98	80.85	3.83	89.95	7.50	82.39	6.17
Aurora B	Aurora B	80.32	5.91	54.18	0.67	40.59	3.19	16.54	3.19	40.42	3.39	15.50	2.30
CAMK-1	Calmodulin-dependent protein kinase	102.09	6.59	97.49	6.37	119.96	2.25	127.51	1.34	110.87	6.24	108.18	2.87
CDK2/ cyclin A	Cyclin-dependent protein kinase 2	102.38	1.00	104.46	7.36	106.28	1.24	101.54	3.04	103.11	2.00	106.45	1.00
CHK1	Checkpoint kinase 1	93.12	1.20	98.92	1.00	119.16	4.11	169.69	0.46	106.20	1.51	141.04	2.00
CHK2	Checkpoint kinase 2	86.68	1.60	81.53	4.69	89.29	3.62	72.04	0.59	83.67	1.81	58.04	1.07
CK1	Casein kinase 1	113.50	4.91	106.56	7.46	109.79	1.00	102.49	7.44	111.67	1.93	100.20	3.51
CK2	Casein kinase 2	97.83	1.52	101.16	5.24	98.33	4.26	78.63	5.20	87.68	5.13	73.55	3.55
CSK	C-terminal Src kinase	105.61	3.02	98.38	2.00	99.39	3.13	108.25	8.73	96.83	0.12	94.87	8.12
DYRK1A	Dual-specificity, tyrosine-phosphorylated and regulated kinase 1A	87.89	1.12.	65.60	0.25	39.18	1.00	10.33	1.45	57.15	1.35	13.77	3.03
EF2K	Elongation factor 2 kinase	80.45	5.81	88.71	1.13	101.33	2.00	123.61	8.58	94.63	1.83	120.55	9.21
ERK8	Extracellular-signal-regulated kinase	96.21	5.43	101.84	4.02	72.90	4.69	57.95	3.14	77.49	9.27	48.46	5.59
GSK3b	Glycogen synthase kinase 3	89.62	8.33	57.14	2.00	61.52	5.20	42.61	9.16	71.57	7.02	41.64	2.54
IKKβ	IKB kinase β	95.21	3.44	92.54	1.67	103.12	2.00	17.77	2.03	97.01	2.37	71.81	3.28
JNK/ SAPK1c	c-Jun N-terminal kinase	103.99	2.98	102.15	5.73	92.05	2.00	79.59	5.33	92.45	8.03	77.44	0.34
JNK3	c-Jun N-terminal kinase 3	87.72	2.08	83.13	2.36	72.16	3.93	62.20	2.00	72.95	2.92	50.87	4.32
LCK	Lymphocyte kinase	100.45	2.05	97.49	8.64	95.16	8.17	82.58	3.16	88.64	0.69	80.79	0.51
MAPK2/ ERK2	Mitogen-activated protein kinase 2	88.50	2.21	87.15	1.10	115.42	2.00	123.90	6.30	104.83	0.79	125.32	8.44
MAPKAP-K1a	MAPK-activated protein kinase 1a	102.57	3.67	82.38	0.54	140.32	8.25	111.55	2.00	114.86	1.00	132.30	1.87
MAPKAP-K1b	MAPK-activated protein kinase 1b	79.68	3.81	79.56	4.00	73.43	4.80	43.00	5.15	74.81	8.70	62.05	1.61
MAPKAP-K2	MAPK-activated protein kinase 2	108.51	6.93	103.21	3.00	100.62	0.83	56.93	2.41	95.36	2.00	79.93	3.82
MAPKAP-K3	MAPK-activated protein kinase 3	79.99	2.26	68.58	8.60	60.20	1.00	4.05	1.59	67.03	0.50	42.64	1.00
MARK3	MAP/microtubule affinity regulating kinase 3	164.31	1.00	105.36	1.00	159.54	2.00	158.35	5.55	130.59	1.00	82.28	2.00

Table 1 (*Continued*)

