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The inhibition of TNF- α induced NF- κ B activation by marine natural products

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1
2 **Abstract**
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7 The deregulated activation of NF- κ B is associated with cancer development and
8 inflammatory diseases. With the aim to find new NF- κ B inhibitors, we purified and
9 characterized compounds from extracts of the Fijian sponge *Rhabdastrella globostellata*, the
10 crinoid *Comanthus parvicirrus*, the soft corals *Sarcophyton* sp. nov. and *Sinularia* sp., and the
11 gorgonian *Subergorgia* sp. after an initial screening of 266 extracts from different marine
12 origins.
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21 Results obtained show that selected purified compounds had a cytotoxic effect on the human
22 leukaemia cell line K562, inhibited both TNF- α -induced NF- κ B-DNA binding as well as
23 TNF- α induced I κ B α degradation and nuclear translocation of p50/p65. Furthermore, we
24 observed inhibition of NF- κ B activation induced by an overexpression of TNFR1, TRAF2,
25 TRADD, or IKK β . Interestingly, natural products inhibited IKK β kinase as well as the 26S
26 proteasome proteolytic activity.
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42 **Keywords:**
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44 NF- κ B; marine natural product; anti-cancer drug discovery
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1. Introduction

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5 Nuclear factor κ B (NF- κ B) is an inducible transcription factor that regulates the transcription
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7 of genes involved in a wide range of immune and inflammatory cell functions [1, 2]. While
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9 normal activation of NF- κ B is required for cell survival and immunity, it is now well
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11 established that the deregulated activation of NF- κ B is associated with cancer, inflammation,
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13 and a wide range of other pathologies [3-11]. Because of its severe implications in a large
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15 number of diseases, NF- κ B has recently become a major target in drug discovery [4, 7, 8, 12].
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18 In non-stimulated cells, NF- κ B is sequestered in the cytoplasm in an inactive form, bound to
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20 the NF- κ B inhibitor I κ B [13]. Several factors are known to activate NF- κ B, including the
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22 exposure to cytokines, to carcinogens, or to physical or oxidative stress [14]. The extra-
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24 cellular signals converge on the TNF- α receptor1 (TNFR1), and trigger the recruitment of the
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26 TNFR1-associated death domain protein (TRADD) and of the TNF-receptor-associated factor
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28 2 protein (TRAF2) to TNFR1. This leads to the ubiquitination of the receptor-interacting
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30 protein (RIP) and to the activation of the transforming growth factor β (TGF β)-activated
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32 kinase 1 (TAK1) and of the I κ B kinase complex IKK (kinase of I κ B). Upon phosphorylation
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34 by IKK, I κ B is rapidly degraded and NF- κ B is released to translocate to the nucleus, where it
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36 binds to its target DNA sequences to initiate gene transcription [1, 3, 4, 8, 15-19].
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42 A large number of natural and synthetic compounds are currently being investigated for NF-
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44 κ B inhibitory activity [20-22]. Marine natural products, in particular, have recently been
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46 recognized as a promising source of NF- κ B inhibitors [20, 23, 24]. With the aim to find new
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48 potent NF- κ B inhibitors, we screened 220 extracts from Fijian marine organisms including
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50 algae, sponges, cnidarians, ascidians, and echinoderms collected by the Institute of Applied
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52 Sciences (IAS) Marine Collection (University of the South Pacific, Suva, Fiji) between 1997
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54 and 2003. Since marine microorganisms have become widely recognized as a particularly
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1 promising source of novel bioactive natural products [25-28], we also screened 43 extracts
2 from microalgae and cyanobacteria provided by the Culture Collection of Algae and Protozoa
3 (CCAP) (Dunstaffnage Marine Laboratory, Oban, U.K.) and from bacteria isolated from
4 Scottish and Costa Rican marine sediments and cultured in our laboratories. Notably, marine
5 microorganisms are rich sources of polyunsaturated fatty acids (PUFA) and of carotenoids,
6 which are likely to interfere with the activation of NF- κ B through their strong anti-oxidant
7 activity [29-34].

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9 The relationship between the taxonomy, the habitat, and the metabonomics of the source
10 organisms and the NF- κ B inhibitory potential of the crude extracts included in our large-scale
11 screening is discussed. This type of information can be of extremely high value in the
12 development of more efficient and more sustainable sampling and screening strategies in NF-
13 κ B-related drug discovery. A first insight into the relationship between the metabonomics of
14 marine organisms and the NF- κ B inhibitory potential of the secondary metabolites produced
15 by the latter was obtained by analyzing the fatty acid (FA) composition of the microalgae
16 included in our screening by gas chromatography (GC).

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18 Five extracts from Fijian marine organisms with strong NF- κ B inhibitory activity were
19 selected based on the availability of supplementary biomass and purified by assay-guided
20 fractionation in order to identify the chemical structure and the mechanism of action of the
21 compounds responsible for the bioactivity of the source extracts. The five selected samples
22 include the extracts isolated from a crinoid (*Comanthus parvicirrus*, FJ01-199), from a
23 sponge (*Rhabdastrella globostellata*, FJ97125D114), from two soft corals (*Sarcophyton* sp.
24 nov., FJ03-147 and *Sinularia* sp., FJ01-111), and from a gorgonian (*Subergorgia* sp., FJ01-
25 160). Preliminary results on the NF- κ B-inhibitory potential of the two naphthopyrones 6-
26 methoxy-comaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) isolated from an
27 extract of the crinoid *Comanthus parvicirrus* (FJ01-199) were presented by Folmer *et al.* in
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2008 [35]. As described in [35], both naphthopyrones (1) and (2) inhibit TNF- α -induced NF- κ B activation in a dose-dependent manner (MIC = 300 μ M), and their mechanisms of action involve the inhibition of the enzymatic activity of the kinase IKK β and of the 26S proteasome. Additional results on the mechanism of action of (1) and (2) downstream of I κ B degradation are included in the present paper, together with the results obtained on the mechanisms of action of the bioactive compounds isolated from the four remaining extracts FJ97125D114, FJ03-147, FJ01-111, and FJ01-160.

2. Materials and methods

2.1. Chemicals and biological material

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Unless specified otherwise, all the chemicals were purchased from Sigma-Aldrich (Bornem, Belgium). RPMI 1640 cell culture medium and the antibiotic-antimycotic solution were purchased from Bio-Whittaker (Fisher Scientific) (Tournai, Belgium). Foetal calf serum (FCS) was purchased from Perbio (Erembodegem, Belgium). TNF- α was dissolved at 10 μ g/mL in 1x PBS supplemented with 0.5% (w/v) BSA according to the manufacturer's instructions and stored at 4°C until being used. Synthetic astaxanthin (Sigma-Aldrich (Bornem, Belgium)) (catalogue # A9335) was dissolved in DMSO at a concentration of 20 mg/mL and stored in the dark at 4°C until being used. The chemical solvents (HPLC-grade) used for chromatography were purchased from Rathburn Chemicals (Glasgow, U.K.). NMR solvents were purchased from Sigma-Aldrich (Poole, U.K.).

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Methanol extracts from 220 marine organisms collected around the Fiji islands (18°00'N, 175°00'E) between 1997 and 2003, including 102 sponges, 9 ascidians, 12 echinoderms, 81 cnidarians, and 16 algae, were kindly provided by the IAS Marine Collection of the

1 University of the South Pacific, Suva (Fiji). The extracts (1 to 5 mg, D.W.) were prepared in
2 DMSO at a concentration of 20 mg/mL for the biological assays. Strains of bacteria were
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4 isolated from marine sediment collected in 2004-2005 at 0 to 20 m depth along the coast of
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6 Dunstaffnage Bay, Oban (U.K.) (56°27'N, 5°25'W) and along the coast of Costa Rica
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8 (Gandoca-Manzanillo Wildlife Reserve, Caribbean Sea (9°37'N, 82°36'W), Cahuita National
9
10 Park, Caribbean Sea (9°43'N, 82°48'W), Las Baulas Marine National Park, Pacific Ocean
11
12 (9°34'N, 85°07'W), and Cabo Blanco Natural Absolute Reserve, Pacific Ocean (10°18'N,
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14 85°50'W)), using a grab sampler or a shovel depending on the collection depth. The sediment
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16 samples were maintained on ice during the transportation to the laboratory and stored at 4°C
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18 until processing. The sediment samples were inoculated in 10⁻¹ to 10⁻³ dilutions on marine
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20 agar (Scottish samples) or on M1 agar (10g soluble starch, 4g yeast extract, 2g peptone, 18g
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22 agar, 33g Instant Ocean[®] sea salt (Spectrum Brands, Inc. (Salzbach, Germany)) per litre of
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24 distilled water, pH 7.6) supplemented with 10 µg/mL nalidixic acid (Sigma-Aldrich
25
26 (Louisville, MO, U.S.A.)) and 50 µg/mL nystatin (Sigma-Aldrich (Louisville, MO, U.S.A.))
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28 (Costa Rican samples) and cultured at room temperature. The phylogenetic analysis of the
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30 bacteria was performed by Gram staining and by 16S rDNA sequencing followed by FASTA
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32 (European Bioinformatics Institute) database search [36]. The 16S rDNA sequencing was
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34 performed by Dr. David Green at the SAMS Dunstaffnage Marine Laboratory (Oban, U.K.)
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36 and by Macrogen Inc. (Seoul, Korea) (Scottish samples), by Godofredo Solano at INBio
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38 (Santo Domingo de Heredia, Costa Rica) (Costa Rican samples), using the 27f primer
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40 (5'AGAGTTTGATCMTGGCTCAG3') (INBio (Santo Domingo de Heredia, Costa Rica))
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42 and an ABI BigDye[™] terminator system on an Applied Biosystems[®] sequencer (Applied
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44 Biosystems (Foster City, CA, U.S.A.)). Strains of the marine cyanobacterium *Phormidium*
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46 *foveolarum* originating from Corsica, France (CCAP 1446/8) and of the freshwater
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48 cyanobacterium *Fischerella muscicola* originating from Allahabad, India (CCAP 1427/1)
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were obtained from the Culture Collection of Algae and Protozoa (CCAP) at the SAMS Dunstaffnage Marine Laboratory (Oban, U.K.) and cultured in Provasoli-enriched artificial seawater (ASW) medium and in BG11 medium, respectively (protocols from the CCAP online catalogue available at www.sams.ac.uk). Strains of the microalgae *Chlorococcum submarinum* (Chlorophyta) originating from brackish water in Merseyside, U.K. (CCAP 213/10), *Chlamydomonas raudensis* (Chlorophyta) originating from Lake Bonney, Antarctica (CCMP 1619, from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (West Boothbay Harbor, ME, U.S.A.), *Chlamydomonas noctigama* (Chlorophyta) originating from a meadow lake in the former Czechoslovakia (CCAP 11/17), and *Chlamydomonas hedleyi* (Chlorophyta) originating from seawater in the Florida Keys, U.S.A. (ATCC 50216, from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) were obtained from CCAP. CCMP 1619 is identical to SAG 49.72 at the Culture Collection of Algae (SAG) of the University of Göttingen (Göttingen, Germany) and to UW0241 at the University of Washington Culture Collection (UW) in Seattle, WA (U.S.A.), and CCAP 11/17 is also known as UTEX 2289 at the Culture Collection of Algae of the University of Texas, Austin (UTEX) in Austin, TX (U.S.A.). The microalgae were cultured in 3N-BBM+W culture medium (protocol from the CCAP catalogue) at 20°C, except for CCMP 1619, which was cultured at 4°C.

2.2. Analysis of the fatty acid composition of the microalgae included in the large-scale screening

The fatty acids present in the methanol extracts from the microalgae included in our screening for NF- κ B inhibitors were converted to fatty acid methyl esters (FAMES) as described in [37]. Briefly, 5 mg of extract were dissolved in 1 mL of toluene. 2 mL of 1% (v/v) sulfuric acid in

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methanol were added, and the samples were heated for two hours at 100°C in sealed tubes. On cooling, 5 mL of water with 5% (w/v) NaCl were added, and the formed esters were extracted in hexane. Prior to gas chromatography (GC) analysis, the esters were purified by thin layer chromatography on silica G60 plates (Merck Sharp & Dohme Ltd. (Hoddesdon, U.K.)), using hexane/ether/glacial acetic acid 78:20:2 as solvent. The GC analysis was performed on a Shimadzu GC-2014 gas chromatograph (equipped with a split injector and flame ionization detector (Shimadzu Europe, Ltd. (Manchester, U.K.))). Separation was carried out using a polar Zebron ZB-WAX column (30 m x 0.25 mm, 0.25 µm) (Phenomenex, Macclesfield, U.K.), using helium (BOC (Aberdeen, U.K.)) as the carrier gas. The oven temperature was programmed to increase from 160°C to 240°C at a rate of 4°C min⁻¹ and then to hold for 10 min. at 240°C. Data acquisition and analysis were performed using the Shimadzu GC Solution software (Shimadzu Europe, Ltd. (Manchester, U.K.))). Individual fatty acids were identified by reference to authentic GC standards (Supelco 37 component FAME mix (Sigma-Aldrich, Poole, U.K.))).

