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Bupleurum chinense Roots: a Bioactivity-Guided Approach toward Saponin-Type NF-κB Inhibitors

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ABSTRACT

The roots of *Bupleurum chinense* have a long history in traditional medicine to treat infectious diseases and inflammatory disorders. Two major compounds, saikosaponins A and D, were reported to exert potent anti-inflammatory activity by inhibiting NF-κB. In the present study, we isolated new saikosaponin analogues from the roots of *B. chinese* interfering with NF-κB activity *in vitro*. The methanol-soluble fraction of the dichloromethane extract of Radix Bupleuri was subjected to activity-guided isolation yielding 18 compounds, including triterpenoids and polyacetylenes. Their structures were determined by spectroscopic methods as saikogenin D (**1**), prosaikogenin D (**2**), saikosaponins B_2 (**3**), W (**4**), B_1 (**5**), Y (**6**), D (**7**), A (**8**), E (**9**), B_4 (**10**), B_3 (**11**), and T (**12**), saikodiyne A (**13**), D (**14**), E (**15**) and F (**16**), falcarindiol (**17**), and 1-linoleoyl-sn-glycero-3-phosphorylcholine (**18**). Among them, **4**, **15**, and **16** are new compounds, whereas **6**, previously described as a semi-synthetic compound, is isolated from a natural source for the first time, and **13**–**17** are the first reports of polyacetylenes from this plant. Nine saponins/triterpenoids were tested for inhibition of NF-κB signaling in a cell-based NF-κB-dependent luciferase reporter gene model *in vitro*. Five of them (**1**, **2**, **4**, **6**, and **8**) showed strong (> 50 %, at 30 µM) NF-κB inhibition, but also varying degrees of cytotoxicity, with compounds **1** and **4** (showing no significant cytotoxicity) presenting IC_{50} values of 14.0 µM and 14.1 µM in the cell-based assay, respectively.

Introduction

The transcription factor NF-κB plays a key role in the control of genes involved in the innate and adaptive immune response. Its aberrant regulation is implicated in the pathogenesis of several disease states, including inflammation and autoimmune disorders [1]. NF-κB signaling is involved in cancer development and progression [2]. Furthermore, it is responsible for certain kinds of chemoresistance [3]. In consistence with its role as a central mediator in inflammatory responses, NF-κB is implicated in the aging process [4] and the development of various metabolic diseases [5], with beneficial effects observed upon its inhibition [6]. Therefore, the NF-κB signaling pathway appears as a promising drug

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target [7], which is also susceptible to the bioactivity of numerous small molecules from plant origin [8–11].

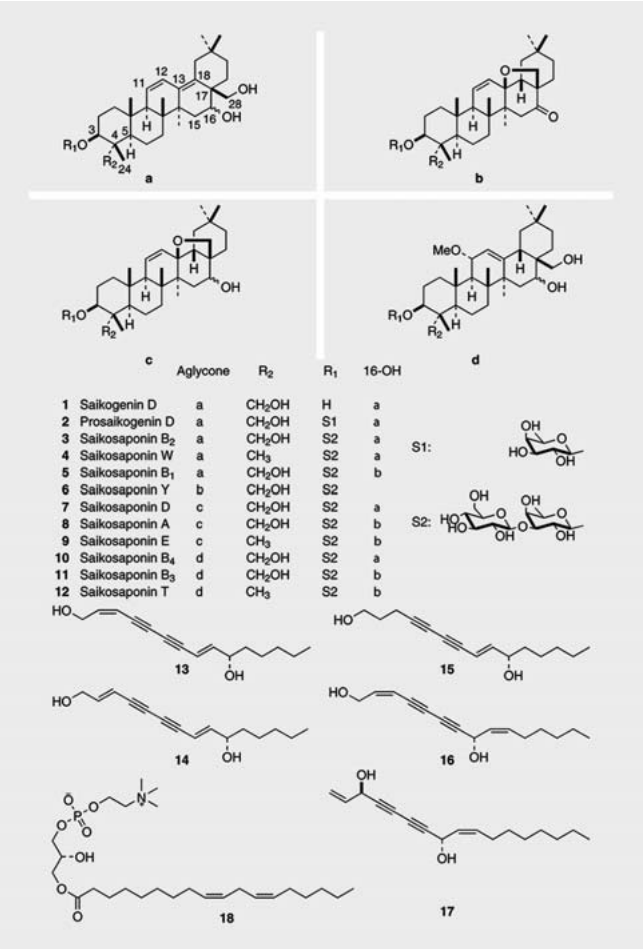
The roots of *Bupleurum chinense* DC (Apiaceae) have a long history in traditional medicine and have been used in China for the treatment of inflammatory disorders and infectious diseases [12, 13]. The main constituents of this plant are triterpenoid saponins ("saikosaponins"), which were reported to have *in vitro* and *in vivo* anti-inflammatory, immunomodulatory [14], and hepatoprotective [15] activities. One of the major constituents, saikosaponin D, and its epimer, saikosaponin A, were reported to inhibit NF-κB activation [16].

However, there are no further studies that address the NF-κB inhibitory activity of additional saikosaponin analogues from this plant. We report here the isolation of 12 saikosaponins/saikogenins, of which nine have been tested for their potential to inhibit NF-κB transactivation in a cell-based *in vitro* luciferase reporter gene assay.