		Kavain (1) (10 μM)	±S.D.	Kavain (1) (50 μM)	±S.D.	Flavokavain A (7) (10 μM)	±S.D.	Flavokavain A (7) (50 μM)	±S.D.	Flavokavain B (8) (10 μM)	±S.D.	Flavokavain B (8) (50 μM)	±S.D.
MKK1	MAPK kinase1 (MEK1)	85.76	5.98	88.09	7.44	82.08	6.70	70.97	2.00	83.72	2.50	91.63	4.00
MNK1	MAPK-integrating kinase 1	84.38	3.00	86.00	7.53	81.26	1.11	72.73	6.34	83.37	2.37	70.94	5.28
MNK2	MAPK-integrating kinase 2	109.91	2.00	100.83	1.77	95.76	5.93	82.49	9.12	88.75	5.75	82.24	1.68
MSK1	Mitogen- and stress-activated protein kinase 1	83.15	0.02	78.48	4.96	94.37	3.45	39.86	2.20	90.25	7.71	76.11	5.32
MST2	Mammalian STE (sterile) 20-like kinase 2	78.13	4.04	81.33	5.56	91.51	2.29	104.01	3.39	78.92	1.31	92.40	9.42
NEK2a	NIMA-related kinase 2a	87.79	8.56	82.88	3.61	80.13	9.20	66.02	1.42	86.14	1.00	63.76	7.15
NEK6	NIMA-related kinase 6	91.11	1.18	95.82	5.55	97.06	6.12	109.64	1.69	100.10	1.31	105.57	1.81
NEK7	NIMA-related kinase 7	89.16	0.77	89.99	6.45	85.98	2.00	84.82	2.00	95.57	5.33	98.77	2.00
p70 S6K	p70 Ribosomal protein S6 kinase	105.64	5.18	96.69	2.18	85.91	4.17	59.64	5.24	83.24	0.20	75.06	0.18
PDK1	3-Phosphoinositide-dependent protein kinase 1	104.69	7.85	90.89	5.25	98.74	2.00	53.42	2.53	96.59	3.25	79.35	3.11
PHK	Phosphorylase kinase	90.07	7.61	86.93	8.87	63.33	7.13	29.92	1.60	62.78	0.35	26.08	2.81
PIM2	PIM 2 kinase	97.43	6.12	94.15	8.08	91.48	6.55	71.90	7.37	84.37	4.36	77.59	5.40
PKA	cAMP-dependent protein kinase A	93.31	9.22	88.50	3.00	69.17	0.32	54.99	8.74	87.30	6.17	63.15	4.15
PKBΔph	cAMP-dependent protein kinase B Δph	81.72	1.78	68.06	7.56	97.11	1.00	87.98	6.46	97.28	3.01	89.28	2.00
PKBb	cAMP-dependent protein kinase B b	96.85	7.23	86.23	7.59	80.05	10.26	62.05	8.22	81.67	2.00	61.09	3.32
PKCa	cAMP-dependent protein kinase C a	107.17	5.68	106.69	10.56	104.37	0.94	106.30	1.12	102.71	0.95	109.22	4.35
PKD1	cAMP-dependent protein kinase D1	86.67	8.63	85.95	5.49	83.30	0.71	80.43	2.52	91.13	2.00	77.98	3.59
PLK1	Polo-like kinase 1	107.28	1.00	96.47	8.31	86.66	7.69	33.56	0.48	87.95	9.46	52.63	3.33
PRAK	p38-Regulated/ activated kinase	91.10	4.31	64.96	7.06	46.21	5.20	9.11	0.31	62.67	6.58	22.21	0.96
PRK2	Protein kinase C-related protein kinase	89.18	1.00	84.04	3.29	83.11	0.96	85.31	3.35	81.34	3.00	77.47	2.24
ROCK-II	Rho-dependent protein kinase II	98.64	7.24	100.08	0.01	95.87	3.49	92.79	3.06	93.38	3.40	88.17	1.74
SAPK2a/ p38	Stress-activated protein kinase 2a	79.04	3.58	81.56	2.00	108.65	1.00	114.71	2.00	92.44	3.16	112.93	1.00

SAPK2b/p38ß2	Stress-activated protein kinase 2b	94.46	2.37	91.28	7.73	99.94	7.84	106.38	4.00	92.35	4.20	95.93	1.92
SAPK3/p38g	Stress-activated protein kinase 3	89.48	0.95	77.99	5.10	114.02	8.16	145.07	0.72	98.46	2.00	119.21	1.00
SAPK4/p38d	Stress-activated protein kinase 4	85.54	1.80	88.02	9.25	137.82	3.81	166.43	3.00	118.93	1.93	143.79	0.94
SGK	Serum- and glucocorticoid-induced kinase	98.50	4.92	82.12	8.62	94.49	4.88	50.41	2.00	97.43	2.00	64.64	8.62
smMLCK	Smooth muscle myosin light chain kinase	97.68	1.61	108.13	3.60	62.57	1.10	40.10	6.16	72.24	0.45	34.06	1.19
Src	Src kinase	106.10	2.56	100.27	5.69	104.19	0.89	70.55	1.00	91.03	5.99	87.62	3.86
SRPK1	Homo sapiens SFRS protein kinase	86.76	0.10	84.73	0.26	75.20	1.22	64.54	8.92	83.07	2.72	65.78	2.23

Concentrations of kava derivatives are indicated. Protein kinase activities are presented as a percentage relative to untreated K562 cells (=100%). Results are expressed as mean ± S.D.

and visualized by autoradiography. In supershift/immunodepletion experiments, the nuclear extracts and labelled probes were incubated in the reaction mixture for 30 min on ice prior a 30 min incubation with 2 μg of antibodies on ice. All antibodies (anti-p50, anti-p52, anti-p65, anti-c-Rel and anti-RelB) were from Santa Cruz.

2.6. Western blot analysis

Cytosolic and nucleic protein extracts were prepared as previously described [23]. Briefly, 2×10^6 cells/mL were pretreated with various concentrations of kava derivatives for 2 h and then exposed to 20 ng/mL of TNFα for various amounts of time. A 10 μg of protein extract were resolved on a 10% SDS-PAGE gel, transferred onto a membrane, blocked with 5% non-fat milk, and probed with specific antibodies against IκBα, p65, and p50 (Santa Cruz) as well as with antibodies against actin, lamin C and tubulin α. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and finally detected by ECL reagent (GE Healthcare).