2.3. Purification and structure elucidation of natural products isolated from methanol extracts from Fijian marine invertebrates

Approximately 1.5 g each of extract from five Fijian marine invertebrates, including the crinoid *Comanthus parvicirrus* (FJ01-199), the sponge *Rhabdastrella globostellata* (FJ97125D114), the soft corals *Sarcophyton* sp. nov. (FJ03-147) and *Simularia* sp. (FJ01-111), and the gorgonian *Subergorgia* sp. (FJ01-160) were provided by the Institute of Applied Sciences Marine Collection (University of the South Pacific, Suva, Fiji). The extracts were purified by assay-guided fractionation using liquid-liquid partitioning (water-methanol-dichloromethane-hexane) followed by high-performance liquid chromatography (HPLC).

1 HPLC separations were carried out using a Gemini reversed-phase HPLC column (C₁₈, 250 x
2 10 mm, 5 μm) (Phenomenex, Macclesfield, U.K.) and an HP 1100 HPLC system (Agilent
3 Technologies, Waldbronn, Germany) with diode array detector, semi-preparative binary pump
4 system, methanol/water gradients). The chemical structures of the isolated compounds were
5 elucidated by nuclear magnetic resonance (NMR) (¹H, ¹³C, ¹H-¹H COSY, HMBC, and
6 HSQC; 400 MHz; CDCl₃) (Varian Ltd. (Oxford, U.K.)), UV, and low and high resolution
7 mass spectroscopy (MS) (Agilent Technologies, Waldbronn, Germany). The purified
8 compounds were stored as dry powder or oil at 4 °C and prepared in DMSO at a concentration
9 of 20 mg/mL for the biological assays.
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24 **2.4. Cancer cell culture**

25 K562 (human chronic myelogenous leukaemia) and Jurkat (T cell leukaemia) cells (Deutsche
26 Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) Braunschweig, Germany)
27 were cultured in RPMI 1640 cell culture medium supplemented with 10% (v/v) FCS and 1%
28 (v/v) antibiotic-antimycotic solution at 37°C and 5% of CO₂.
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39 **2.5. Transient transfection and luciferase reporter gene assay**

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43 Transient transfections of K562 cells were performed as described previously [38]. Five μg of
44 luciferase reporter gene construct containing 5 repeats of a consensus NF-κB site (Stratagene
45 (La Jolla, CA, U.S.A.) and 5 μg *Renilla* luciferase plasmid (Promega, Leiden, Netherlands)
46 were used for each pulse. Following electroporation, the cells were resuspended in growth
47 medium (RPMI 1640 with 10% (v/v) FCS) and incubated at 37°C and 5% of CO₂. Twenty
48 hours after transfection, the cells were harvested and resuspended in growth medium (RPMI
49 1640 with 10% (v/v) FCS) to a final concentration of 10⁶ cells/mL and treated for two hours
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with the crude extracts or the isolated natural products at different test concentrations. The cells were then challenged with 20 ng/mL TNF- α for two hours. 75 μ l Dual-GloTM Luciferase Reagent (Promega, Leiden, Netherlands) were added to the cells for a 10 min. long incubation at 22 °C before firefly luciferase activity was measured. Then, 75 μ l Dual-GloTM Stop & Glo[®] Reagent (Promega, Leiden, Netherlands) were added for 10 min. at 22°C in order to assay *Renilla* activity. Firefly and *Renilla* luciferase activities were measured using an Orion microplate luminometer (Berthold, Vilvoorde, Belgium) 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. The luciferase reporter gene assays were performed as double-blinds (*i.e.* without knowing the nature and the origin of the test samples).

2.6. Cytotoxicity assay

The effects of the crude extracts and of the purified natural products on the viability of K562 test cells during the reporter gene assay were assessed using a CellTiterGlo[®] kit (Promega, Leiden, Netherlands) as described in the manufacturer's protocol.

2.7. Effects of the test compounds on the enzymatic activity of luciferase

The effects of the crude extracts and of the purified natural products on the enzymatic activity of luciferase were assessed by incubating 100 μ L of QuantiLum Recombinant firefly luciferase (Promega, Leiden, Netherlands), diluted 10⁵ times in a 10% BSA solution, with the test compounds at the concentration used in the luciferase assays, for 1h at room temperature.

The enzymatic activity of luciferase at the end of the treatment was measured on a

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luminometer after an addition of 100 μ L of luciferin substrate (SteadyGlo[®] reagent, Promega, Leiden, Netherlands).

2.8. Electrophoretic mobility shift assay (EMSA)

Jurkat cells were resuspended in growth medium (RPMI 1640 with 10% (v/v) FCS) to a final concentration of 10^6 cells/mL and treated for two hours at 37°C and 5% of CO₂ with the test compounds at various concentrations. The cells were then challenged with 20 ng/mL TNF- α for two hours at 37°C and 5% of CO₂. Nuclear extracts were prepared using the method described by Müller *et al.* [39] and stored at -80°C. The oligonucleotide NF- κ B c (consensus NF- κ B site) (5'-AGTTGAGGGGACTTCCAGGC-3') and its complementary sequence (Eurogentec, Seraing, Belgium) were used as probe. The probe was hybridized and labeled with ³²P ATP (MP-Biomedicals, Brussels, Belgium) and the EMSA was performed as published before [39]. Briefly, 10 μ g of nuclear extract was incubated in binding buffer with the ³²P ATP labeled probe for 20 min. The DNA-protein complexes were analyzed by electrophoresis on a 4% native polyacrylamide gel using Tris-glycine buffer, and visualized by autoradiography.

In order to investigate the direct interference, within the nucleus, of the test compounds with the binding of activated NF- κ B to its DNA binding site, 10 μ g of nuclear extract from Jurkat cells treated for two hours with 20 ng/mL TNF- α at 37°C and 5% of CO₂ were incubated for 20 min. on ice with the test compounds prepared in binding buffer at a 10³-fold dilution compared to the MIC observed in the EMSA. The nuclear extracts were then incubated with the ³²P ATP labeled probe for a further 20 min. on ice, and the DNA-protein complexes were analyzed by electrophoresis as described above.

1 In the immuno-depletion experiments, the nuclear extracts and labeled probes were incubated
2 in the reaction mixture for 30 min. on ice prior to a 30 min. incubation on ice with 2 μg of
3 antibodies. All antibodies (anti-p50, anti-p65, anti c-Rel and anti-RelB) were purchased from
4 Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).
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10 11 **2.9. Western blot analysis**

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16 To determine the effects of the test compounds on $\text{TNF}\alpha$ -dependent $\text{I}\kappa\text{B}\alpha$ degradation and on
17 $\text{TNF}\alpha$ -dependent translocation of p65 and p50 into the nucleus, cytosolic and nucleic protein
18 extracts were prepared as previously described [38]. Briefly, 2×10^6 cells/mL were pre-
19 treated with the test compounds at a concentration of 100 $\mu\text{g}/\text{mL}$ for 2 hours and then exposed
20 to 20 ng/mL of $\text{TNF-}\alpha$ for various amounts of time. Ten μg of protein extract were resolved
21 on a 10% SDS-PAGE gel, transferred onto a membrane, blocked with 5% non-fat milk, and
22 probed with specific antibodies against $\text{I}\kappa\text{B}\alpha$, p65, and p50 (Santa Cruz Biotechnology (Santa
23 Cruz, CA, U.S.A.)). Specific antibodies against lamin C (Vision Biosystems Novacastra
24 (Zaventem, Belgium)) and against tubulin- α (Calbiochem (San Diego, CA, U.S.A.)) were used
25 to verify the purity of the cytoplasmic and nuclear extracts and to confirm equal protein
26 loading in each well. The blots were washed, exposed to horseradish peroxidase-conjugated
27 secondary antibodies for one hour and finally detected by ECL reagent (GE Healthcare,
28 Diegem, Belgium). The levels of protein expression (band area \times mean band intensity) were
29 quantified using the software Adobe Photoshop Elements version 2.0 (Adobe Systems Inc.
30 (U.S.A.)).
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54 55 56 **2.10. Fluorescence microscopy**

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Jurkat cells were prepared at a concentration of 10^6 cells/mL in RPMI 1640 cell culture medium with 10% (v/v) FCS. The test compounds were prepared in DMSO at a concentration of 100 mg/mL. 1 μ L of the solution was added to 1 mL of cell suspension to achieve a final concentration of 100 μ g/mL. The mixtures were incubated for two hours at 37°C, with 5% CO₂. After the incubation period, the cells were centrifuged for 5 minutes at 1.2 rcf, washed twice with PBS 1x, and resuspended in 1 mL of PBS 1x supplemented with 5 μ L of Hoechst staining solution (bisbenzimidazole H33258 fluorochrome trichloride) (BD Biosciences (Erembodegem, Belgium)). After 10 minutes of incubation with the Hoechst staining solution in the dark and at room temperature, the cell suspensions were placed onto glass slides and analyzed under an inverted fluorescence microscope (Leica Microsystems (Wetzlar, Germany)) (1000 x magnification; excitation filters: excitation/emission filter cubes: 340-380 nm (UV excitation) - 400-425 nm (blue emission) and 450-490 nm (blue excitation) - 510-515 nm (green emission)). The background fluorescence of Jurkat cells was assessed using non-treated cells. The images taken with the different filters were merged using the ImageJ software [40].

2.11. Co-transfection experiments

The effects of the test compounds on TNFR1-, TRADD-, TRAF2-, and IKK β -induced NF- κ B activation was analysed by luciferase reporter gene assay as described above, using K562 cells transfected with the reporter gene plasmids pNF κ B-consensus and pRhLuc, as well as with either of the pTNFR1, pTRADD, pTRAF2, or pIKK β expression vector plasmids. The pTNFR1, pTRADD, pTRAF2, and pIKK β expression vector plasmids were generous gifts from Prof. Bharat Aggarwal (University of Texas M.D. Anderson Cancer Research Centre, Houston, TX, U.S.A.). Each compound was tested in two independent experiments.

2.12. IKK β protein kinase assays

The effects of the test compounds on the kinase activity of IKK β were investigated using a K-LISA IKK β inhibition assay kit (Calbiochem, San Diego, CA, U.S.A.) as described in the manufacturer's protocol. The IKK β inhibitor Calbio IV provided with the K-LISA kit was used (at a concentration of 1 μ M) as a positive control. Each compound was tested in three independent experiments.

2.13. Effects of the test compounds on the chymotrypsin-like protease activity of the 26S proteasome

The effects of the test compounds on the chymotrypsin-like protease activity of the 26S proteasome were evaluated using a Proteasome-GloTM assay kit (Promega, Leiden, Netherlands). 100 μ L of K562 leukaemia cells prepared at a concentration of 2×10^5 cells/mL in RPMI 1640 culture medium supplemented with 10% FBS were treated for two hours at 37°C with 100 μ g/mL of the test compounds. 100 μ L of Proteasome-GloTM reagent containing luciferase and the proteasome substrate succinyl-leucine-leucine-valine-tyrosine-aminoluciferin were then added to each well, and after 10 min. of shaking at room temperature the luminescence was measured on a luminometer. The proteasome-inhibiting peptide aldehyde MG-132 (Calbiochem (San Diego, CA, U.S.A.)) was used as a positive control. A viability assay using the Cell-Titer Glo[®] kit (Promega, Leiden, Netherlands) as described in the manufacturer's protocol was run in parallel with the proteasome assay in order to standardize the results of the proteasome assay to the number of live cells in each sample at the end of the assay. Each compound was tested in four independent experiments.

2.14. Statistical analysis

The results presented in this paper were obtained from at least two independent experiments.

The data were analyzed for statistical significant differences using the Student's t-test. The results are expressed as the mean \pm SD. P-values below 0.05 (*) or 0.01 (**) were considered as statistically significant.

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3. Results

3.1. Large-scale screening of marine natural products for NF- κ B inhibitory activity

The large-scale screening of extracts from marine organisms was performed using luciferase reporter gene assays. In parallel with the luciferase reporter gene assays, all the samples were screened for their cytotoxicity on the K562 test cells over the incubation period of the luciferase reporter gene assays. All the experiments were performed at a single test concentration of 100 μ g/mL, and in a double-blind manner. Taxonomic and bioactivity data were only exchanged after completion of the screening assays. The quantitative results of the large-scale screening were arbitrarily translated to a qualitative scale, based on observations made during the investigation of the NF- κ B inhibitory potential of kava derivatives [41]. Extracts achieving a ≤ 1.2 fold (17%) decrease in NF- κ B-induced luciferase activity, compared to the negative control, are classified as neutral in terms of NF- κ B inhibitory activity. Extracts achieving a 1.2-1.8 fold (17-44%) decrease in NF- κ B-induced luciferase activity, compared to the negative control, are classified as mild inhibitors of NF- κ B activation. Extracts achieving a 1.8-2.3 fold (44-57%) decrease in NF- κ B-induced luciferase activity, compared to the negative control, are classified as substantial inhibitors of NF- κ B activation. Extracts achieving a ≥ 2.3 fold (57%) decrease in NF- κ B-induced luciferase activity, compared to the negative control, are classified as strong inhibitors of NF- κ B activation. Samples classified as strong NF- κ B-inhibitors were evaluated for their *in vitro* effects on the enzymatic activity of luciferase. The extracts that, during the time frame of the screening assays, were shown to induce $\geq 25\%$ cell death or to provoke $\geq 25\%$ decrease in enzymatic activity of luciferase, in comparison with the negative control, have been grouped together as samples leading to false positive results in the luciferase reporter gene assay used

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for the large-scale screening. Samples enhancing the TNF- α induced activation of NF- κ B by $\geq 25\%$ are classified as inducers of NF- κ B. The results of the large-scale screening, at a phylum level, are shown in figure 1. The genus and strain-specific results of the screening can be found in the supplementary material. As shown in figure 1, 18% of the tested extracts strongly inhibited TNF- α induced NF- κ B activity at a concentration of 100 μ g/mL. The most active groups of marine organisms included in the screening were the algae (40% hit rate of strong (≥ 2.3 fold decrease) NF- κ B inhibitory activity), the chordates (34% hit rate), and the bacteria (33% hit rate). Three of the bacterial extracts, all isolated from *Pseudomonas* sp. strains, were shown to stimulate TNF- α induced NF- κ B activity at the test concentration of 100 μ g/mL.