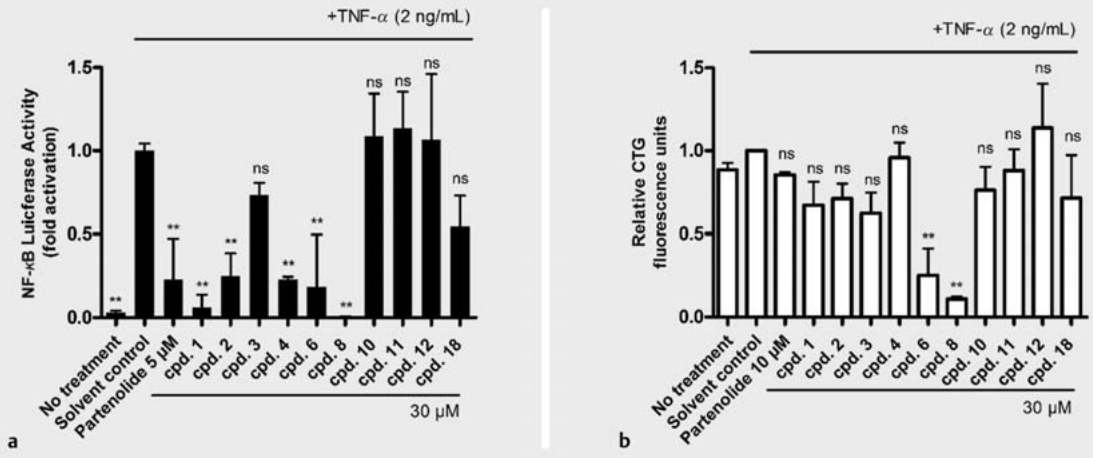
Results and Discussion

The dichloromethane (DCM) extract of *B. chinense* roots inhibited NF-κB-driven gene expression in a cell-based *in vitro* luciferase reporter assay at a concentration of 10 µg/mL by 83%. The MeOH soluble part of the DCM extract was separated by column chromatography (CC) on MCI® gel. Aliquots of the collected fractions were combined based on the TLC profiles into 10 pooled fractions that were tested for NF-κB inhibition. Activity was detected in pooled fractions 5–8, with the highest activity in pooled fraction 6 (91% inhibition, 10 µg/mL), and a moderate activity in pooled fractions 5 (47% inhibition at 10 µg/mL) and 7 (52% inhibition at 10 µg/mL) (**Fig. 1S**, Supporting Information). From MCI fractions belonging to the pooled fractions 4, 5 and 6, 18 compounds were isolated (▶ **Fig. 1**), including one sapogenin (**1**), saponins (**2–12**), polyines (**13–17**), and a phospholipid (**18**). Their structures were determined by 1D and 2D NMR (COSY, HSQC, HMBC) and mass spectrometry (HR-ESI-MS), and by comparison of the spectral data with literature values. Data regarding the inhibition of the NF-κB transactivation activity by pooled fractions 1–10 are shown in **Fig. 1S** of the supporting information. Also spectroscopic and spectrometric data for the identification of known compounds (**1–3, 5, 7–14**, and **17–18**) are provided in the supporting information.

Compound **6** was obtained as a white amorphous powder. Its HR-ESI-MS displayed a quasimolecular ion peak at 779.4567 $[M + H]^+$, which, together with ^{13}C NMR data provided a molecular formula of $C_{42}H_{66}O_{13}$. The ^1H NMR spectrum showed the presence of a *cis*-olefinic group, two anomeric protons, and six tertiary methyl groups. Through interpretation of the 2D NMR data, all proton and carbon resonance were assigned. HMBC correlation was observed between one of the olefinic protons (δ 5.94, H-11) and a tertiary carbon (δ 85.6, C-13), which in turn showed HMBC correlation with a methylene group (δ 76.3, CH_2-28), suggesting an ether linkage between them. This indicated a triterpenoid aglycone similar to that of saikosaponins A (**8**) or D (**7**) [17]. The NMR resonances belonging to the rings A and B of compound **6** were almost superimposable to that of compounds **7** and **8**. The major difference was the existence in compound **6** of a ketone group (δ 214.8, C-16) instead of a hydroxylated methine. This was supported by the HMBC correlations of this ketone carbon with H-15, H-22, and H-28, as well as lower field shifts for C-14 ($\Delta\delta_C$ − 6.6 Hz), C-15 ($\Delta\delta_C$ − 9.9 Hz), C-17 ($\Delta\delta_C$ − 11.3 Hz), and C-18 ($\Delta\delta_C$ − 4.2 Hz). Therefore, the aglycone part of compound **6** was determined as 13,28-epoxy-3β,23-dihydroxy-olean-11-en-16-one [18]. The remaining 12 carbon signals, together with two anomeric protons at δ 4.53 (*d, J* = 7.7 Hz) and 4.38 (*d, J* = 7.7 Hz), suggested the existence of two sugar units. Further analysis of the resonance data revealed a β-glucopyranose and a β-fucopyranose. As only D-configuration of both sugars have been reported in saikosaponins, we tentatively assigned the D-configuration based on biosynthetic considerations. HMBC correlations between signals at δ 4.53 (H-1′′) and δ 85.2 (C-3′) suggested the connection of β-glucopyranose to C-3′ of the β-fucopyranose moiety. The connection of the sugar chain to C-3 of the aglycone was deduced from the HMBC correlation between signals at δ 4.38 (H-1′) and δ 83.0 (C-3). The sugar sequence was supported by fragment ions at *m/z* 617 $[M+H-Glc]^+$, 471 $[M+H-Glc-Fuc]^+$, and 453 $[M+H-Glc-Fuc-H_2O]^+$. Therefore, the structure of compound **6** is established as 13,28-epoxy-23-hydroxy-olean-11-en-16-one-3β-yl *O*-β-D-glucopyranosyl(1 → 3)-β-D-fucopyranoside. Com-