2.7. Protein kinase assays

A panel of 52 protein kinases was used in the kinase assays (Table 1). All protein kinase assays (25 μL) were carried out at room temperature (21 °C) and were linear with respect to time and enzyme concentrations under the conditions used. The assays were initiated with MgATP and performed for 30 min using a Biomek 2000 Laboratory Automation Workstation in a 96-well format (Beckman Instruments). The assays were stopped by the addition of 5 μL of 0.5 M orthophosphoric acid and then harvested onto P81 Unifilter plates using a wash buffer of 50 mM orhophosphoric acid before being counted for radioactivity. IKKβ (5–20 m-units), was diluted in 50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1 mg/mL BSA and assayed against the substrate peptide LDDRHDSGLDSMKDEEY in an incubation buffer containing 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/mL BSA, 300 μM substrate peptide, 10 mM magnesium acetate, and 0.05 mM [γ-^{33}P]ATP (500–1000 cpm/pmol). All the other kinases were assayed as described previously [24]. Values ≤20% relative to untreated K562 cells (=100%) were considered significant.

2.8. Statistics

Data were expressed as mean ± S.D. and analyzed by the Student's *t*-test. *P*-values below 0.001 were considered as statistically significant.

3. Results

3.1. Effect of kava derivatives on human leukaemia cell viability

In order to determine potential toxic effects of kava derivatives, K562 leukaemia cells were incubated with various

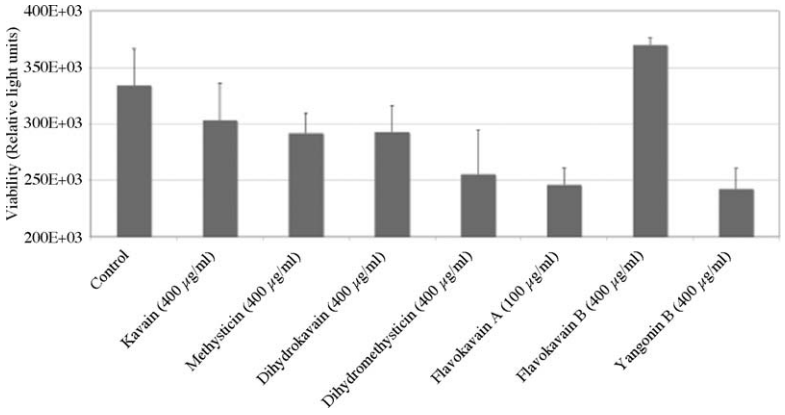


Fig. 2 – Effect of kava compounds on K562 viability. Cells were treated with kavain (A), methysticin (B), dihydrokavain (C), dihydromethysticin (D), yangonin (E), flavokavain A (F), or flavokavain B (G) for 4 h. Cell death was determined using CellTiterGlo kit. Assays were repeated four times.

concentrations of kava derivatives for 4 h. Results confirm that Kava derivatives did not affect the viability of leukaemia cells in our experimental conditions (Fig. 2). In the absence of toxic effects, we decided to investigate the effect of the kava compounds on the NF-κB signalling pathway.

3.2. *Inhibition of TNFα-induced transcriptional activity of NF-κB by kava derivatives*

We next examined the effect of the kava derivatives on the inhibition of TNFα-induced transcriptional activity of NF-κB, using a luciferase reporter gene assay. Transfected cells were pre-treated for 2 h with different concentrations of kava derivatives and challenged for 2 h with 20 ng/mL of TNFα. The results showed that none of the tested kava derivatives had any effect on basal NF-κB transcriptional activity (Fig. 3). All tested compounds reduced TNFα-induced NF-κB activation in a concentration-dependent manner. A decrease in transcriptional activity of NF-κB was achieved at a concentration of 400 μg/mL by kavain (**1**) (1.7 mM) (two-fold), methysticin (**2**) (1.5 mM) (two-fold), dihydrokavain (**3**) (1.7 mM) (3.2-fold), and dihydromethysticin (**4**) (1.5 mM) (2.3-fold) (Fig. 3A–D). Yangonin (**5**) achieved a reduction of 1.6-fold of the transcriptional activity of NF-κB at the highest test concentration of 400 μg/mL (1.5 mM) (Fig. 3E) whereas flavokavain A (**7**) and flavokavain B (**8**) inhibited the TNFα-induced transcriptional activity of NF-κB at a concentration of 100 μg/mL (0.3 mM) by 1.9- and 2.1-fold, respectively (Fig. 3F and G).

3.3. *Inhibition of TNFα-induced NF-κB-DNA binding by kava derivatives*

The effects of kava derivatives on the TNFα-induced NF-κB-DNA binding were confirmed by EMSA. K562 cells were pre-treated for 2 h with different concentrations of kava derivatives and treated for 2 h with 20 ng/mL of TNFα. The results showed that kavain (**1**), dihydrokavain (**3**), flavokavain A (**7**), and flavokavain B (**8**) inhibited TNFα-induced NF-κB-DNA binding in a concentration-dependent manner (Fig. 4). Complete inhibition of TNFα-induced NF-κB-DNA binding was achieved at 200 μg/mL (870 μM) for kavain (**1**) and dihydrokavain (**3**), at 100 μg/mL (320 μM) for flavokavain A (**7**), and at 50 μg/mL (175 μM) for flavokavain B (**8**). Methysticin (**2**), dihydromethysticin (**4**), and yangonin (**5**) did not achieve complete inhibition of TNFα-induced NF-κB–DNA binding at any of the tested concentrations. In order to generalize our findings, kava compounds were also tested on TNF-stimulated Jurkat T cells (Fig. 4H). In order to characterize NF-κB binding activities, we realized immunodepletion assays. K562 cells were treated with TNFα and nuclear factors were analyzed by EMSA. The use of NF-κB-specific antibodies demonstrates the presence of p50/p65 and p65/p65 dimers (Fig. 4I).