3.2. Fatty acid composition of the microalgae screened for NF- κ B-inhibitory activity

The fatty acid (FA) composition of the microalgae included in the large-scale screening for NF- κ B inhibitors is presented in table 1.

As shown in table 1, there is little difference in FA composition between the extracts from the *Chlorococcum* strain and the extracts from the *Chlamydomonas* strains. All the extracts contain high amounts (up to 81% of the organisms' total FA composition) of the three saturated fatty acids palmitic acid (16:0), stearic acid (18:0), and myristic acid (14:0). The only noticeable difference is that the *Chlorococcum* extract is proportionally richer in myristic (14:0) and palmitic (16:0) acid than the *Chlamydomonas* extracts.

At an *intra*-genus level, on the other hand, more important differences in FA composition can be observed. Amongst the extracts from the three *Chlamydomonas* strains, the Antarctic strain CCMP 1619 is the only one that accumulates the ω -3 poly-unsaturated fatty acid (PUFA) linolenic acid in high concentrations (12% of the organism's total FA composition). CCMP

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1619 and CCAP 11/17 accumulate large amounts of ω -9 mono-unsaturated FAs oleic acid (18:1) and nervonic acid (24:1), respectively, while ATCC 50216 is the only one to accumulate high levels (14%) of saturated heptadecanoic acid (17:0). The three *Chlamydomonas* strains included in the study accumulate similar concentrations of ω -6 PUFA linoleic acid (18:2) (0 to 4% of the organisms' total FA composition) and of ω -9 mono-unsaturated FA eicosenoic acid (20:1) (2 to 6% of the organisms' total FA composition).

3.3. Chemical identity and NF- κ B-inhibitory potential of marine natural products isolated from Fijian extracts shown to inhibit TNF- α -induced transcriptional activity of NF- κ B.

Extracts from five Fijian marine invertebrates, including the crinoid *Comanthus parvicirrus* (FJ01-199), the sponge *Rhabdastrella globostellata* (FJ97125D114), the soft corals *Sarcophyton* sp. nov. (FJ03-147) and *Sinularia* sp. (FJ01-111), and the gorgonian *Subergorgia* sp. (FJ01-160), were kindly provided by the IAS Marine Collection (University of the South Pacific, Suva, Fiji) for further investigation of the chemical structure and mechanism of action of the bioactive compounds. Following the bioassay-guided purification of the extracts by chromatography, the chemical structures of the major bioactive compounds were elucidated by NMR, UV, and mass spectrometry. The extract from the crinoid *Comanthus parvicirrus* yielded two major bioactive compounds, namely the naphthopyrones 6-methoxycomaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) previously identified by Smith and Sutherland [42]. The extract from the sponge *Rhabdastrella globostellata* yielded the three bioactive triterpenoids stelletin A (**3**), stelletin B (**4**), and stelliferin riboside (**5**) previously isolated from various species of marine sponges [43-47]. The two known cembranoids 3,4-epoxy,13-oxo,7*E*,11*Z*,15-cebratriene (**6**) and 3,4-epoxy,13-oxo,7*E*,11*E*,15-

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cembratriene (7) [48] were identified as the major bioactive compounds of the extract isolated from the soft coral *Sarcophyton* sp. nov. The major bioactive compound isolated from the extract of the gorgonian *Subergorgia* sp. was identified as astaxanthin (8), a carotenoid produced by several marine bacteria and algae and accumulated from their diet by wide range of marine organisms [31, 49, 50]. Because of the difficulty to isolate pure astaxanthin (8) from the *Subergorgia* extract, synthetic astaxanthin (8) was tested in parallel with the impure natural astaxanthin (8). The extract from the soft coral *Sinularia* sp. yielded the cyclodepsipeptide jaspilakinolide (9) as the major bioactive compound. Jaspilakinolide (9) is normally isolated from sponges, rather than from soft corals [51], suggesting that the *Sinularia* sp. extract included in our study was probably contaminated with spongean material on which the soft coral had been encrusted.

The effects of the marine natural products 1-9 on TNF- α -induced transcriptional activity of NF- κ B was examined using a luciferase reporter gene assay on pNF- κ BLuc K562 cells. The results are presented in figure 2. As shown in figure 2, none of the tested compounds has any effect on basal NF- κ B transcription. All the compounds reduce TNF- α induced NF- κ B activation in a concentration-dependent manner. All the compounds, except (5), achieved a strong decrease in the transcriptional activity of NF- κ B at a concentration of 100 μ g/mL, which is equivalent to 216 μ M for (3) and (4), 331 μ M for (6) and (7), 168 μ M for both natural and synthetic (8), and 141 μ M for (9). (5) showed little NF- κ B inhibitory activity, even at the highest test concentration of 100 μ g/mL, which corresponds to 159 μ M. Due to its poor bioactivity in the luciferase reporter gene assay, (5) was discarded from the remaining bioassays.

3.4. Cytotoxic effect of NF- κ B-inhibiting natural products isolated from Fijian marine organisms on the human leukaemia cell line K562

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2 The viability of K562 cells exposed to 100 $\mu\text{g}/\text{mL}$ of the Fijian marine natural products **1-4**
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4 and **6-9** over the 4 hours long test period of the luciferase reporter gene assay is shown in
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6 figure **2K**. The results indicate that none of the tested compounds severely affects the viability
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8 of K562 leukaemia cells in the test conditions of the luciferase reporter gene assay, and
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10 cytotoxicity of the test compounds can be excluded as a potential source of false positive
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12 results.
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19 **3.5. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine organisms** 20 21 **on the enzymatic activity of luciferase**

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26 The effects of the Fijian marine natural products **1-4** and **6-9** on the enzymatic activity of
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28 luciferase are shown in figure 2K. The results indicate that none of the tested Fijian marine
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30 natural products severely affects the enzymatic activity of luciferase at the concentrations at
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32 which a strong decrease in luciferase activity was observed in the luciferase reporter gene
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34 assays (100 $\mu\text{g}/\text{mL}$), confirming that the results obtained in the luciferase assay are unbiased
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36 by effects of the test compounds on the enzymatic activity of luciferase.
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44 **3.6. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine organisms** 45 46 **on TNF- α -induced NF- κ B-DNA binding**

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51 The inhibition of TNF- α -induced NF- κ B activation by the Fijian marine natural products **3-4**
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53 and **6-9** was confirmed by EMSA in Jurkat T-cells. As shown in figure 3, all the tested
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55 compounds achieve complete inhibition of TNF- α -induced binding of NF- κ B to its DNA
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57 binding site at a concentration of 100 $\mu\text{g}/\text{mL}$, which corresponds to 216 μM for **(3)** and **(4)**,
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1 331 μ M for (6) and (7), 168 μ M for (8), and 141 μ M for (9). At a concentration of 50 μ g/mL,
2 the inhibition is either very weak, or completely absent.
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4 In order to investigate the direct interferences, at the nuclear level, of compounds 1-4 and 6-9
5 with the binding of activated NF- κ B to DNA, Jurkat cells were treated with TNF- α in order to
6 induce the activation of NF- κ B. The nuclear extracts were incubated with the test compounds
7 prepared in binding buffer. The nuclear extracts from the treated cells were incubated with the
8 32 P ATP-labeled NF- κ B c probe, and the DNA-protein complexes were analyzed by
9 electrophoresis as described above. As shown in figure 4, 1-4, (6), (7), and (9), but not (8)
10 (natural or synthetic) have a direct inhibitory effect on the binding of NF- κ B to its DNA
11 binding site. Since inhibiting the transcriptional activity of NF- κ B by targeting the binding of
12 NF- κ B to its DNA binding site requires the access of the bioactive molecule into the cells,
13 and into the nuclei, we analyzed whether the NF- κ B-inhibiting Fijian marine natural products
14 were capable of penetrating into the nucleus of Jurkat cells, using fluorescent microscopy. As
15 shown in figure 5, all the marine natural products tested in the present survey are naturally
16 fluorescent. Yellow or green fluorescence is, as a matter of fact, a widespread property of
17 marine natural products [52]. In terms of chemical ecology, the fluorescence of marine
18 secondary metabolites provides cellular and tissue protection against UV radiation, and
19 fluorescent natural products are frequently used by numerous marine organisms for
20 underwater signaling [52]. The fluorescence of the compounds was used to illustrate that the
21 test compounds are able to permeate Jurkat leukaemia cells and to penetrate into the latter's
22 nuclei in less than two hours. Background fluorescence of untreated cells was negligible
23 compared to the fluorescence emitted by the treated cells (figure 5).
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3.7. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine organisms on TNF- α induced I κ B α degradation and on TNF- α induced nuclear translocation of p50/p65

In order to further investigate the effects of the Fijian marine natural products **3**, **4**, and **6-9** on the TNF- α induced activation of NF- κ B, Western blot analyses of cytosolic and nucleic protein extracts from Jurkat cells pre-treated with the Fijian marine natural products for two hours and treated with TNF- α for different amounts of time were performed. The blotted membranes were analyzed using primary antibodies against I κ B α , p50, and p65. The results show that all the tested Fijian marine natural products prevent TNF- α -induced degradation of I κ B α and inhibit the nuclear translocation of p50 and p65 (figure 6).

3.8. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine organisms on NF- κ B activation induced by an overexpression of TNFR1, TRAF2, TRADD, or IKK β

In order to further investigate the mechanism of action of the marine NF- κ B inhibitors **1-4** and **6-9**, co-transfection-based luciferase reporter gene assays were performed. K562 leukaemia cells were co-transfected with the pNF κ B-Luc and phRL-TK plasmids, together with either one of the pTNFR1, pTRAF2, pTRADD, or pIKK β plasmids. As shown in figure 7A, at the MIC value observed in the EMSA (100 μ g/mL), none of the tested Fijian marine natural products significantly inhibits the NF- κ B activity induced by the over-expression of the TNF- α receptor TNFR1, or of the TRAF2 or TRADD proteins. However, at the same test concentration of 100 μ g/mL, **(1)** and **(7)** significantly ($p < 0.01$) inhibited the NF- κ B activity

1 induced by the over-expression of IKK β . (2), (4), (6) and (9) were shown to be weak ($p<0.05$)
2 inhibitors of IKK β -induced NF- κ B activation. (8) did not have any substantial effect on the
3 NF- κ B activation induced by an overexpression of IKK β .
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10 **3.9. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine organisms** 11 **on the kinase activity of IKK β** 12 13

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17 The potential of the NF- κ B inhibitors 1-4 and 6-9 to inhibit the kinase activity of IKK β was
18 assessed using an IKK β kinase assay. The results indicate that all the tested compounds
19 inhibit the kinase activity of IKK β in a concentration-dependent manner. Nevertheless, the
20 IKK β inhibitory potential of (3) and (4), and of (8) was very weak, even at the highest test
21 concentration (200 μ g/mL). (2), (6), and (9) only induced a significant ($p<0.01$) inhibition of
22 IKK β at, and above the test concentrations of 200 μ g/mL (606 μ M), 100 μ g/mL (331 μ M),
23 and 200 μ g/mL (282 μ M), respectively. (1) and (7) were the strongest IKK β inhibitors
24 amongst the tested compounds. (1) and (7) inhibited IKK β in a significant ($p<0.01$) manner at
25 a concentration of 50 μ g/mL, which corresponds to 145 μ M for (1) and to 165 μ M for (7)
26 (figure 7B). These results are in agreement with the results obtained in the luciferase reporter
27 gene assay performed with K562 cells co-transfected with a pIKK β expression vector plasmid
28 (figure 7A).
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51 **3.10. Effects of NF- κ B-inhibiting natural products isolated from Fijian marine** 52 **organisms on the proteolytic activity of the 26S proteasome** 53 54 55 56 57 58 59 60 61 62 63 64 65

1 The last component of the NF- κ B activation pathway investigated as a potential target for the
2 NF- κ B inhibiting natural products isolated from Fijian marine organisms was the 26S
3 proteasome, which is known to degrade poly-ubiquitinated I κ B, thereby releasing the active
4 p50/p65 dimer for nuclear translocation. The results indicate that, at the MIC value observed
5 in the EMSA (100 μ g/mL), (**1**) was the only tested compound shown to significantly ($p < 0.01$)
6 inhibit the proteolytic activity of the 26S proteasome. The proteasome-inhibiting potential of
7 (**1**) (1.6 fold decrease in proteolytic activity at a test concentration of 303 μ M) is, however,
8 very weak in comparison with the commercially available proteasome-inhibiting peptide
9 aldehyde MG-132 [53], which was shown decrease the proteolytic activity of the 26S
10 proteasome by a 10 fold at a concentration of 10 μ M) (figure 7C).
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4. Discussion