▶ **Fig. 1** Structures of compounds **1–18** isolated from *B. chinense*.



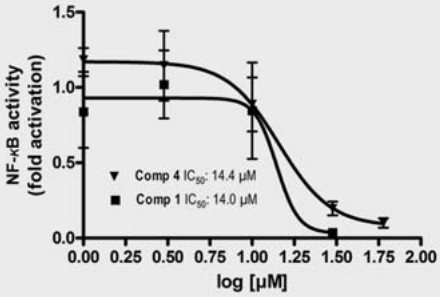
▶ **Fig. 2** **a** NF-κB inhibition by selected compounds isolated from *B. chinense* detected in an *in vitro* cell-based luciferase assay. Luciferase activity (relative units to the internal control CTG fluorescence) are presented as mean ± SD (n = 3, Dunnett's test, $^{*}p < 0.05$, $^{***}p < 0.001$, ns: not significant) and are normalized to the solvent control DMSO (0.1 %). **b** Cells were stained with CTG 1 h before treatment of cells with the indicated compounds for 4 h. Relative fluorescence units were measured directly before measuring the NF-κB-dependent luciferase activity and are displayed here normalized to the solvent control DMSO (0.1 %). Data are expressed as mean ± SD (n = 3, unpaired t-test, $^{*}p < 0.05$, $^{***}p < 0.001$, ns: not significant).

pound **6** was once reported as semi-synthetic derivative from saikosaponin D, but never as a natural product [18]. It is now assigned the trivial name saikosaponin Y.

Compound **4** was obtained as a white amorphous powder. A molecular formula of $C_{42}H_{68}O_{12}$ was derived from its HR-ESI-MS and ^{13}C NMR data. The UV spectrum showed strong absorption maxima at 245 (sh), 251, and 260 (sh) nm, indicating the existence of an heteroannular diene [19]. This was further supported by the ^{1}H NMR signals of *cis*-olefinic protons at δ 6.45 and 5.58, and ^{13}C NMR signals at δ 137.2, 132.7, 127.1, and 126.7. Through interpretation of the 2D NMR data, all proton and carbon resonances were assigned. The ^{13}C NMR data of the aglycone part were nearly superimposable to that of saikosaponin B_2 (**3**) [17], except for the upper field shift for C-4 ($\Delta\delta_C$ − 3.7 ppm), and downfield shifts for C-3 ($\Delta\delta_C$ 7.2 ppm), C-5 ($\Delta\delta_C$ 8.5 ppm), C-24 ($\Delta\delta_C$ 3.7 ppm), and C-23 ($\Delta\delta_C$ 36.9 ppm), which revealed the absence of a 23-hydroxyl group in compound **4** [17]. At the same time, upper field shifts were observed in the ^{1}H NMR for H-3 ($\Delta\delta_H$ − 0.46 ppm) and H-5 ($\Delta\delta_H$ − 0.42 ppm), owing to the releasing of steric hindrance from 23-hydroxyl group. When compared with saikosaponin E (**9**), these atoms, together with other atoms belonging to the ring A of the pentacyclic skeleton of compound **4**, shared nearly identical chemical shifts with their counterpart in compound **9** [17]. Therefore, the aglycone of compound **4** was determined to be 16-*epi*-saikogenin C [19]. The remaining 12 carbon signals, together with two anomeric protons at δ 4.55 (*d*, J = 7.7 Hz) and 4.32 (*d*, J = 7.7 Hz), could be readily assigned to the sugar moiety. As in the case of compound **6**, through further analysis of the NMR data, the existence of a β-glucopyranose and a β-fucopyranose was determined, and the configuration of both of them was also tentatively assigned as D for biogenetic reasons. Their sequence was elucidated by HMBC correlations at δ 4.55 (H-1′′)/δ 84.9 (C-3′) and δ 3.10 (H-3′)/δ 105.6 (C-1′′), which suggest the connection of β-glucopyranose to C-3′ of the β-fucopyranose moiety. HMBC correlations at δ 3.62 (H-3)/δ 105.6 (C-1′) and δ 4.32 (H-1′)/δ 83.1 (C-3) supported the connection of the sugar chain to C-3 of the aglycone. This linking pattern was supported by mass fragment peaks at *m/z* 747 $[M+H\text{-}H_2O]^+$, 585 $[M+H\text{-}H_2O\text{-}Glc]^+$, and 439 $[M+H\text{-}H_2O\text{-}Glc\text{-}Fuc]^+$. Therefore, the structure of compound **4** is established as 16α,28-dihydroxyolean-11,13(18)-diene-3β-yl *O*-β-D-glucopyranosyl-(1 → 3)-β-D-fucopyranoside. To our knowledge, it is reported here for the first time and is given the trivial name saikosaponin W.