3.4. *Kava compounds inhibit TNFα-induced IκBα degradation and NF-κB translocation*

As activation of NF-κB is initiated by degradation of the natural inhibitor IκB, we next assessed integrity of IκBα as well as the translocation of p50 and p65 to the nucleus by Western blot analysis. In TNFα-stimulated Jurkat cells, IκBα degradation occurs between 5 and 60 min whereas nuclear translocation of p50 and p65 subunits of NF-κB is observed 15 min after TNFα treatment (Fig. 5). In cytosolic and nuclear protein extracts of Jurkat cells pre-treated with kavain (**1**), flavokavain A (**7**), or flavokavain B (**8**) before TNFα challenge, results show that all three compounds completely prevent TNFα-induced degradation of IκBα and the subsequent translocation of p50 and p65 to the nucleus (Fig. 5A and B). Actin was used as a loading control. Purities of nuclear and cytoplasmic extracts are shown with lamin C (nucleus) and tubulin α (cytoplasm) Western blots.

3.5. *Kava compounds inhibit protein kinase activities*

Kavain (**1**), flavokavain A (**7**), and flavokavain B (**8**) were tested for their effects on various protein kinases by selectivity screening (Table 1). Results show that Kavain (**1**) did not have any substantial effect on any of tested protein kinases at test

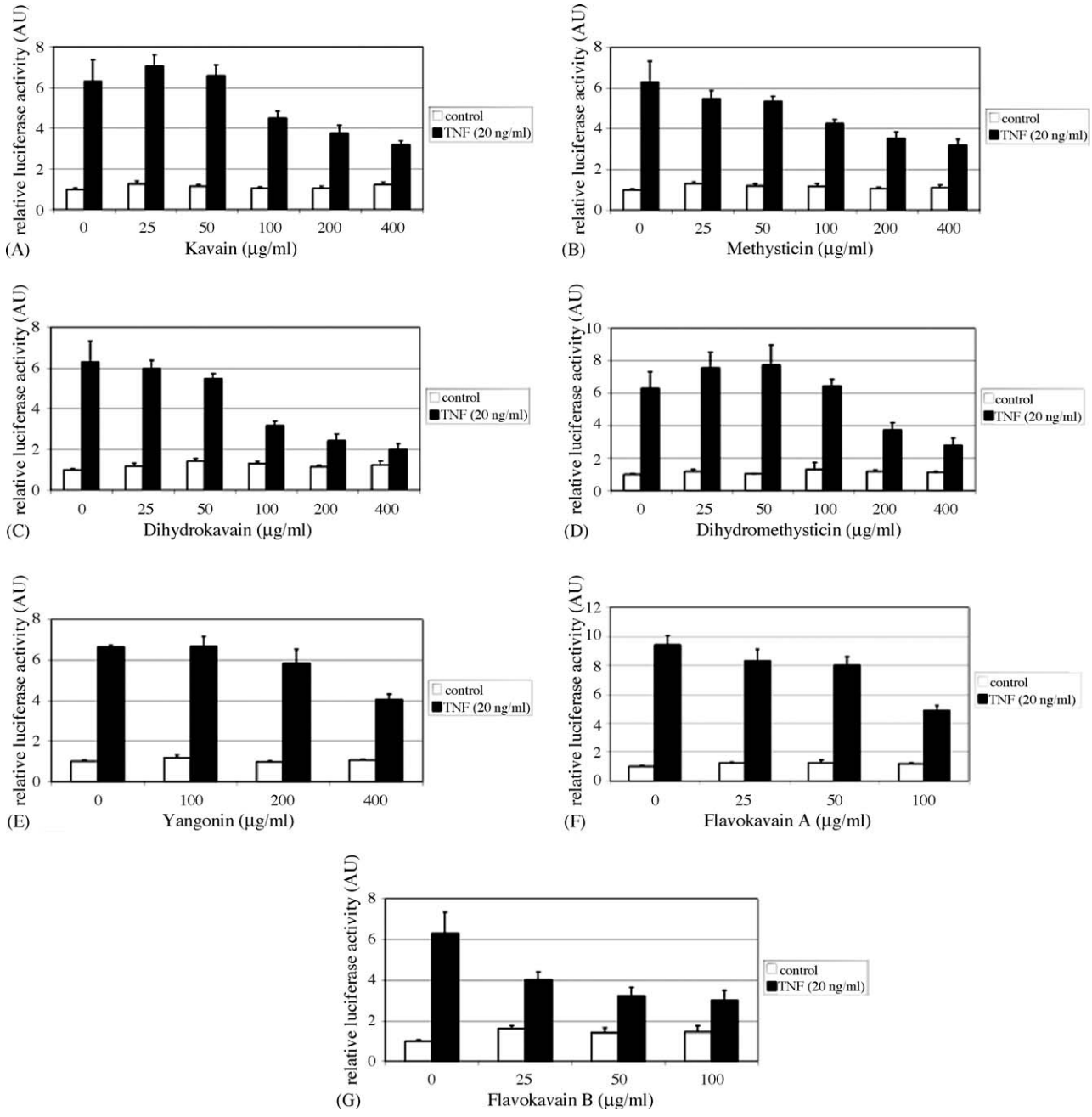
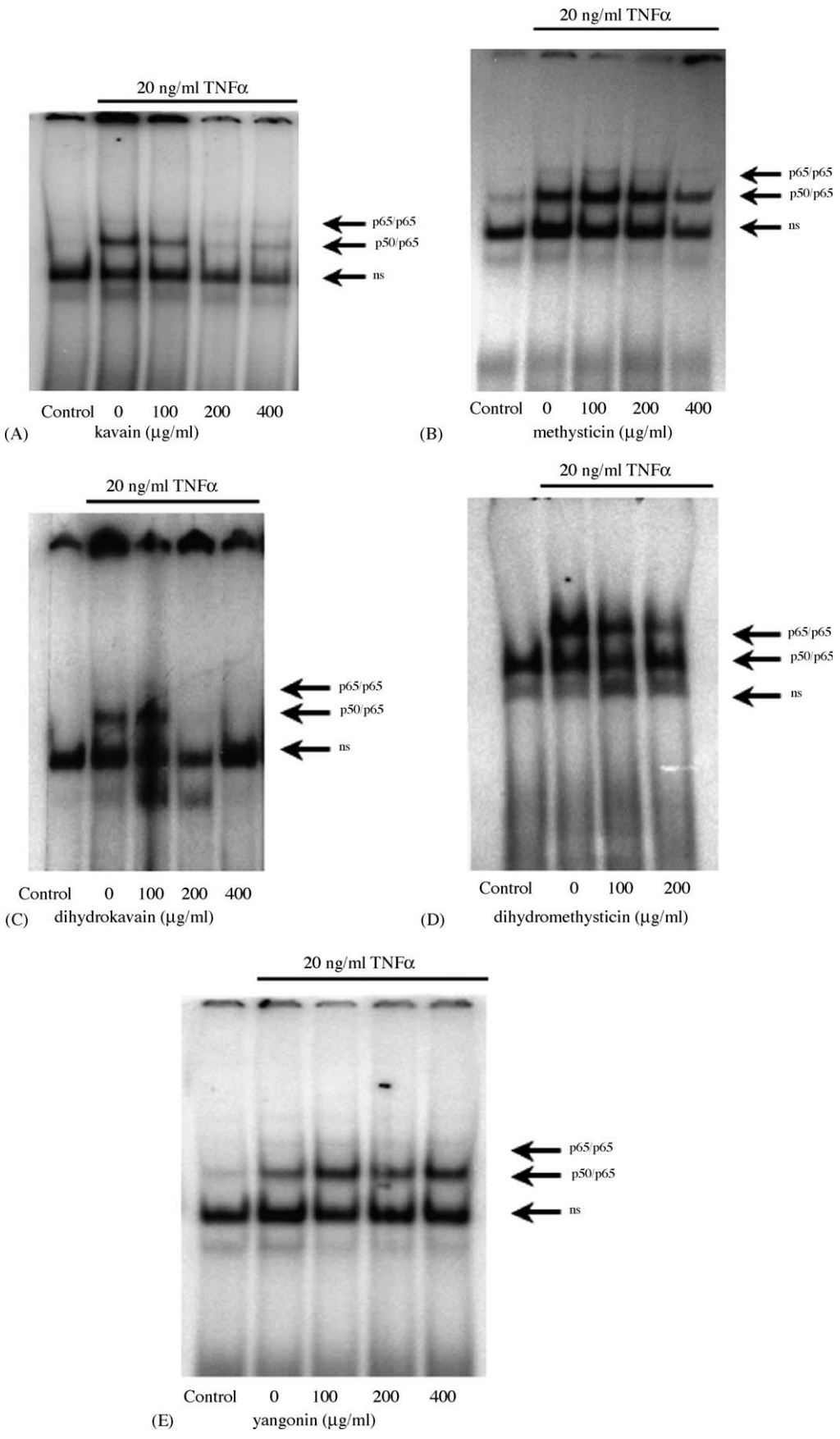