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5 The aim of the present study was to search for novel inhibitors of NF- κ B, a transcription
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7 factor that has recently become a major target in drug discovery due to its severe implications
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9 in cancer development, inflammation, and many other diseases [7, 8, 54, 55]. Out of the 263
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11 extracts from marine organisms screened for their potential to inhibit TNF- α induced NF- κ B
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13 activation, 18% had a strong NF- κ B inhibitory potential at a concentration of 100 μ g/mL.
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15 This high hit rate does not come as a surprise, as marine natural products have recently been
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17 recognized as promising candidates for NF- κ B inhibition [20, 56, 57]. The two NF- κ B-
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19 inhibiting marine natural products macrolide bryostatin 1 isolated from a bryozoan [56] and
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21 salinosporamide A isolated from a bacterium [24] are currently in clinical trials [58]. Several
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23 ecological reasons why marine natural products may inhibit NF- κ B have been established
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25 [20, 21, 59-69].
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32 In our study, the most abundant sources of NF- κ B inhibitors were algae, chordates, and
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34 bacteria. The algae not only had the highest hit rate in terms of percentage of extracts with
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36 strong NF- κ B inhibitory potential, they were also the source of extracts with the highest NF-
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38 κ B inhibitory strength at the single test concentration of 100 μ g/mL. Within most phyla, a
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40 good correlation between the taxonomy of the source organisms and the NF- κ B inhibitory
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42 potential of the extracts could be observed. In order to gain further insight into the possible
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44 correlations between the taxonomy, metabonomics, and ecological properties of the source
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46 organisms and the NF- κ B inhibitory potential of the extracts, we evaluated the FA
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48 composition of the microalgal samples included in the screening. Algae are known to be rich
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50 in FAs [70-72], and their FA composition can, to a certain extent, be used as fingerprints for
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52 their taxonomy or for the environmental conditions of their habitat [32, 34, 70]. Little
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54 difference could be seen between the FA composition of the two genera. Among the
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Chlamydomonas samples, however, noticeable differences in the FA composition could be observed. While the three tested strains of *Chlamydomonas* are almost indistinguishable in terms of their phylogeny [73], they originate from very different ecosystems. The Antarctic strain CCMP 1619 was the only one to accumulate the ω -3 PUFA linolenic acid (18:3) at a high concentration (12% of the total FA composition). The concentrations of ω -6 PUFA linoleic acid (18:2) and of the ω -9 mono-unsaturated FA eicosenoic acid (20:1) were similar from one species to another. Our results are in accord with Poerschmann *et al.*, who showed that the unsaturation index of FAs in algae and bacteria tends to be higher in colder climates than in warmer climates [70, 74]. ω -3 PUFAs, but not ω -6 PUFAs, mono-unsaturated FAs or saturated FAs, have been shown to be potent NF- κ B inhibitors [75-80]. One would hence have expected the CCMP 1619 extract, which has a twelve times higher concentration in ω -3 PUFA than the remaining extracts, to stand out as the strongest NF- κ B inhibitor. However, our results disagreed with this hypothesis, as all the microalgal extracts strongly inhibited TNF- α induced NF- κ B activation, without any strong correlation between the bioactivity and the taxonomy of the source organisms or the FA composition of the extracts. A hypothetical explanation which has not been evaluated in our study, for the overall strong NF- κ B inhibitory activity of the tested microalgal extracts, could be an ubiquitously high concentration of NF- κ B-inhibiting carotenoids. High concentrations of carotenoids, and of astaxanthin in particular, have been previously reported in *Chlamydomonas*, *Chlorococcum*, and other microalgae [50, 73, 81], and most carotenoids are potent antioxidants [30] [82], which renders them capable of interfering with NF- κ B activation [83].

The high hit rate observed for chordate-derived extracts is novel and highly promising, as the only natural products isolated from this phylum and reported as NF- κ B inhibitors to date is the quinone 3-demethylubiquinone Q₂ from the ascidian *Aplidium glabrum* [84].

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At the end of the large-scale screening, five extracts from the IAS Marine Collection (University of the South Pacific, Suva, Fiji) were selected to pursue an in-depth investigation of the chemical characteristics and of the mechanisms of action of the natural products responsible for the NF- κ B-inhibitory activity observed in the crude extracts. Together, the extracts yielded eight compounds with diverse chemical structures which inhibited TNF- α induced NF- κ B activation in a concentration-dependent manner, with MIC values as low as 141 μ M. All the bioactive compounds except asthaxanthin (**8**) were shown to directly interfere with the binding of NF- κ B to DNA. Upstream of the nuclear translocation of NF- κ B, IKK β was shown to be the major molecular target for most of the tested compounds. The naphthopyrones 6-methoxy-comaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) isolated from the echinoderm *Comanthus parvicirrus* and the isomalabaricanes stelletin A (**3**) and stelletin B (**4**) isolated from the sponge *Rhabdastrella globostellata* possess lactone rings with α,β -unsaturated carbonyl groups which may be responsible for the latter's interferences with IKK β and with the binding of NF- κ B to DNA as described previously for other NF- κ B-inhibiting natural products [21]. 6-methoxy-comaparvin-5-methyl ether (**1**) was the only compound shown to strongly inhibit the proteolytic activity of the 26S proteasome at the test concentration of 100 μ g/mL. The carotenoid astaxanthin (**8**) isolated from the gorgonian *Subergorgia* sp., as well as its synthetic counterpart, were the only NF- κ B-inhibiting compound included in the our study for which no molecular target could be pinpointed. Most likely, the mechanism of action of the NF- κ B inhibitor astaxanthin (**8**) is through its strong antioxidant potential, although the latter hypothesis was not tested in the present study. Astaxanthin is, indeed, a very potent antioxidant and free-radical scavenger [30, 82, 85], and oxidative stress is known to be implicated in the activation of NF- κ B [83, 86, 87].

We conclude that marine algae, bacteria and invertebrates are a highly promising and hitherto largely unrecognized source of NF- κ B inhibitors. Furthermore, NF- κ B-inhibiting marine

1 natural products target a wide range of molecules along the NF- κ B activation pathway.
2 Hence, marine natural products can be viewed as a treasure-case for anticancer, anti-
3 inflammatory, and other NF- κ B-related drug discovery. Unfortunately, marine drug
4 discovery, especially when based on marine invertebrate-derived natural products, has been
5 dogged since its early days by the difficulty to supply sufficient biomaterial for drug
6 development [20]. Our results, which show that the cultured algae and bacteria are amongst
7 the richest sources of NF- κ B inhibitors, provide some hope that this hurdle will soon become
8 surmountable, as large amounts of active biomass of these organisms can be obtained through
9 culture and fermentation. Further extracts identified in our large-scale screening assay as
10 sources of NF- κ B inhibitors are currently being investigated in order to determine the
11 chemical and biological properties of the natural products responsible for the NF- κ B
12 inhibitory activity observed in the crude extracts.
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References