The remaining triterpenoids were identified by comparison of their spectral data with literature values as saikogenin D (**1**) [17], prosaikogenin D (**2**) [20], saikosaponins B_2 (**3**) [17], B_1 (**5**) [17], D (**7**) [17], A (**8**) [17], E (**9**) [17], B_4 (**10**) [17], B_3 (**11**) [17], and T (**12**) [21].

Nine of these compounds (**1**, **2**, **3**, **4**, **6**, **8**, **10**, **11**, and **12**) present in bioactive pooled fractions 5 and 6 were tested at 30 µM for their NF-κB inhibitory activity *in vitro* (▶ **Fig. 2a**). At 30 µM, compound **6** markedly reduced fluorescence of the vital dye (cell tracker green [CTG]), indicating cytotoxicity and therefore a false positive result of NF-κB inhibition in the cell-based luciferase assay. Compound **8** (saikosaponin A) showed a strong decrease of NF-κB-dependent luciferase gene expression *in vitro*. However, it also showed a significant reduction of CTG fluorescence at 30 µM, indicating cytotoxicity of this compound. Compounds **10**–**12** showed no NF-κB inhibition and did not show any cytotoxicity. Interestingly, compounds **1**–**4** showed NF-κB inhibitory activities, but no or low cytotoxicity (▶ **Fig. 2**). For compounds **1** and **4**, which show strong NF-κB inhibition at 30 µM, IC_{50} values were determined. Compound **1** possesses an IC_{50} val-



▶ **Fig. 3** Concentration-response curves of NF-κB inhibition by *B. chinese* compound **1** and compound **4**.

ue of 14.0 μM and compound **4** an IC_{50} value of 14.4 μM in the NF-κB-dependent luciferase reporter gene assay *in vitro* (▶ **Fig. 3**).

Some preliminary structure-activity relationships can be deduced regarding the aglycone moiety. Both of the highly cytotoxic saponins (**6** and **8**, type "b" and "c" ▶ **Fig. 1**) possess an ether bridge between C-13 and C-28 of the aglycone part. The three NF-κB inactive saponins (**10**–**12**, type "d" ▶ **Fig. 1**), which are artifacts from the isolation process [22], have a methoxy group at C-11 in common. The NF-κB active and less cytotoxic compounds (**1**–**4**, type "a" ▶ **Fig. 1**) possess a heteroannular 11,13 (18)-diene system [22].

Compounds **1**–**3** showed a mild decrease in CTG fluorescence, indicating low cytotoxicity at 30 μM (without reaching significance for **1**). Their activities in terms of inhibiting NF-κB transactivation seem to correlate inversely with the number of sugars attached at C-3 of the aglycone. The aglycone saikogenin D (**1**) was the strongest inhibitor (91 % inhibition) with no significant cytotoxicity. Compound **2**, identified as prosaikogenin D, with one sugar (fucose) attached, was less active (75 % inhibition). Compound **3**, identified as saikosaponin B2, and possessing one more sugar (glucose), was even less active (20 % inhibition). Compound **4** (saikosaponin W), the C-23-deoxyanalogue of compound **3** (saikosaponin B2), was one of the most active, decreasing NF-κB activation by 76.9 ± 1.4 %, with no indication of cytotoxicity at 30 μM. These data suggest, in the case of type "a" saponin/triterpenoid, that NF-κB inhibition is favorably influenced by a shorter (or no) sugar chain at aglycone C-3 position, and no C-23 hydroxyl group.

Compound **8,** which is one of the two major saponins of Radix Bupleuri, can be transformed into its prosapogenin and genuine sapogenin after oral administration. Its incubation with intestinal flora led also mainly to genuine sapogenin. But when incubated in gastric juice, type "a" saponins turned out to be the major products [23]. Actually, Chinese pharmacopoeia includes vinegar processing (being fried in vinegar) of Radix Bupleuri. Due to the heated acidic condition, this process can lead to a sharp increase of the content of type "a" and concomitant decrease of type "c" saponins [24]. The NF-κB inhibition observed for compounds **1, 2**, and **4** (type "a") lend support to this processing method.

Besides one saponin (**6**), fractions belonging to pooled fraction 6 also contained a small amount of compound **18**, obtained as a colorless gum, whose strong affinity to both normal and reversed stationary phases indicated amphiphilic properties. Its 1D and 2D NMR spectra suggested a 1,3-substituted glycerol, an esterified linoleic acid, and a choline moiety [25]. The remaining phosphate unit was identified by positive ESI-MS/MS, which showed a quasi-molecular ion (*m/z* 520) and a specific fragment at *m/z* 184 characteristic of a protonated phosphorylcholine head group [26]. Therefore, compound **18** was identified as a lysophosphatidylcholine (Lyso-PC) [25]. This is the first time that a lysophosphatidylcholine is isolated from the genus *Bupleurum*. Compound **18** showed moderate NF-κB inhibition (44 %) at 30 μM.