Fig. 3 – Inhibition of TNFα-induced NF-κB activation by kava derivatives. Luciferase activity of pNF-kBLuc K562 cells pre-treated for 2 h with different concentrations (in μg/mL) of kavain (A), methysticin (B), dihydrokavain (C), dihydromethysticin (D), yangonin (E), flavokavain A (F), or flavokavain B (G), and treated for 2 h with 20 ng/mL of TNFα. The results of the luciferase NF-κB reporter gene assay are given as a ratio of the luminescence measured for the firefly luciferase divided by the luminescence measured for the *Renilla* luciferase. The negative control refers to the basal transcription activity of NF-kB in absence of any test substance. Results shown as mean ± S.D. of eight individual measurements. $^{**}P < 0.001$ (compared to TNFα treated control).

concentrations ranging from 0 to 50 μM. Flavokavain A (**7**) and flavokavain B (**8**) substantially decrease the kinase activity of PRAK (p38-regulated/activated kinase), DYRK1A (dual-specificity tyrosine-phosporylated and regulated kinase 1A) and Aurora B. Interestingly, flavokavain A (**7**) additionally inhibits IKKβ as well as MAPKAP-K3 (MAPK-activated protein kinase 3) at a concentration of 50 μM.

4. Discussion

NF-κB is a transcription factor involved in numerous inflammatory and cancer-related diseases and it has hence become a major target in drug discovery [9]. Kava (*P. methysticum*) has a long history of application in ethnopharmacology for numer-


20 ng/ml TNFα
p65/p65
p50/p65
ns
Control 0 100 200 400
(A) kavain (μg/ml)
20 ng/ml TNFα
p65/p65
p50/p65
ns
Control 0 100 200 400
(B) methysticin (μg/ml)
20 ng/ml TNFα
p65/p65
p50/p65
ns
Control 0 100 200 400
(C) dihydrokavain (μg/ml)
20 ng/ml TNFα
p65/p65
p50/p65
ns
Control 0 100 200
(D) dihydromethysticin (μg/ml)
20 ng/ml TNFα
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(E) yangonin (μg/ml)

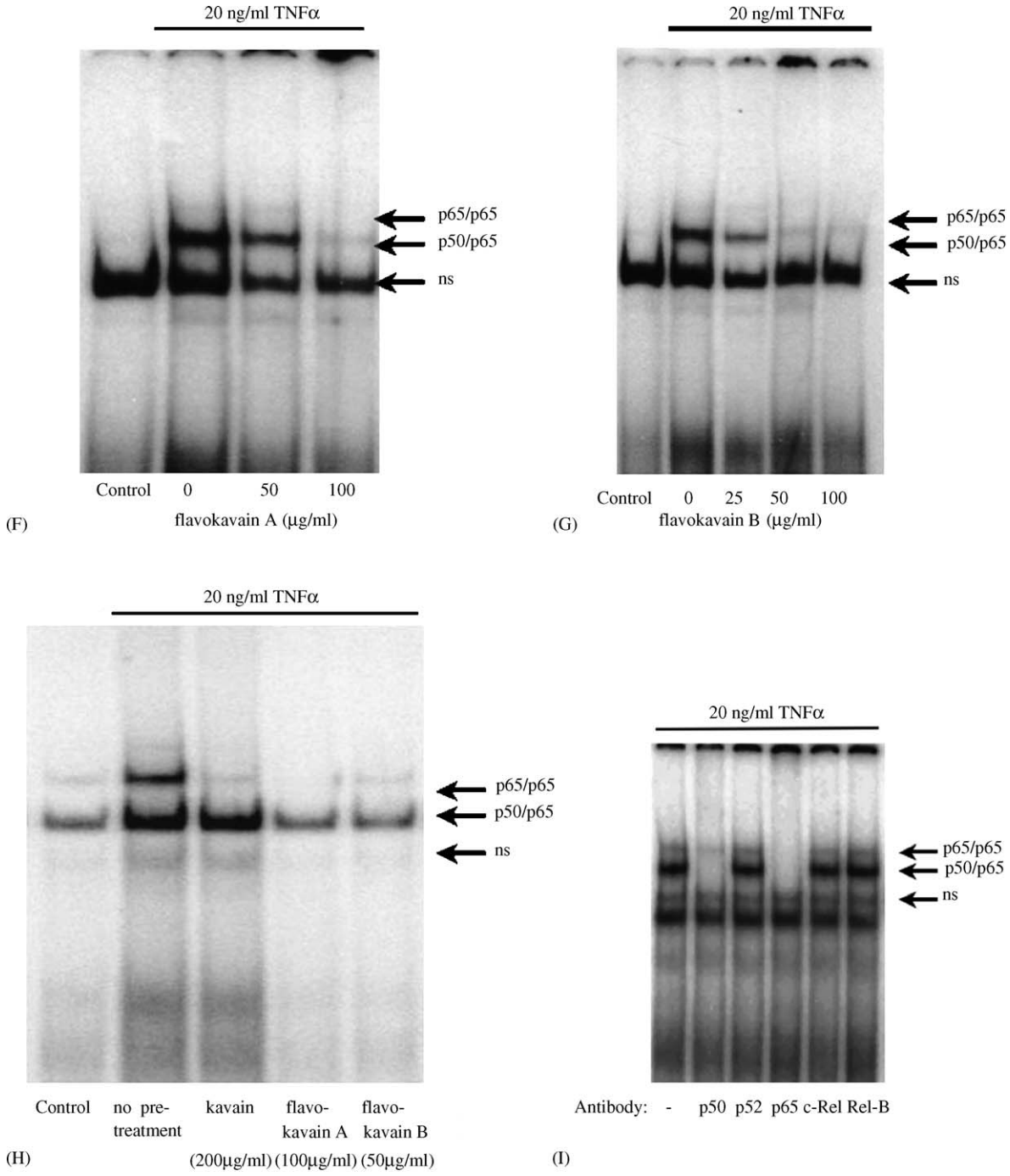

Fig. 4 – Inhibition of TNFα-induced NF-κB–DNA binding activity by kava derivatives. Binding activity of NF-κB to the κB binding site. K562 cells were pre-treated for 2 h with different concentrations (in μg/mL) of kavain (A), methysticin (B), dihydrokavain (C), dihydromethysticin (D), yangonin (E), flavokavain A (F), or flavokavain B (G), before being treated for 2 h with 20 ng/mL of TNFα. Jurkat cells were pre-treated for 2 h with different concentrations of kava compounds as indicated (H). EMSA experiments were performed by incubating 10 μg of nuclear extract for 20 min with an oligonucleotide probe containing the consensus NF-κB binding site C-κB. For supershift/immunodepletion experiments, the nuclear extracts and labelled probes were incubated in the reaction mixture for 30 min on ice prior a 30 min incubation with 2 μg of anti-p50, anti-p52, anti-p65, anti-c-Rel and anti-RelB antibodies (I) (ns: non specific).

ous diseases, many of which are likely to be NF-κB-dependent pathologies [14–17].