- 1
2 [1] Garcia-Pineros AJ, Lindenmeyer MT, Merfort I. Role of cysteine residues of p65/NF-
3 kappa B on the inhibition by the sesquiterpene lactone parthenolide and-N-ethyl
4 maleimide, and on its transactivating potential. *Life Sciences* 2004;75:841-56.
5
6 [2] Mayo MW, Baldwin AS. The transcription factor NF-kappa B: control of oncogenesis
7 and cancer therapy resistance. *Biochimica et Biophysica Acta-Reviews on Cancer*
8 2000;1470:M55-M62.
9
10 [3] Soysa NS, Alles N. NF-κB functions in osteoclasts. *Biochemical and Biophysical*
11 *Research Communications* 2009;378:1-5.
12 [4] Baud V, Karin M. Is NF-κB a good target for cancer therapy? Hopes and pitfalls.
13 *Nature Reviews Drug Discovery* 2009;8:33-40.
14 [5] Ghosh S, Hayden MS. New regulators of NF-κB in inflammation. *Nature Reviews*
15 *Immunology* 2008;8:837-48.
16 [6] Ocio E, Mateos MV, Maiso P, Pandiella A, San-Miguel JF. New drugs in multiple
17 myeloma: mechanisms of action and phase I/II clinical findings. *Lancet* 2008;9:1157-
18 65.
19 [7] Sakar F, Li Y. NF-κB: a potential target for cancer chemoprevention and therapy.
20 *Frontiers in Bioscience* 2008;13:2950-9.
21 [8] Aggarwal BB, Takada Y, Shishodia S, Gutierrez AM, Oommen OV, Ichikawa H, et
22 al. Nuclear transcription factor NF-kappa B: role in biology and medicine. *Indian*
23 *Journal of Experimental Biology* 2004;42:341-53.
24 [9] Karin M. Nuclear factor-kB in cancer development and progression. *Nature*
25 2006;441:431-6.
26 [10] Senftleben U, Karin M. The IKK/NF-kappa B pathway. *Critical Care Medicine*
27 2002;30:S18-26.
28 [11] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB
29 functions as a tumour promoter in inflammation-associated cancer. *Nature*
30 2004;431:461-6.
31 [12] Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death--a
32 new approach to cancer therapy. *J Clin Invest* 2005;115:2625-32.
33 [13] Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: A treasure trove for
34 drug development. *Nature Reviews Drug Discovery* 2004;3:17-26.
35 [14] Garg A, Aggarwal BB. Nuclear transcription factor-kappa B as a target for cancer
36 drug development. *Leukemia* 2002;16:1053-68.
37 [15] Pande V, Sharma RK, Inoue JI, Otsuka M, Ramos MJ. A molecular modeling study of
38 inhibitors of nuclear factor kappa-B (p50) - DNA binding. *Journal of Computer-Aided*
39 *Molecular Design* 2003;17:825-36.
40 [16] Sun ZW, Andersson R. NF-kappa b activation and inhibition: A review. *Shock*
41 2002;18:99-106.
42 [17] Blonska M, Shameshakar P, Kobayashi M, Zhang D, Sakurai H, Su B, et al. TAK1 is
43 recruited to the tumor necrosis factor-alpha (TNF-alpha) receptor 1 complex in a
44 receptor-interacting protein (RIP)-dependent manner and cooperates with MEKK3
45 leading to NF-kappa B activation. *Journal of Biological Chemistry* 2005;280:43056-
46 63.
47 [18] Shim J, Changchun X, Paschal A, Bailey S, Rao P, Hayden MS, et al. TAK1, but not
48 TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes*
49 *& Development* 2005;19:2668-81.
50 [19] Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives.
51 *Trends in Cell Biology* 2001;11:372-7.
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [20] Folmer F, Jaspars M, Diederich M. Marine natural products as targeted modulators of
2 the transcription factor NF- κ B. *Biochemical Pharmacology* 2008;75:603-17.
- 3 [21] Bremner P, Heinrich M. Natural products as targeted modulators of the nuclear factor-
4 kappa B pathway. *Journal of Pharmacy and Pharmacology* 2002;54:453-72.
- 5 [22] Zambre AP, Kulkarni V, Padhye S, Sandur SK, Aggarwal BB. Curcumin analogs
6 targeting TNF-induced NF- κ B activation and proliferation in human leukemic KBM-5
7 cells. *Bioorganic and Medicinal Chemistry* 2006;14:7196-204.
- 8 [23] Amigo M, Paya M, Braza-Boils A, De Rosa M, Terencio MC. Avarol inhibits TNF-
9 alpha generation of NF-kappaB activation in human cells and in animal models. *Life
10 Sciences* 2008;82:256-64.
- 11 [24] Ahn KS, Sethi G, Chao TH, Neuteboom ST, Chaturvedi MM, Palladino MA, et al.
12 Salinosporamide A (NPI-0052) potentiates apoptosis, suppresses osteoclastogenesis,
13 and inhibits invasion through down-modulation of NF- κ B-regulated gene products.
14 *Blood* 2007;110:2286-95.
- 15 [25] Jensen PR, Mincer TJ, Williams PG, Fenical W. Marine actinomycete diversity and
16 natural product discovery. *Antonie Van Leeuwenhoek* 2005;87:43-8.
- 17 [26] Kobayashi J, Ishibashi M. Bioactive metabolites of symbiotic marine microorganisms.
18 *Chemical Reviews* 1993;93:1753-69.
- 19 [27] Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR. Marine natural products.
20 *Natural Product Reports* 2006;23:26-78.
- 21 [28] Jaspars M, Lawton L. Cyanobacteria - a novel source of pharmaceuticals. *Current
22 Opinions on Drug Discovery and Development* 1998;1:77-84.
- 23 [29] Cardozo KH, Guaratini T, Barros MP, Falcao VR, Tonon AP, Lopes NP, et al.
24 Metabolites from algae with economical impact. *Comparative Biochemistry and
25 Physiology C: Toxicology & Pharmacology* 2006;29:29.
- 26 [30] Naguib YM. Antioxydant activities of astaxanthin and related carotenoids. *Journal of
27 Agricultural and Food Chemistry* 2000;48:1150-4.
- 28 [31] Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara A, Saito T, et al. Structure
29 and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and
30 astaxanthin biosynthetic pathway proposed at the gene level. *Journal of Bacteriology*
31 1995;177:6575-84.
- 32 [32] Berge JP, Barnathan G. Fatty acids from lipids of marine organisms: molecular
33 biodiversity, roles as biomarkers, biologically active compounds, and economical
34 aspects. *Advances in Biochemical Engineering and Biotechnology* 2005;96:49-125.
- 35 [33] Patnayak S, Sree A. Screening of bacterial associates of marine sponges for single cell
36 oil and PUFA. *Letters on Applied Microbiology* 2005;40:358-63.
- 37 [34] Zhukova NV, Aizdaicher NA. Fatty acid composition of 15 species of marine
38 microalgae. *Phytochemistry* 1995;39:351-6.
- 39 [35] Folmer F, Harrison W, Tabudravu J, Jaspars M, Aalbersberg W, Feussner K, et al.
40 NF- κ B inhibiting naphthopyrones from the Fijian echinoderm *Comanthus parvicirrus*.
41 *Journal of Natural Products* 2008;71:106-11.
- 42 [36] Pearson WR. Rapid and sensitive sequence comparison with FASTP and FASTA.
43 *Methods in Enzymology* 1990;183:63-98.
- 44 [37] Christie W. Preparation of ester derivatives of fatty acids for chromatographic
45 analysis. *In: Advances in lipid methodology - Two*. pp69-111. Dundee: Oily Press,
46 1993.
- 47 [38] Duvoix A, Delhalle S, Blasius R, Schnekenburger M, Morceau F, Fougere M, et al.
48 Effect of chemopreventive agents on glutathione S-transferase P1-1 gene expression
49 mechanisms via activating protein 1 and nuclear factor kappaB inhibition.
50 *Biochemical Pharmacology* 2004;68:1101-11.
- 51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [39] Müller MM, Schreiber E, Schaffner W, Matthias P. Rapid test for in vivo stability and
2 DNA-Binding of mutated octamer binding-proteins with mini-extracts prepared from
3 transfected cells. *Nucleic Acids Research* 1989;17:6420-.
- 4 [40] Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. *Biophotonics*
5 *International* 2004;11:36-42.
- 6 [41] Folmer F, Blasius R, Morceau F, Tabudravu J, Dicato M, Jaspars M, et al. Inhibition
7 of TNF α -induced activation of nuclear factor κ B by kava (*Piper methysticum*)
8 derivatives. *Biochemical Pharmacology* 2006;71:1206-18.
- 9 [42] Smith IR, Sutherland MD. Pigments of Marine Animals. 11. Angular naphthopyrones
10 from crinoid *Comanthus parvicirrus Timorensis*. *Australian Journal of Chemistry*
11 1971;24:1487-9.
- 12 [43] Lv F, Deng Z, Li J, Fu H, van Soest RW, Proksch P, et al. Isomalabaricane-type
13 compounds from the marine sponge *Rhabdastrella aff. distincta*. *Journal of Natural*
14 *Products* 2004;67:2033-6.
- 15 [44] McCormick JL, McKee TC, Cardellina JH, 2nd, Leid M, Boyd MR. Cytotoxic
16 triterpenes from a marine sponge, *Stelletta* sp. *Journal of Natural Products*
17 1996;59:1047-50.
- 18 [45] Su JY, Meng YH, Zeng LM, Fu X, Schmitz FJ. Stelletin A, a new triterpenoid
19 pigment from the marine sponge *Stelletta tenuis*. *Journal of Natural Products*
20 1994;57:1450-1.
- 21 [46] Tasdemir D, Mangalindan GC, Concepcion GP, Verbitski SM, Rabindran S, Miranda
22 M, et al. Bioactive isomalabaricane triterpenes from the marine sponge *Rhabdastrella*
23 *globostellata*. *Journal of Natural Products* 2002;65:210-4.
- 24 [47] Tabudravu JN, Jaspars M. Stelliferin riboside, a triterpene monosaccharide isolated
25 from the Fijian sponge *Geodia globostellifera*. *Journal of Natural Products*
26 2001;64:813-5.
- 27 [48] Ravi BN, Faulkner DJ. Cembranoid diterpenes from a South Pacific soft coral. *Journal*
28 *of Organic Chemistry* 1978;43:2127-31.
- 29 [49] Osterlie M, Bjerkgeng B, Liaaen-Jensen S. Accumulation of astaxanthin all-*E*, 9*Z*, and
30 13*Z* geometrical isomers and 3 and 3' *R S* optical isomers in rainbow trout
31 (*Oncorhynchus mykiss*) is selective. *Journal of Nutrition* 1999;129:391-8.
- 32 [50] Liu BH, Lee YK. Composition and biosynthetic pathways of carotenoids in the
33 astaxanthin-producing green alga *Chlorococcum* sp. *Biotechnology Letters*
34 1999;21:1007-10.
- 35 [51] Crews P, Manes LV, Boehler M. Jaspilakinolide, a cyclodepsipeptide from the marine
36 sponge *Jaspis* sp. *Tetrahedron Letters* 1986;27:2797-800.
- 37 [52] Bandaranayake W. The nature and role of pigments of marine invertebrates. *Natural*
38 *Product Reports* 2006;23:223-55.
- 39 [53] Banerjee D, Liefshitz A. Potential of the proteasome inhibitor MG-132 as an
40 anticancer agent, alone and in combination. *Anticancer Research* 2001;21:3941-7.
- 41 [54] Haefner B. NF-kappa B: arresting a major culprit in cancer. *Drug Discovery Today*
42 2002;7:653-63.
- 43 [55] Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention
44 and treatment of cancer. *Cancer Letters* 2004;206:193-9.
- 45 [56] Terracciano S, Rodriquez M, Aquino M, Monti MC, Casapullo A, Riccio R, et al.
46 Chemistry and biology of anti-inflammatory marine natural products: molecules
47 interfering with cyclo-oxygenase, NF- κ B and other unidentified targets. *Current*
48 *Medicinal Chemistry* 2006;13:1947-69.
- 49 [57] Haefner B. Drugs from the deep: marine natural products as drug candidates. *Drug*
50 *Discovery Today* 2003;8:536-44.
- 51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [58] Molinski T, Dalisay DS, Lievens SL, Saludes JP. Drug development from natural
2 products. *Nature Reviews Drug Discovery* 2009;8:69-85.
- 3 [59] Escoubas JM, Briant L, Montagnani C, Hez S, Devaux C, Roch P. Oyster IKK-like
4 protein shares structural and functional properties with its mammalian homologues.
5 *FEBS Letters* 1999;453:293-8.
- 6 [60] Romano G, Russo GL, Buttino I, Ianora A, Miralto A. A marine diatom-derived
7 aldehyde induces apoptosis in copepod and sea urchin embryos. *Journal of*
8 *Experimental Biology* 2003;206:3487-94.
- 9 [61] Lisowska K, Witkowski JM. Viral strategies in modulation of NF-kappaB activity.
10 *Archivum Immunologiae et Therapiae Experimentalis* 2003;51:367-75.
- 11 [62] Hiscott J, Kwon H, Genin P. Hostile takeovers: viral appropriation of the NF-kappa B
12 pathway. *Journal of Clinical Investigation* 2001;107:143-51.
- 13 [63] Gauthier MJ, Degnan BM. The transcription factor NF-kappaB in the demosponge
14 *Amphimedon queenslandica*: insights on the evolutionary origin of the Rel homology
15 domain. *Development Genes and Evolution* 2008;218:23-32.
- 16 [64] Merlo E, Freudenthal R, Romano A. The I kappa B kinase inhibitor sulfasalazine
17 impairs long-term memory in the crab *Chasmagnathus*. *Neuroscience* 2002;112:161-
18 72.
- 19 [65] Povelones M, Tran K, Thanos D, Ambron RT. An NF-kappaB-like transcription factor
20 in axoplasm is rapidly inactivated after nerve injury in *Aplysia*. *Journal of*
21 *Neurosciences* 1997;17:4915-20.
- 22 [66] Wang XW, Tan NS, Ho B, Ding JL. Evidence for the ancient origin of the NF-kB/IkB
23 cascade: Its archaic role in pathogen infection and immunity. *Proceedings of the*
24 *National Academy of Sciences of the USA* 2006;103:4204-9.
- 25 [67] Muller WE, Gamulin V, Rinkevich B, Spreitzer I, Weinblum D, Schroder HC.
26 Ubiquitin and ubiquitination in cells from the marine sponge *Geodia cydonium*.
27 *Biological Chemistry Hoppe-Seyler* 1994;375:53-60.
- 28 [68] Schlezinger JJ, Blickarz CE, Mann KK, Doerre S, Stegeman JJ. Identification of NF-
29 kappaB in the marine fish *Stenotomus chrysops* and examination of its activation by
30 aryl hydrocarbon receptor agonists. *Chemical and Biological Interactions*
31 2000;126:137-57.
- 32 [69] Pancer Z, Rast JP, Davidson EH. Origins of immunity: transcription factors and
33 homologues of effector genes of the vertebrate immune system expressed in sea urchin
34 coelomocytes. *Immunogenetics* 1999;49:773-86.
- 35 [70] Colombo ML, Rise P, Giavarini F, De Angelis L, Galli C, Bolis CL. Marine
36 macroalgae as sources of polyunsaturated fatty acids. *Plant Foods for Human*
37 *Nutrition* 2006;61:67-72.
- 38 [71] Bhakoo M, Herbert RA. Fatty acid and phospholipid composition of five
39 psychrotrophic *Pseudomonas* spp. grown at different temperatures. *Archives in*
40 *Microbiology* 1980;126:51-5.
- 41 [72] Harwood J, Pettitt T, Jones A. Lipid metabolism. *In: Biochemistry of algae and*
42 *cyanobacteria*. Oxford: Oxford University Press, 1988.
- 43 [73] Pocock T, Lachance MA, Proeschold T, Priscu JC, Kim SS, Huner NPA.
44 Identification of a psychrophilic green alga from Lake Bonney Antarctica:
45 *Chlamydomonas raudensis* Ettl. (UWO 241) Chlorophyceae. *Journal of Phycology*
46 2004;40:1138-48.
- 47 [74] Poerschmann J, Spijkerman E, Langer U. Fatty Acid Patterns in *Chlamydomonas* sp.
48 as a Marker for Nutritional Regimes and Temperature under Extremely Acidic
49 Conditions. *Microbial Ecology* 2004;48:78-89.
- 50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [75] Novak T, Babcock T, Jho D, Helton W, Espat N. NF-kappaB inhibition by omega -3
2 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. *American*
3 *Journal of Physiology* 2003;284:84-9.
- 4 [76] Okamoto M, Misunobu F, Ashida K. Effects of dietary supplementation with omega-3
5 fatty acids compared with omega-6 fatty acids on bronchial asthma. *International*
6 *Medicine* 2000;39:107-11.
- 7 [77] Aronson W, Glaspy J, Reddy S, Reese D, Heber D, Bagga D. Modulation of omega-
8 3/omega-6 polyunsaturated fatty acid ratios with dietary fish oils in men with prostate
9 cancer. *Urology* 2001;58:283-8.
- 10 [78] Cheng W, Lii C, Chen H, Lin TT, Liu K. Contribution of conjugated linoleic acid to
11 the suppression of inflammatory responses through the NF-kappaB pathway. *Journal*
12 *of Agricultural and Food Chemistry* 2004;52:71-8.
- 13 [79] Lee J, Zhao L, Youn H, Weatherill A, Tapping R, Feng L, et al. Saturated fatty acid
14 activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with
15 Toll-like receptor 6 or 1. *Journal of Biological Chemistry* 2004;279:16971-9.
- 16 [80] Mishra A, Chandhary A, Sethi S. Oxidized omega-3 fatty acids inhibit NF-kappa B
17 activation via a PPARalpha-dependent pathway. *Arteriosclerosis, thrombosis, and*
18 *vascular biology* 2004;24:1621-7.
- 19 [81] Liu BH, Lee YK. Effect of total secondary carotenoids extracts from *Chlorococcum* sp
20 on *Helicobacter pylori*-infected BALB/c mice. *International Immunopharmacology*
21 2003;3:979-86.
- 22 [82] Takamatsu S, Hodges TW, Rajbhandari I, Gerwick WH, Hamann MT, Nagle DG.
23 Marine natural products as novel antioxidant prototypes. *Journal of Natural Products*
24 2003;66:605-8.
- 25 [83] Bowie A, O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: a
26 reassessment of the evidence in the light of recent discoveries. *Biochemical*
27 *Pharmacology* 2000;59:13-23.
- 28 [84] Fedorov S, Radchenko O, Shubina L, Balaneva N, Bode A, Stonik VA, et al.
29 Evaluation of Cancer-Preventive Activity and Structure-Activity Relationships of 3-
30 Demethylubiquinone Q2, Isolated from the Ascidian *Aplidium glabrum*, and its
31 Synthetic Analogs. *Pharmaceutical Research* 2006;23:70-81.
- 32 [85] Shahidi F, Metusalach B, Brown J. Carotenoid pigments in seafood and aquaculture.
33 *Critical Reviews in Food Science* 1998;38:1-67.
- 34 [86] Bours V, Bonizzi G, Bentires-Alj M, Bureau F, Piette J, Lekeux P, et al. NF-kappaB
35 activation in response to toxic and therapeutic agents: role in inflammation and
36 cancer treatment. *Toxicology* 2000;153:27-38.
- 37 [87] Shrivastava A, Aggarwal BB. Antioxidants differentially regulate activation of
38 Nuclear Factor-kB, Activator Protein-1, c-Jun Amino-Terminal Kinases, and
39 apoptosis induced by Tumor Necrosis Factor: Evidence that JNK and NF-kB
40 activation are not linked to apoptosis. *Antioxidants & Redox Signaling* 1999;1:181-91.
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Fig. 1. Distribution of NF- κ B inhibitory activity and cytotoxicity of crude extracts across different phyla of marine organisms, as listed in table 2. The screening for NF- κ B inhibitory activity, using a luciferase reporter gene assay, includes a total of 263 crude extracts (A) isolated from marine organisms belonging to the phyla Porifera (102 representatives) (B), Cnidaria (81 representatives) (C), Echinodermata (12 representatives) (D), Chordata (9 representatives) (E), Chloro-, Phaeo-, and Rhodophyta (grouped under Algae) (20 representatives) (F), or Firmicutes, Bacteroidetes, Cyano-, Actino-, or Proteobacteria (grouped under Bacteria) (39 representatives) (G). Each extract was assayed in duplicates, at a single test concentration of 100 μ g/mL. The cytotoxicity and the effects of the extracts on the enzymatic activity of luciferase were assessed at the same test concentration. Strong NF- κ B inhibition is defined as ≥ 2.3 fold decrease in NF- κ B induced luciferase activity, in comparison with control cells. Substantial NF- κ B inhibition refers to a 1.8-2.3 fold decrease in luciferase activity. Mild NF- κ B inhibition refers to a 1.2-1.8 fold decrease in luciferase activity. Neutral in terms of NF- κ B inhibitory activity refers to a ≤ 1.2 fold decrease in luciferase activity. A cytotoxic sample is defined as a sample inducing $\geq 25\%$ cell death, in comparison with the control. An inhibitor of the enzymatic activity of luciferase is defined as an inducer of $\geq 25\%$ decrease in enzymatic activity of luciferase, in comparison with the control. Extracts that enhanced the TNF- α induced NF- κ B activity by $\geq 25\%$ are classified as NF- κ B inducers.