Finally, four C15 polyacetylenes (**13**–**16**), and one C17 polyacetylene (**17**) were isolated in very small amounts. Their structures were determined through interpretation of NMR and MS spectra as well as by comparison with reference compounds. This is the first time polyacetylenes were isolated from *B. chinense*.

Compounds **13**–**16** were isolated as amorphous solids. Compound **13** was identified as saikodiyne A [27]. Present ^{13}C NMR experiments showed a different shift for C-4 than previously reported. The corrected value is outlined in the supporting information. Compound **14** was identified as a reduced derivative of saikodiyne B [27]. Compound **17** was identified as falcarindiol, a C17 polyacetylene [28].

Compounds **15** and **16** are both reported for the first time. Compound **15** was obtained as a white amorphous solid. The HR-ESI-MS, ^{13}C NMR, and HSQC data indicated a molecular formula of $C_{15}H_{22}O_2$. As in the case of compound **13** and **14**, ^{13}C NMR spectrum of **15** suggested the presence of four acetylene carbon signals, at δ 84.2, 75.3, 66.2, and 64.1 ppm. Comparison of ^{13}C NMR spectra of **15** with those of **13** and **14** suggested the existence of a five-membered aliphatic chain, whose connection to the alkene C-10 was supported by the HMBC correlations at δ 4.08 (H-10)/δ 37.9 (C-11), δ 1.48 (H-11)/δ 72.6 (C-10), δ 1.48 (H-11)/δ 150.7 (C-9). The missing of one of the two pairs of alkene protons in **15** at lower field coincided with the appearing of two vicinal methylene signals at δ 2.42, δ 1.73 (CH_2-2 and CH_2-3). At the same time, up-field shifts were observed for their vicinal hydroxymethylene protons (HO-CH_2-). Taking the solvent effect into consideration, the NMR data of **15** are highly superimposable to that of virol C, except for the signals of the longer aliphatic chain in virol C [29, 30]. By comparing their optical rotation, the configuration of **15** was determined as 10*S* [29]. Therefore, the structure of compound **15** was elucidated as (10*S*,8*E*)-pentadeca-8-en-4,6-diyne-1,10-diol. We assigned the trivial name saikodiyne E to compound **15**.

Compound **16** was obtained as a white amorphous solid. The HR-ESI-MS, ^{13}C NMR, and HSQC data indicated a molecular formula of $C_{15}H_{22}O_2$. The ^{13}C NMR spectrum of **16** suggested the presence of four acetylene carbon signals, at δ 85.1, 79.4, 75.1, and 69.0 ppm. Like compounds **13** and **14**, compound **16** possessed two olefinic bonds. One of the major differences revealed by the HSQC spectrum was the presence of a lower field hydroxymethine proton (δ 5.19, d, J = 8.3 Hz, H-8) in **16,** while in both **13** and **14**, a higher field hydroxymethine proton (δ 4.21, p, J = 5.5 Hz, H-10) was observed. The splitting pattern of the hydroxymethine proton at δ 5.19, together with the HMBC correlations at δ 5.47 (H-9)/δ 28.5 (C-11), δ 2.13 (H-11)/δ 134.1 (C-10), δ 2.13 (H-11)/δ 129.8 (C-9) revealed the shifting of the double bond from Δ^8 in **13** and

14 to Δ^9 in **16**. The NMR data of **16** are highly superimposable to that of another C17 analogue, (2*Z*,8*S*,9*Z*)-heptadeca-2,9-dien-4,6-diyne-1,8-diol [31]. By comparison of their optical rotations, the configuration of **16** was determined as 8*S* [31]. Therefore, the structure of compound **16** was elucidated as (2*Z*,8*S*,9*Z*)-pentadeca-2,9-dien-4,6-diyne-1,8-diol. Compound **16** was named saikodiyne F.

Due to their limited amounts, compounds **13**–**17** have not been tested for their bioactivity.

In summary, this study examined nine triterpenoids (**1**–**4, 6, 8**, and **10**–**12**) for their potential to inhibit NF-κB transactivation activity *in vitro* in a cell-based NF-κB-driven luciferase reporter gene model in HEK293 cells. Six of these compounds showed NF-κB inhibition. However, four of them appeared also to be cytotoxic, especially the type "b" and "c" saponins **6** and **8**. Compound **4**, a new saponin belonging to type "a" presented the best NF-κB inhibitory activity in relation to its cytotoxicity. Several polyacetylenes, including two new congeners, have been isolated in trace amounts for the first time from *B. chinense*. These findings enhance the understanding of this plant as an anti-inflammatory herbal medicine.