The present study provides an insight into the effects of the chemical structure of kava derivatives on their NF-κB inhibitory activity. First, the reduction of the cinnamyl chain from the enolides kavain (**1**) and methysticin (**2**) to their dihydro counterparts dihydrokavain (**3**) and dihydromethysticin (**4**), respectively, does not have any substantial effect on

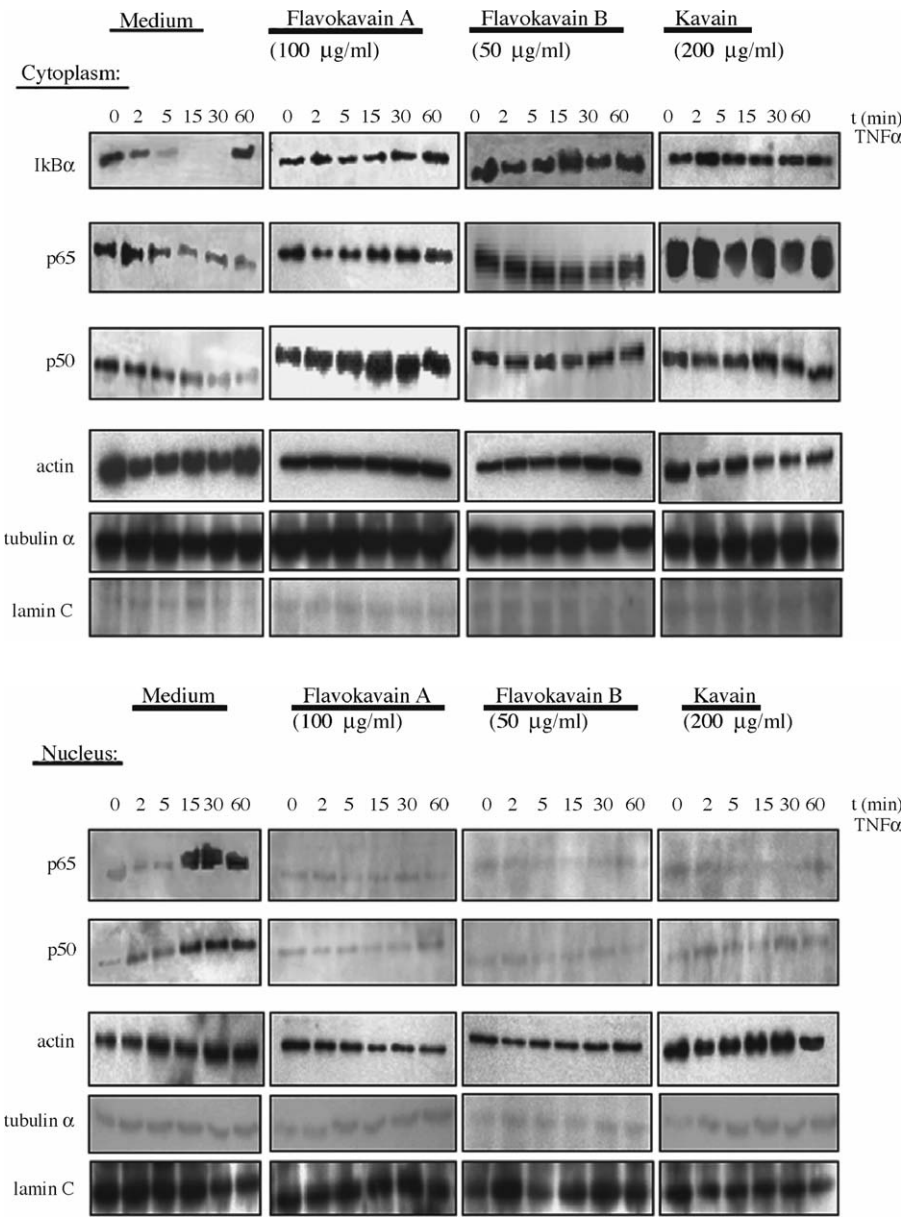

Fig. 5 – Effect of kavain (1), flavokavain A (7), or flavokavain B (8) on IκBα degradation and NF-κB translocation. Kavain (1), flavokavain A (7), and flavokavain B (8) inhibit TNFα-induced degradation of IκBα and the consequent translocation of p50 and p65 into the nucleus. Jurkat cells (2×10^6 cells/mL) were incubated with 200 μg/mL kavain (1), 100 μg/mL flavokavain A (7), or 50 μg/mL flavokavain B (8) for 2 h at 37 °C, treated with 20 ng/mL TNFα for the indicated times at 37 °C, and then tested for IκBα, p50, and p65 in cytosolic fractions and for p50 and p65 in nuclear fractions by Western blot analysis. Equal protein loading was evaluated by β-actin. Purities of nuclear and cytoplasmic extracts were demonstrated with α-tubulin and lamin C Western blots.

the activity of the compounds, suggesting that the bioactivity of the compounds is mainly determined by non-conformational molecular features. Second, since kavain (1) and dihydrokavain (3) were shown to be more potent than methysticin (2) and dihydromethysticin (4) in inhibiting TNFα-induced NF-κB activation, one can suggest that the functional groups of the B ring form the pharmacophore of NF-κB-inhibitory kava-derived enolides, whereas the ketal group on the A ring of methysticin (2) and of dihydromethysticin (4) has no effect on the bioactivity of the molecules or even hinders it partially or completely. The weak bioactivity observed in yangonin (5) suggest that the methoxy-group of the A ring of this molecule hinders the latter's bioactivity. This is in accord with the observations by Schijlen et al. [25] who found that flavonoids and chalcones with unsubstituted aromatic rings or with hydroxyl- and methyl-substituted aromatic rings tend to have a strong anticancer potential, whereas compounds with aromatic rings solely substituted with a methoxy group tend to lack anticancer activity. The results show that, amongst the tested kava chalcones,

flavokavain B (**8**) has a stronger NF-κB inhibitory potential than flavokavain A (**7**). Again, the observed difference in bioactivity may result from the mono-substitution of the B ring of flavokavain A (**7**) with a methoxy group.