Fig. 2. NF- κ B-inhibitory potential of marine natural products isolated from Fijian extracts shown to inhibit TNF- α -induced transcriptional activity of NF- κ B. Luciferase activity of pNF- κ BLuc K562 cells pre-treated for 2 hours with different concentrations (in μ g/mL) of 6-methoxy comaparvin-5-methyl ether (ComME) (A), 6-methoxy comaparvin (Com) (B),

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 stellettin A (StA) (C), stellettin B (StB) (D), stelliferin riboside (E), 3,4-epoxy,13-oxo,7E,11Z,15-cembratriene (CemEZ) (F), 3,4-epoxy,13-oxo,7E,11E,15-cembratriene (CemEE) (G), natural/synthetic astaxanthin (AstN/AstS) (H/I), or jasplakinolide (Jasp) (J), and treated for 2 hours with 20 ng/mL of TNF- α . The results in A-J 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 in A-J refers to the transcription activity of NF- κ B in absence of any test compound. Effects of the test compounds, at a concentration of 100 μ g/mL, on the viability of the K562 test cells and on the enzymatic activity of luciferase over the incubation period of the luciferase reporter gene assay, in comparison with the effects of the compounds on NF- κ B-induced luciferase gene expression under the same treatment conditions as for A-J (K). Results shown as mean + S.D. of eight individual measurements. * and ** refer to $p < 0.05$ and $p < 0.01$, respectively, in comparison with the control.

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Fig. 3. Inhibition of TNF- α -induced NF- κ B-DNA binding by NF- κ B-inhibiting natural products isolated from Fijian marine organisms. Jurkat cells were pre-treated for two hours with different concentrations (in μ g/mL) of stellettin A (A), stellettin B (B), 3,4-epoxy,13-oxo,7E,11Z,15-cembratriene (C), 3,4-epoxy,13-oxo,7E,11E,15-cembratriene (D), natural astaxanthin (E), synthetic astaxanthin (F), or jasplakinolide (G), before being treated for two hours with 20 ng/mL of TNF- α . For the immunodepletion assay, the nuclear extracts and labelled probes were incubated in the reaction mixture for 30 min. on ice with 2 μ g of anti-p50, anti-p65, anti-c-Rel, and anti-Rel-B antibodies (H).

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Fig. 4. Direct interferences of stellettin A (StA), stellettin B (StB), 6-methoxy-comaparvin-5-methyl ether (ComME), 6-methoxy-comaparvin (Com), 3,4-epoxy,13-oxo,7E,11Z,15-cembratriene (CemEZ), 3,4-epoxy,13-oxo,7E,11E,15-cembratriene (CemEE), natural

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astaxanthin (AstN), synthetic astaxanthin (AstS), and jasplakinolide (Jasp) with the binding of activated NF- κ B to DNA. Jurkat cells were treated with 20 ng/mL of TNF- α for two hours. 10 μ g of nuclear extract were incubated for 20 min. with 5 μ L of binding solution containing 100 ng/mL of test compound, followed by a 20 min. long incubation with the radioactively labeled probe. The control (Contr.) refers to nuclear extracts treated with no test compound.

Fig. 5. Fluorescent microscopy based visualization of the distribution of stellettin A (**A**), stellettin B (**B**), 6-methoxy-comaparvin-5-methyl ether (**C**), 6-methoxy-comaparvin (**D**), 3,4-epoxy,13-oxo,7*E*,11*Z*,15 cembratriene (**E**), 3,4-epoxy,13-oxo,7*E*,11*E*,15 cembratriene (**F**), and jasplakinolide (**G**) in Jurkat leukaemia cells after a two hours long incubation with the test compounds at a concentration of 100 μ g/mL. The background fluorescence of untreated Jurkat cells is shown as a reference (**H**). Column I: Jurkat cells permeated by the naturally fluorescent test compounds (340-380 nm excitation filter, 400-425 nm emission filter). Column II: Visualisation of the Hoechst-stained cellular nuclei (450-490 nm excitation filter, 510-515 nm emission filter). Column III: Merge of the image in column I with the image in column II. Each slide corresponds approximately to an area of 10x10 μ m².

Fig. 6. Effect of stellettin A (**3**), stellettin B (**4**), 3,4-epoxy,13-oxo,7*E*,11*Z*,15 cembratriene (**6**), 3,4-epoxy,13-oxo,7*E*,11*E*,15 cembratriene (**7**), natural astaxanthin (**8**), and jasplakinolide (**9**) on TNF- α -induced I κ B α degradation and nuclear translocation of NF- κ B in Jurkat leukaemia cells. Jurkat cells (2×10^6 cells/mL) were incubated for 2 hours at 37°C (5% CO₂) with 100 μ g/mL of the test compounds. The cells were then treated with 20 ng/mL TNF- α and incubated at 37°C (5% CO₂) for the indicated times. The cytosolic and nuclear extracts were tested by Western blot analysis for the presence of I κ B α , p50, and p65. Equal protein loading and purity of cytosolic/nuclear extracts were evaluated by tubulin- α (cytosolic) and

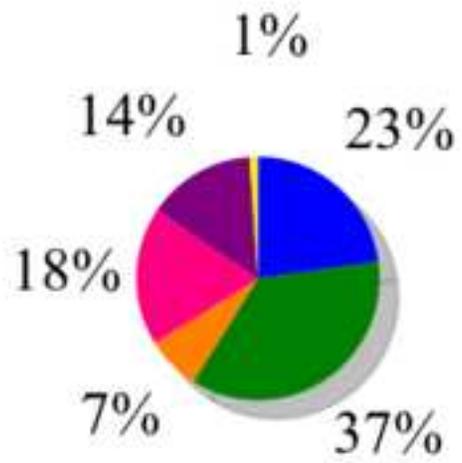
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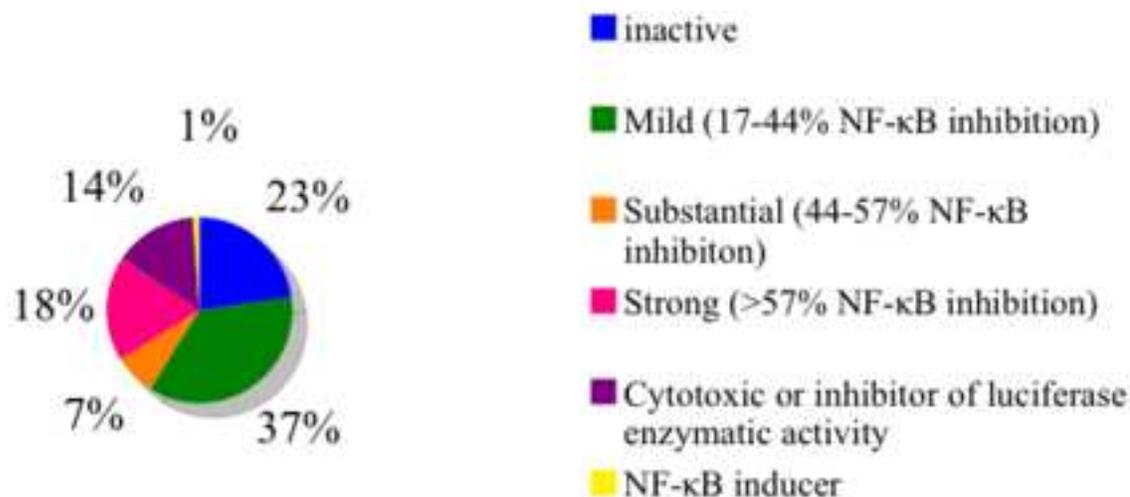
lamin C (nuclear) Western blots. One of two independent experiments is depicted. The level of protein expression (band area x mean band intensity; shown in bold under the individual Western blots) is given as a ratio over the expression of tubulin α (cytosolic extracts) or of lamin C (nuclear extracts).

Fig. 7. Effects of stelletin A (StA), stelletin B (StB), 6-methoxy-comaparvin-5-methyl ether (ComME), 6-methoxy-comaparvin (Com), 3,4-epoxy,13-oxo,7E,11Z,15-cembratriene (CemEZ), 3,4-epoxy,13-oxo,7E,11E,15-cembratriene (Cem7EE), natural astaxanthin (AstN), or jaspalakinolide (Jasp) on NF- κ B activation induced by the over-expression of TNFR1, TRAF2, TRADD, or IKK β in K562 cells (**A**), on the kinase activity of IKK β (**B**), or on the proteolytic activity of the 26S proteasome in K562 cells (**C**) at the indicated test concentrations. In (**A**), the results are given as a ratio of the luminescence measured for the firefly luciferase divided by the luminescence measured for the *Renilla* luciferase, and the negative control (Contr.) refers to the transcription activity of NF- κ B in absence of any test substance. In (**B**), (N.E.) refers to a negative control in absence of IKK β , while (Contr.) refers to a negative control in presence of IKK β , but in absence of any test compound. Calbio IV[®] (CAL) (1 μ M) was used as a positive control. In (**C**), the results are shown as a ratio of the firefly luciferase activity measured in the proteasome assay over the firefly luciferase activity measured in a viability assay run in parallel with the proteasome assay. The negative control refers to the proteolytic activity of the 26S proteasome in absence of any test compound. MG132 (Z-Leu-Leu-Leu-CHO) (2 to 100 μ M) was used as a positive control. All the results shown as mean + S.D. of at least two individual measurements. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, compared to the negative control.

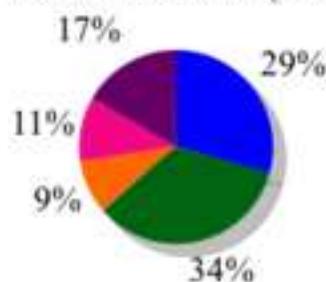
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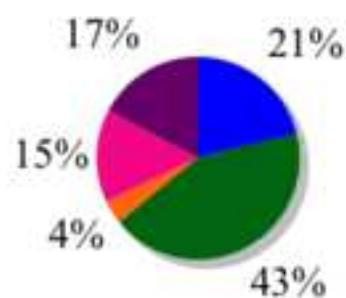




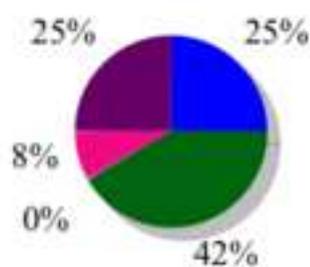
(A) Total collection (263 samples)



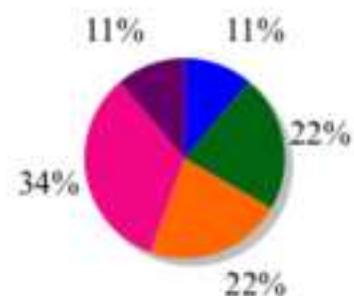
(B) Porifera (102 samples)



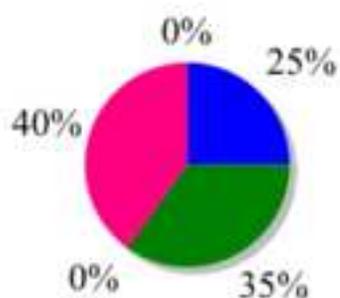
(C) Cnidaria (81 samples)



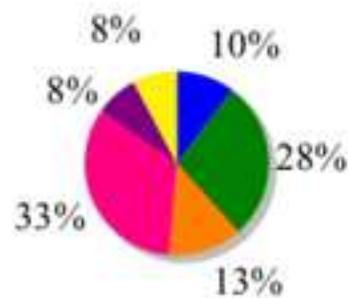
(D) Echinodermata (12 samples)