Materials and Methods

General experimental procedures

All solvents were obtained from VWR Chemicals and Carl Roth. TLC was performed on Silica gel 60_{F254} and Silica gel 60 RP-18_{F254s} plates (Merck): detection with a mixture of 1% vanillin and 10% sulfuric acid in dehydrated ethanol. Open CC was carried out with MCI CH-P 20P resin (Mitsubishi Chemical), RP-18 silica gel (25–40 µm, Fuji silica), Sephadex LH-20 (GE Healthcare), and silica gel (15–40 µm, Merck) as stationary phases. Semi-preparative HPLC experiments were performed with L-6200A intelligent pump (at a flow rate of 3 mL/min) and L-4500 DAD detector (Merck-Hitachi) equipped with LicroCART 10 × 250 mm column packed with LiChrospher 100 RP-18 (particle size: 10 µm). Analytical HPLC was conducted on an Agilent 1100 system using a Zorbax SB-C18 Narrow Bore (3.5 µm) 2.1 × 150 mm column (Agilent). LC-ESI-MS analyses were carried out using the same column on a Dionex Ultimate 3000 UHPLC coupled with a Thermo LTQ XL linear ion trap mass spectrometer equipped with an H-ESI II probe in the positive and negative mode. Accurate masses were measured on a LC/FTMS system consisting of an Exactive Orbitrap mass spectrometer (ThermoFisher) coupled to a ThermoFisher Accela U-HPLC system. ^{1}H, ^{13}C, and 2D NMR spectra (COSY, HSQC, and HMBC) were recorded in CD_3OD at 25 °C on a Unity 600 (Varian), and an Avance 700 spectrometer (Bruker). Chemical shifts are expressed in δ (ppm) with the solvent peak used as reference.

Plant material

Roots of *B. chinense* were obtained from two sources. Material-I, used for screening, was purchased from Plantasia (Batch No. 100093; Oberndorf, Austria). Material-II, used for preparative isolation, was obtained from the Bavarian State Research Center for Agriculture (LfL-Bayern, Freising-Weihenstephan). Voucher specimens (No.: IPW_Bup-chin_2008 and IPW_Bup-chin_2010) are kept at the Institute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Graz.

Extraction and isolation

For screening, 10 g of Material-I were pulverized and extracted by accelerated solvent extraction with DCM and MeOH: preheat 1 min, static 5 min, flush 150%, purge 60 sec, cycles 3, pressure 68.9 bar, temperature 44 °C for DCM and 68 °C for MeOH.

For large-scale isolation, 900 g of Material-II was pulverized and subsequently percolated with DCM (10 L). After evaporation to dryness (< 40 °C), the DCM extract (36 g) was then partitioned twice between n-hexane and 90% MeOH (1.5 : 1, v/v), yielding 24.7 g of a defatted, MeOH soluble part, which was subsequently fractionated by CC (Ø: 5.5 cm, L: 55 cm), using MCI CHP-20P resin as the stationary phase and an MeOH-water gradient (40% to 100%, v/v) as the mobile phase, to afford 101 fractions (Fr. 1 to Fr. 101, 700 mL each). After the fractions had been evaporated to dryness, they were transferred into small vials, and reconstituted to 10 mL. For bioassay, 30 µL were taken from each vial, and combined according to their TLC profiles to form 10 pooled fractions, tested for NF-κB inhibition.

MCI fractions belonging to the NF-κB inhibitory pooled fractions 5 and 6 were subjected to Sephadex LH-20 (35% MeOH in water) chromatography, in order to separate polyacetylenes from dominant saponins. The saponin containing subfractions were then subjected to RP-18 (Ø: 4 cm, L: 45 cm; eluted with 60–70% MeOH water solution, v/v) and silica gel (Ø: 3 cm, L: 30–50 cm; eluted with n-hexane-ethylacetate, 3 : 1 or 2.2 : 1, v/v) open CC, and finally semi-preparative RP-18 HPLC (35%, 40%, 45%, or 50% MeCN) to afford a sapogenin and eleven saponins, respectively: **1** (2.4 mg) [17], **2** (2.5 mg) [20], **3** (1.5 mg) [17], **4** (2.4 mg), **5** (0.6 mg) [17], **6** (1.3 mg) [18], **7** (5.7 mg) [17], **8** (4.7 mg) [17], **9** (4.4 mg) [17], **10** (20.7 mg) [17], **11** (6.2 mg) [17], and **12** (8.5 mg) [21]. Fractions belonging to pooled fraction 4, which also contained polyacetylenes, were processed in the same way. The polyacetylene enriched subfractions were purified directly by semi-preparative HPLC (isocratic 50% or 69% MeCN) to afford five polyacetylenes, respectively: **13** (0.8 mg) [27], **14** (0.8 mg) [27], **15** (0.9 mg), **16** (0.5 mg). and **17** (0.9 mg) [32]. The isolation of **18** (2.4 mg) was achieved through RP-18 SPE (80% MeOH) and semi-preparative RP-18 HPLC (60% MeCN) [25]. Among them, saponins **1**–**5** and **7**–**12** were isolated from the combined fractions 37, 38, 39, and 40 (460 mg) belonging to the fifth pooled fraction; polyacetylenes **13**–**15** were isolated from the combined fractions 33, 34, 35, and 36 (490 mg) belonging to the fourth and fifth pooled fractions; polyacetylene **16** was isolated from the combined fractions 31 and 32 (663 mg) belonging to the fourth pooled fraction; polyacetylene **17** was isolated from the combined fractions 41, 42, 43, and 44 (370 mg) belonging to the fifth pooled fraction; saponin **6** and lysophosphatidylcholine **18** were isolated from the combined fractions 47 and 48 (210 mg) belonging to the sixth pooled fraction. According to HPLC profiles and NMR spectra, all compounds tested for bioactivity are of at least 95% purity.