Several flavonoids, which compromise a group of polyphenolics with a wide distribution throughout the plant kingdom [25], were previously reported as specific NF-κB inhibitors, usually by preventing IκB degradation [11]. Chalcones, which are biogenetic precursors of the flavonoids [13], were also recently reported as potent NF-κB inhibitors [26–28]. In the majority of plants, the biosynthetic pathway rapidly proceeds to the formation of flavonoids, without any accumulation of chalcones [13]. Some plants, however, such as the tomato plant, also accumulate chalcones as end-products of their shikimate/acetate metabolic pathway [13,25]. Synthetic 2′-hydroxychalcone and the natural trihydroxychalcone isoliquiritigenin were shown to specifically inhibit IκBα degradation [29].

Zi and Simoneau [30] recently reported the anticancer activity of the kava compounds flavokavain A, B and C but not kavain to cause strong anti-proliferative and apoptotic effects in human bladder cancer cells. Flavokavain A treatment induces a loss of mitochondrial membrane potential and release of cytochrome C. The authors observed a decrease in Bcl-X_L as well as a decrease in the association of BCl-X_L to Bax accompanied by an increase in active Bax protein. According to Zi and Simoneau, Bax should be responsible in part for the pro-apoptotic effect of flavokavain A in bladder tumours. In addition a down-regulation of anti-apoptotic surviving and X-linked inhibitor of apoptosis (XIAP) was also documented.

Hashimoto et al. [31] on the other hand published that kava extracts showed a strong inhibitory effect against TNFα release in okadaic acid challenged BALB/3T3 cells and lipopolysaccharide-treated mice. Kapadi et al. [32] documented the cancer chemopreventive effect of kava by using an Epstein–Barr virus early antigen activation protocol in Raji Burkitt lymphoma cells. At a concentration of 100 μg/mL, herbal extract from *P. methysticum* root and rhizome strongly inhibited TPA-induced EBV-EA activation. The phorbol ester TPA like TNFα is able to induce numerous cell signalling pathways including the NF-κB cell signalling. Our results are in agreement with those previously published and allow a better understanding of the anti-cancer activity described for kava extracts.

Multiple cell signalling pathways are inhibited by kava extracts. Overexpression of Aurora B, a chromosomal passenger protein, accelerates the proliferation of BJ glioblastoma cells as shown by Jung et al. [33]. Many of the proteins like Aurora B that regulate mitosis are aberrantly expressed in tumour cells when compared to their normal counterparts. Small molecule inhibitors as potential novel anti-cancer drugs are currently being developed and the inhibiting activity of flavokavain A (**7**) observed in this study could contribute to the development of novel inhibitory compounds. DYRKs are an emerging family of dual-specificity kinases that play key roles in cell proliferation, survival, and development [34]. Kelly and Rahmani [35] showed that DYRK1A prolongs the kinetics of ERK activation by interacting with Ras, B-Raf, and MEK1 to facilitate the formation of a Ras/B-Raf/MEK1 multiprotein complex. Flavokavains could therefore destabilize this complex and reduce inflammatory signalling efficiency.

p38 regulated/activated protein kinase PRAK functions downstream of p38alpha and p38beta in mediating the signalling of the p38 pathway. The p38 mitogen-activated protein kinase pathway plays an important role in cellular responses to inflammatory stimuli and environmental stress [36]. In our hands, flavokavain A (**7**) and flavokavain B (**8**) inhibit PRAK kinase activity and could contribute to the anti-inflammatory effect we demonstrate for these kava compounds.

MAPKAP-K3 (MAPK-activated protein kinase 3) is another downstream kinase activated by p38 MAPK essential for the induction of the antiapoptotic protein Bcl-2 by IGF-I through the nuclear transcription factor cAMP-response element-binding protein in PC12 cells as shown by Pugazhenthi et al. [37]. Inhibition of this protein kinase could be in part responsible for the apoptotic mechanisms induced by kava compounds previously described by Zi and Simoneau [30].

Finally favokavain A (**7**) inhibits IKKβ, which is responsible in part for the phosphorylation of IκB. This inhibition was previously shown to prevent phosphorylation and subsequent degradation of IκB. The exact mechanism of action of the kava-derived NF-κB-inhibitor kavain (**1**) remains to be investigated as this compound inhibits transcriptional activity, NF-kB binding to target DNA sequences, IκB degradation and NF-κB translocation to the nucleus without affecting protein kinase activity. The results show that, amongst the tested kava derivatives, kava-chalcones are significantly more potent inhibitors of TNFα-induced NF-κB activation than their lactone counterparts, which gives a new insight into the pharmaceutical potentials of kava-derived chalcones, as kavalactones have long been considered as the only bioactive compounds responsible for the ethnopharmacological properties of kava [13–16].

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