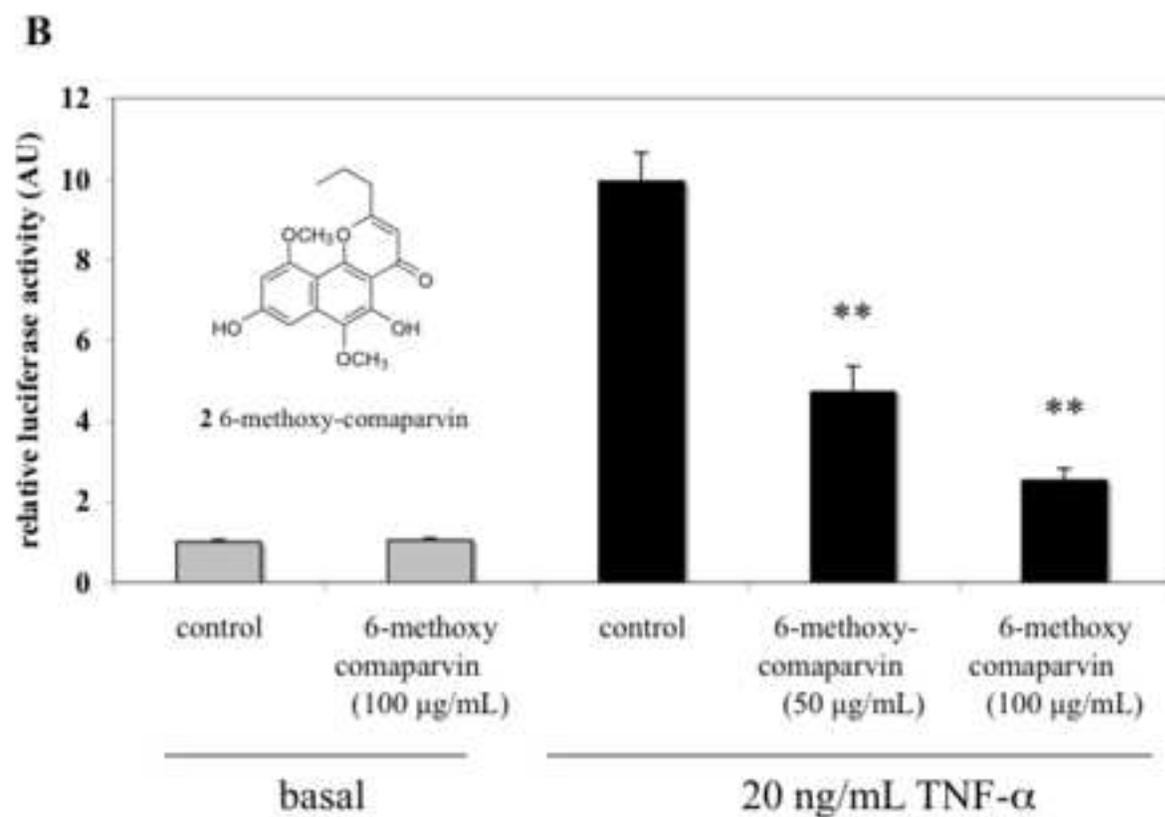
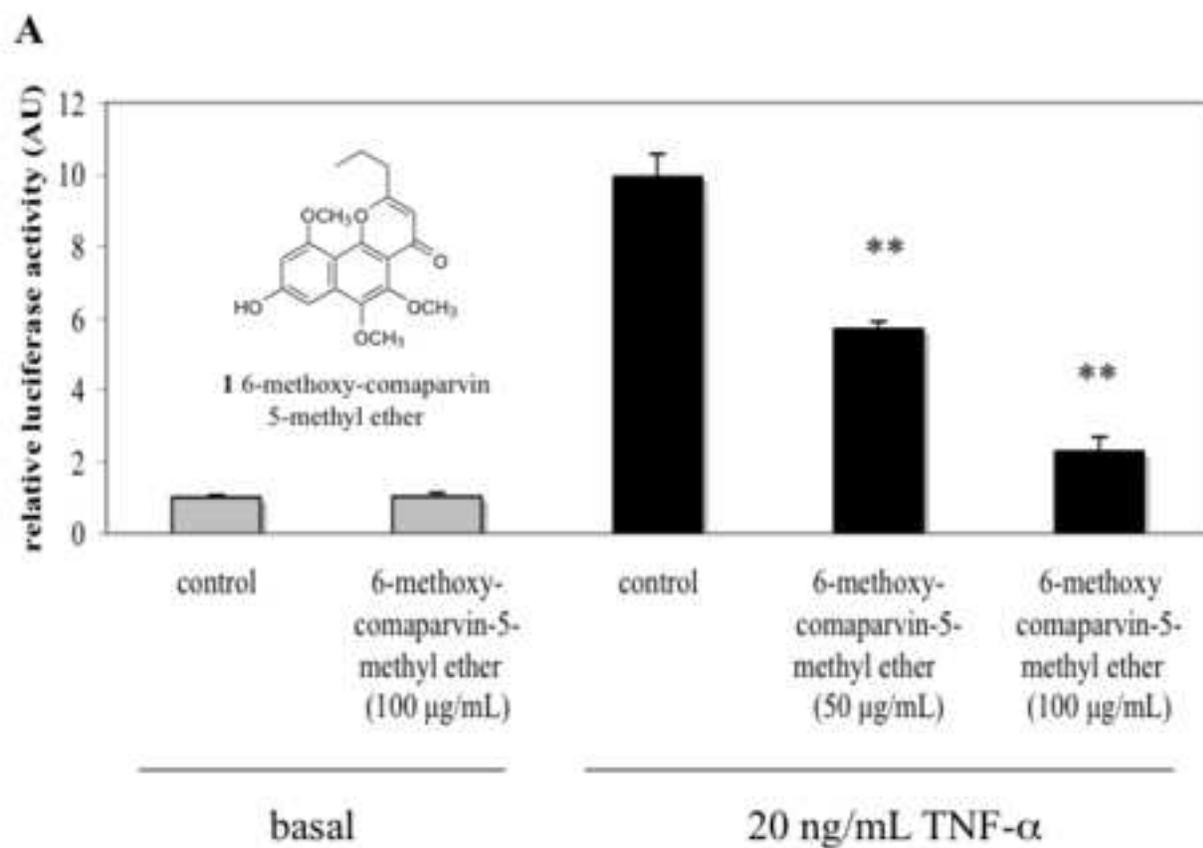
(E) Chordata (9 samples)



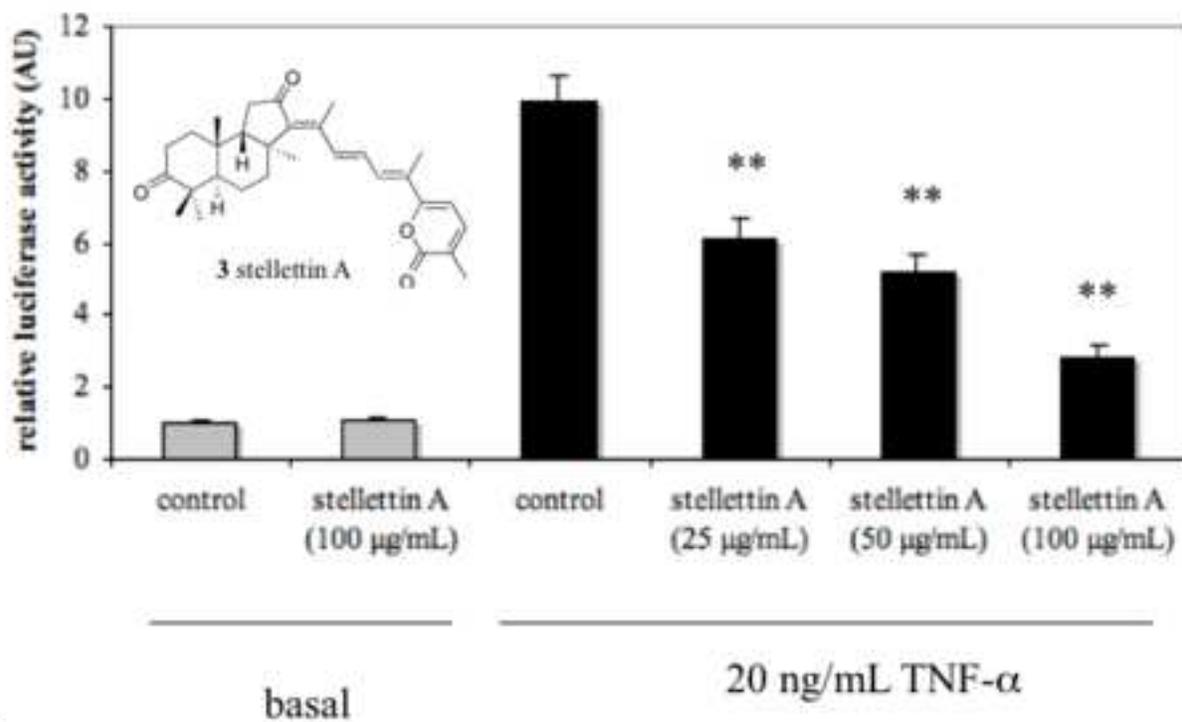
(F) Algae (20 samples)



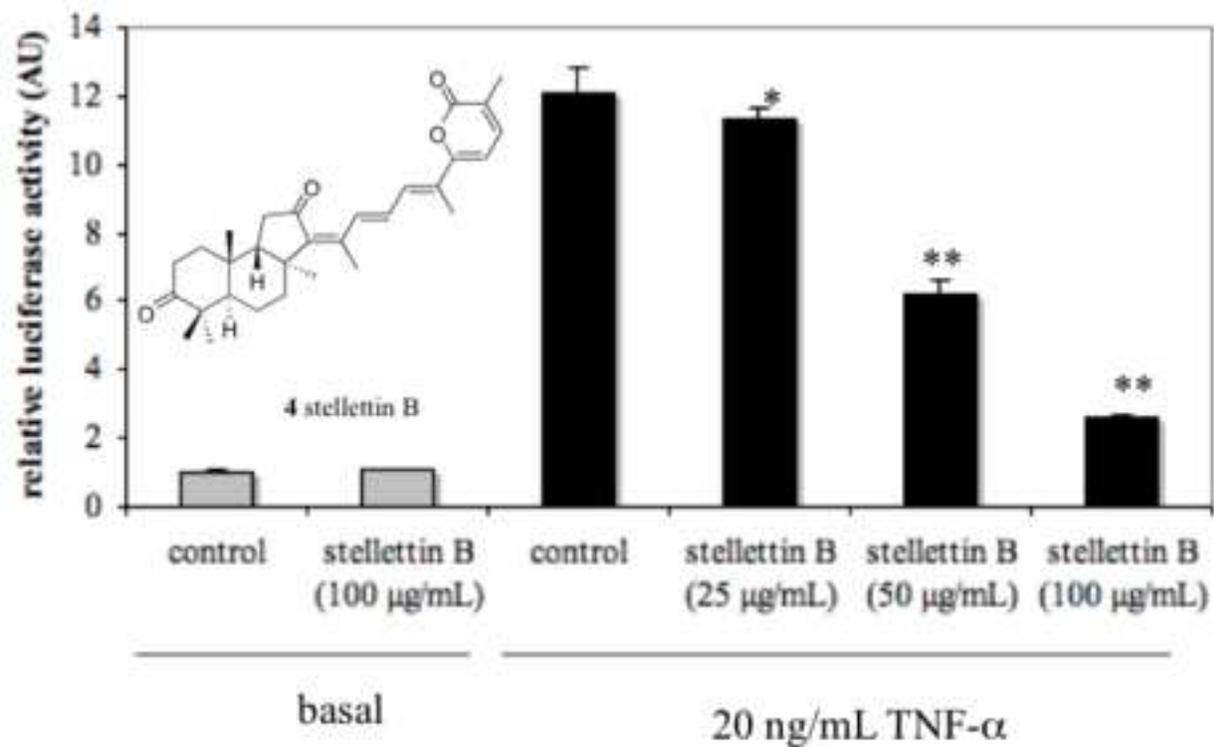
(G) Bacteria (39 samples)