16α,28-dihydroxyoleana-11,13(18)-diene-3β-yl O-β-D-glucopyranosyl-(1 → 3)-β-D-fucopyranoside (**4**, *saikosaponin W*): white amorphous powder; $[\alpha]_D^{20}$ − 29.0 (*c* 0.03, MeOH); UV (MeCN/H_2O) λ_{max}:

▶ **Table 1** ^{1}H and ^{13}C NMR spectroscopic data (in CD_3OD) of compounds **4** and **6**[a], *J* in Hz.

	4		**6**	
C	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	1.88 (1H) 1.02 (1H)	39.4	1.87 (1H) 0.94 (1H)	39.2
2	1.95 (1H) 1.75 (1H)	27.0	1.96 (1H) 1.80 (1H, *d*, *J* = 14.2)	26.1
3	3.18 (1H, *dd*, *J* = 11.7,4.5)	90.6	3.63 (H-3)	83.0
4		40.3		44.1
5	0.85 (1H)	56.7	1.21 (1H)	48.0
6	1.64 (1H) 1.46 (1H)	19.4	1.54 (2H)	18.1
7	1.38 (2H)	33.4	1.50 (1H) 1.14 (1H)	32.0
8		41.9		43.0
9	1.99 (1H, *br s*)	54.8	1.91 (1H)	53.8
10		37.5		37.1
11	5.58 (1H, *dd*, *J* = 10.7,1.2)	127.1	6.04 (1H, *d*, *J* = 10.4)	134.6
12	6.45 (1H, *dd*, *J* = 10.8,2.9)	126.7	5.53 (1H, *dd*, *J* = 10.3,3.1)	129.6
13		137.2		85.6
14		42.3		50.9
15	1.93 (1H) 1.41 (1H)	32.0	2.79 (1H, *d*, *J* = 14.5) 1.80 (1H, *d*, *J* = 14.2)	45.4
16	4.03 (1H, *t*, *J* = 3.2)	69.1		214.8
17		45.4		57.4
18		132.7	2.28 (1H, *dd*, *J* = 14.2,3.3)	56.3
19	2.48 (1H, *d*, *J* = 14.7) 1.69 (1H, *d*, *J* = 14.8)	39.4	1.55 (1H) 1.43 (1H)	40.0
20		33.2		32.4
21	1.56 (1H) 1.30 (1H)	35.8	1.51 (1H) 1.24 (1H)	36.6
22	2.01 (1H) 1.61 (1H)	24.5	2.11 (1H) 1.26 (1H)	25.1
23	1.06 (3H, *s*)	28.2	3.67 (1H) 3.29 (1H)	64.7
24	0.84 (3H, *s*)	16.5	0.71 (3H, *s*)	12.7
25	0.92 (3H, *s*)	18.6	0.98 (3H, *s*)	18.8
26	0.73 (3H, *s*)	17.5	1.21 (3H, *s*)	20.1
27	1.23 (3H, *s*)	22.1	1.02 (3H, *s*)	20.7
28	3.74 (1H, *d*, *J* = 11.7) 3.26 (1H)	65.1	3.90 (*d*, *J* = 7.9) 3.44 (*d*, *J* = 8.0)	76.3
29	0.85 (3H, *s*)	25.3	0.93 (3H, *s*)	33.8
30	0.97 (3H, *s*)	32.9	0.90 (3H, *s*)	23.5
Fuc				
1′	4.32 (1H, *d*, *J* = 7.7)	106.6	4.38 (1H, *d*, *J* = 7.7)	105.7
2′	3.67 (1H)	71.9	3.65 (1H)	71.9
3′	3.60 (1H, *dd*, *J* = 9.7,3.3)	84.9	3.60 (1H)	85.2
4′	3.85 (1H)	72.4	3.85 (1H)	72.3
5′	3.64 (1H)	71.2	3.66 (1H)	71.3
6′	1.26 (3H, *d*, *J* = 6.4);	16.9	1.27 (3H, *d*, *J* = 6.4);	16.9

continued

▶ **Table 1** *Continued*

	4		6	
C	δ_H	δ_C	δ_H	δ_C
Glc				
1′′	4.55 (1H, *d*, *J* = 7.7)	105.6	4.53 (*d*, *J* = 7.7)	105.7
2′′	3.28 (1H)	75.4	3.29 (1H)	75.4
3′′	3.37 (1H)	77.7	3.36 (1H)	77.7
4′′	3.32 (1H)	71.3	3.33 (1H)	71.2
5′′	3.28 (1H)	77.9	3.28 (1H)	77.9
6′′	3.84 (1H) 3.67 (1H)	62.5	3.84 (1H, *dd*, *J* = 12.0,2.0) 3.68 (1H)	62.4

[a] Measured at 600 MHz (for ^{1}H) and 150 MHz (for ^{13}C).

▶ **Table 2** ^{1}H and ^{13}C NMR spectroscopic data (in CD_3OD) of compounds **15** and **16**[a], *J* in Hz.

	15		16	
C	δ_H	δ_C	δ_H	δ_C
1	3.63 (2H, t, *J* = 6.3)	61.4	4.30 (2H, dd, *J* = 6.4,1.6)	61.1
2	1.73 (2H, p, *J* = 6.7)	32.3	6.23 (1H, dt, *J* = 11.1,6.4)	147.8
3	2.42 (2H, t, *J* = 7.0)	16.6	5.63 (1H, d, *J* = 11.1)	109.1
4		84.2		75.1
5		66.2		79.4
6		75.3		69.0
7		74.1		85.1
8	5.71 (1H, d, *J* = 15.8)	109.0	5.19 (1H, d, *J* = 8.3)	59.0
9	6.24 (1H, dd, *J* = 15.9,5.8)	150.7	5.47 (1H, ddt, *J* = 10.1,8.3,1.6)	129.8
10	4.08 (1H, qd, *J* = 6.2,1.6)	72.6	5.56 (1H, dtd, *J* = 10.9,7.6,1.2)	134.1
11	1.49 (2H, q, *J* = 7.1)	37.9	2.13 (2H, q, *J* = 7.1)	28.5
12	1.41 (1H) 1.32 (1H)	26.2	1.41 (2H, p, *J* = 7.2)	30.1
13	1.30 (2H)	32.9	1.32 (2H)	32.5
14	1.33 (2H)	23.7	1.33 (2H)	23.6
15	0.91 (3H, t, *J* = 6.9)	14.4	0.91 (3H, t, *J* = 7.0)	14.4

[a] Except for ^{13}C NMR of **16** (measured at 175 MHz), all spectra were measured at 600 MHz (for ^{1}H) and 150 MHz (for ^{13}C).

245 (sh), 251, and 260 (sh) nm; ^{1}H and ^{13}C NMR (150 MHz, CD_3OD) data, see ▶ **Table 1**; HR-ESI-MS *m/z* 763.4641 $[M - H]^-$ (calcd for $C_{42}H_{68}O_{12}$: 763.4633).

13,28-epoxy-23-hydroxy-olean-11-en-16-one-3β-yl O-β-D-glucopyranosyl(1 → 3)-β-D-fucopyranoside (**6**, *saikosaponin Y*): white amorphous powder; ^{1}H and ^{13}C NMR (150 MHz, CD_3OD) data, see ▶ **Table 1**; HR-ESI-MS *m/z* 777.4437 $[M - H]^-$ (calcd 777.4425 for $C_{42}H_{65}O_{13}$).

(10S,8E)-pentadeca-8-en-4,6-diyne-1,10-diol (**15**, *saikodiyne E*): white amorphous solid; $[\alpha]_D^{20}$ + 19.1 (*c* 0.05, MeOH). UV (MeCN/H_2O) λ_{max}: 236, 264, 278, 295, 314 nm; ^{1}H and ^{13}C NMR, see ▶ **Table 2**; HR-ESI-MS *m/z* 293.1759 $[M + CH_3COO]^-$ (calcd 293.1753 for $C_{17}H_{25}O_4$).

(2Z,8S,9Z)-pentadeca-2,9-dien-4,6-diyne-1,8-diol (**16**, *saikodiyne F*): white amorphous solid; $[\alpha]_D^{20}$ + 35.1 (*c* 0.03, MeOH). UV (MeCN/H_2O) λ_{max}: 216, 243, 256, 270, 286 nm; ^{1}H and ^{13}C NMR, see ▶ **Table 2**; HR-ESI-MS *m/z* 291.1602 $[M + CH_3COO]^-$ (calcd 291.1596 for $C_{17}H_{23}O_4$).

NF-κB transactivation activity

NF-κB activity was evaluated in HEK293/NF-κB-luc cells (HEK293 cells stably transfected with a NF-κB-responsive luciferase reporter gene Panomics, RC0014) as previously described [33,34]. Cells were loaded with CTG CMFDA (C2925; Invitrogen), a fluorescent probe that is retained inside living cells [11,35], and 4×10^4 cells/well were seeded in 96-well plates. After 24-h incubation, cells

were pretreated as indicated for 30 min and activated with TNF-α (2 ng/mL, Sigma; T-6676) for 4 h. Cells were then lysed with luciferase lysis buffer (Promega; E1531), the luminescence of the firefly luciferase and the fluorescence of stained cells with CTG (also: CMFDA, 5-chloromethylfluorescein diacetate) were quantified with a GeniosPro plate reader (Tecan). The luciferase signal resulting from the NF-κB reporter activation was normalized by the CTG-derived fluorescence to account for potential differences in cell number. The impact of cytotoxicity on cell numbers was evaluated by comparing fluorescence of the cells treated by the solvent vehicle with that of cells treated with the indicated samples. Parthenolide (Sigma-Aldrich; P0667 ≥ 98% HPLC), a known NF-κB inhibitor, was used as positive control (10 µM). Results were normalized to the solvent control DMSO (0.1%).

Statistical analysis and calculation of IC_{50} values

Statistical analysis and nonlinear regression were performed using Prism software (version 4.03; GraphPad Software Inc). To calculate the IC_{50} values, data were curve fitted and non-linear transformed using a sigmoidal dose response with variable slope. Results of bioassay data are expressed as the mean ± standard deviation (SD) of at least three independent experiments performed in quadruplicate. Results of IC_{50} values are expressed with 95% confidence interval (CI). Dunnett's test was used for statistical analyses ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant).

Supporting information

Data regarding the inhibition of the NF-κB transactivation activity by pooled fractions 1–10 are shown in **Fig. 1S** of the supporting information. Also spectroscopic and spectrometric data for the identification of known compounds (**1–3, 5, 7–14**, and **17–18**) are provided in the supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

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