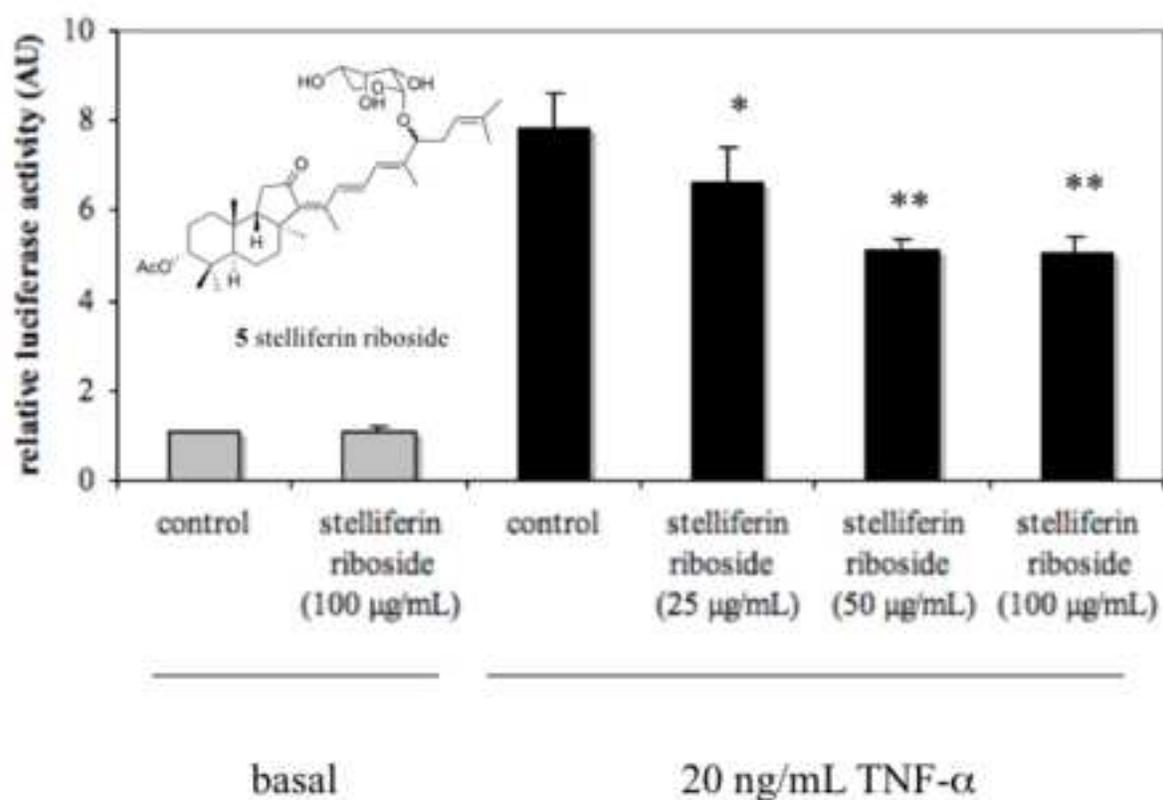
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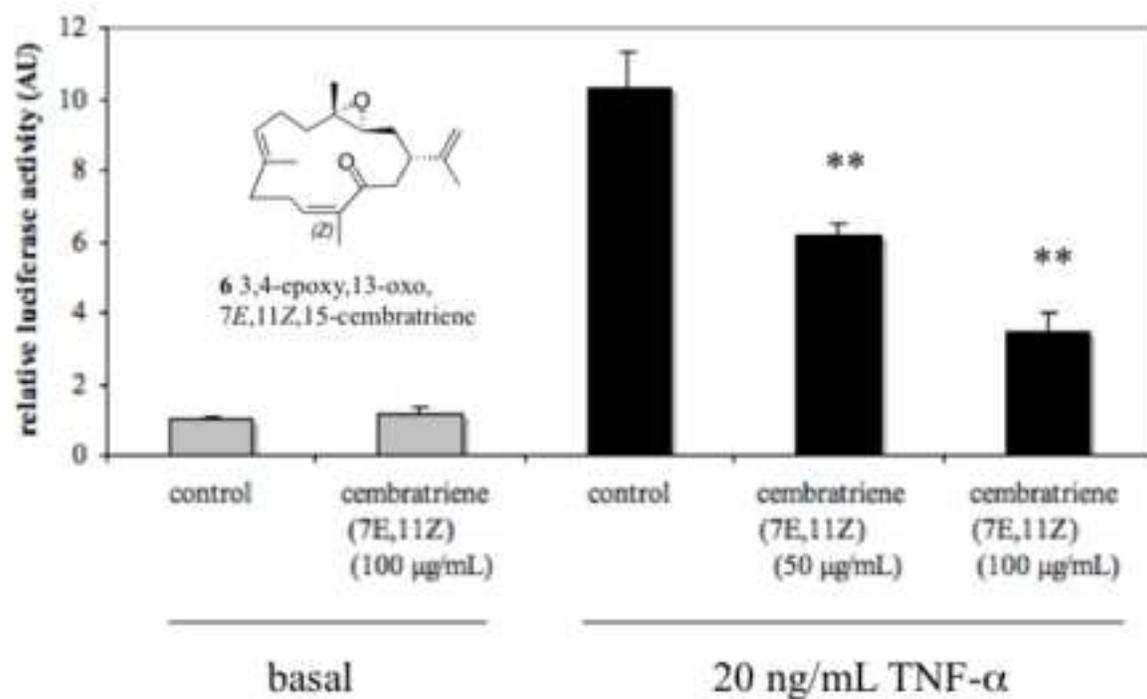
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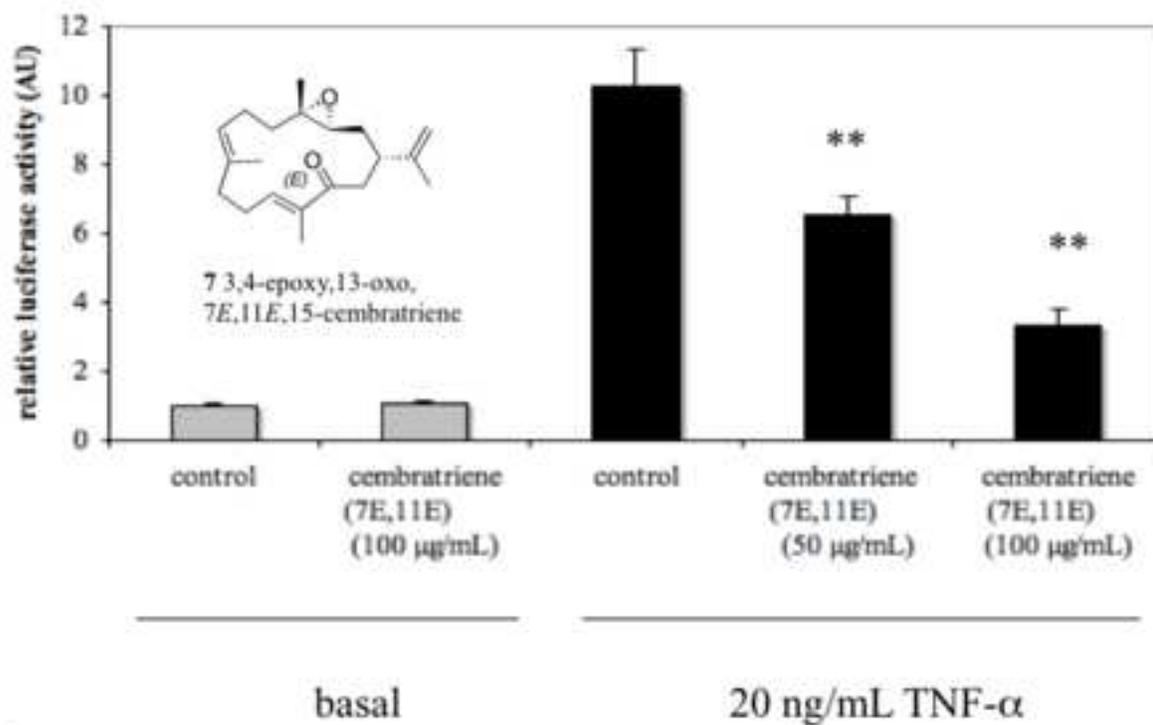
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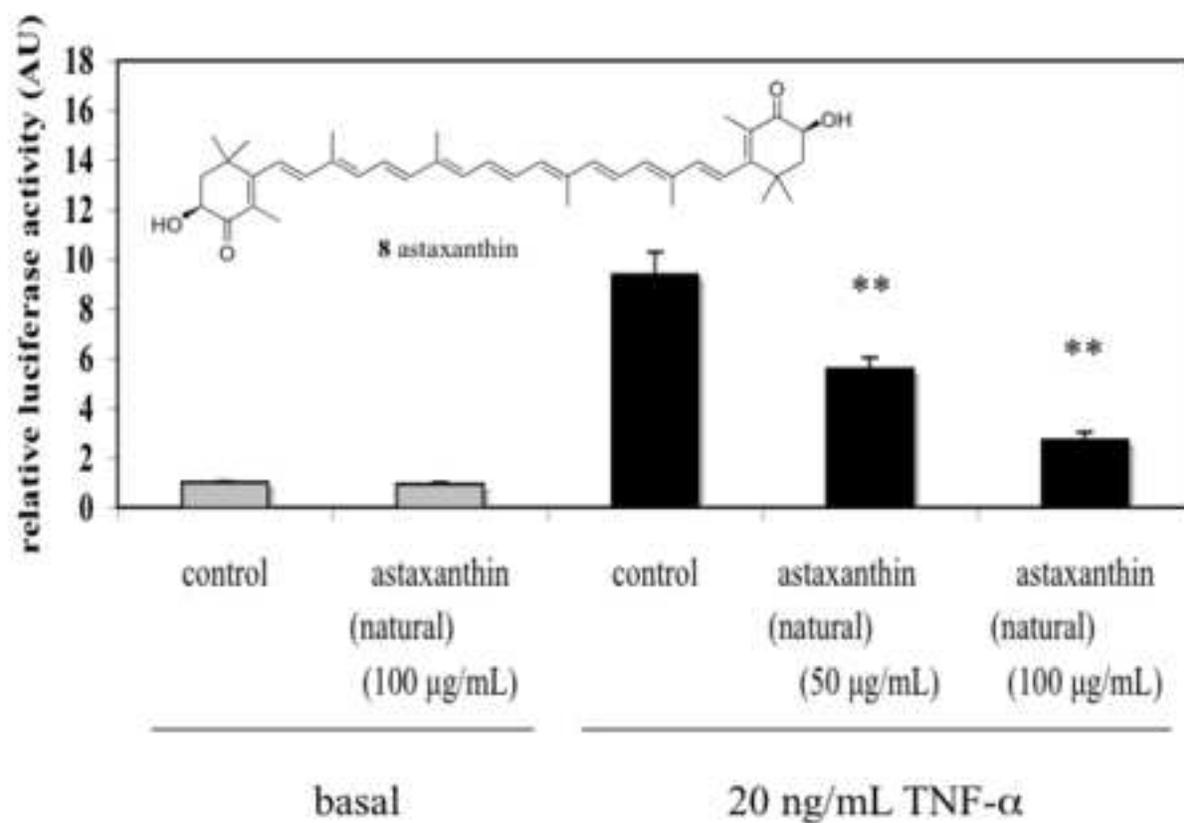
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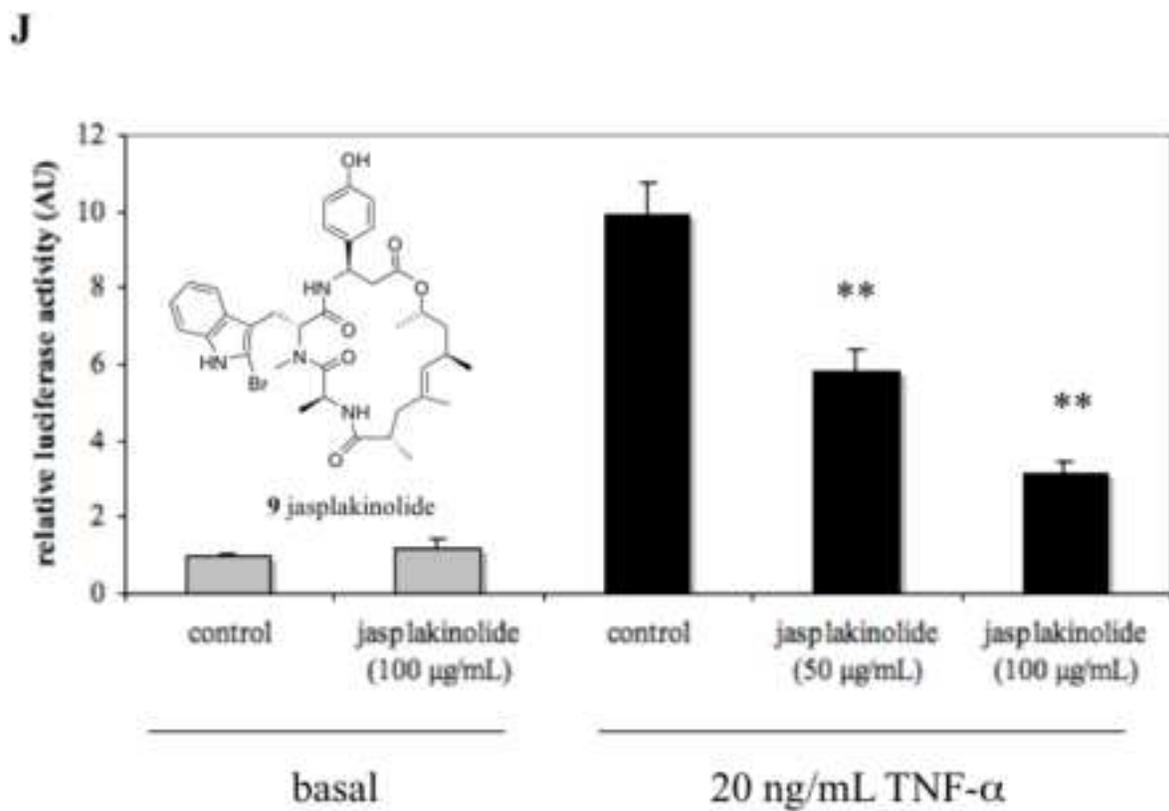
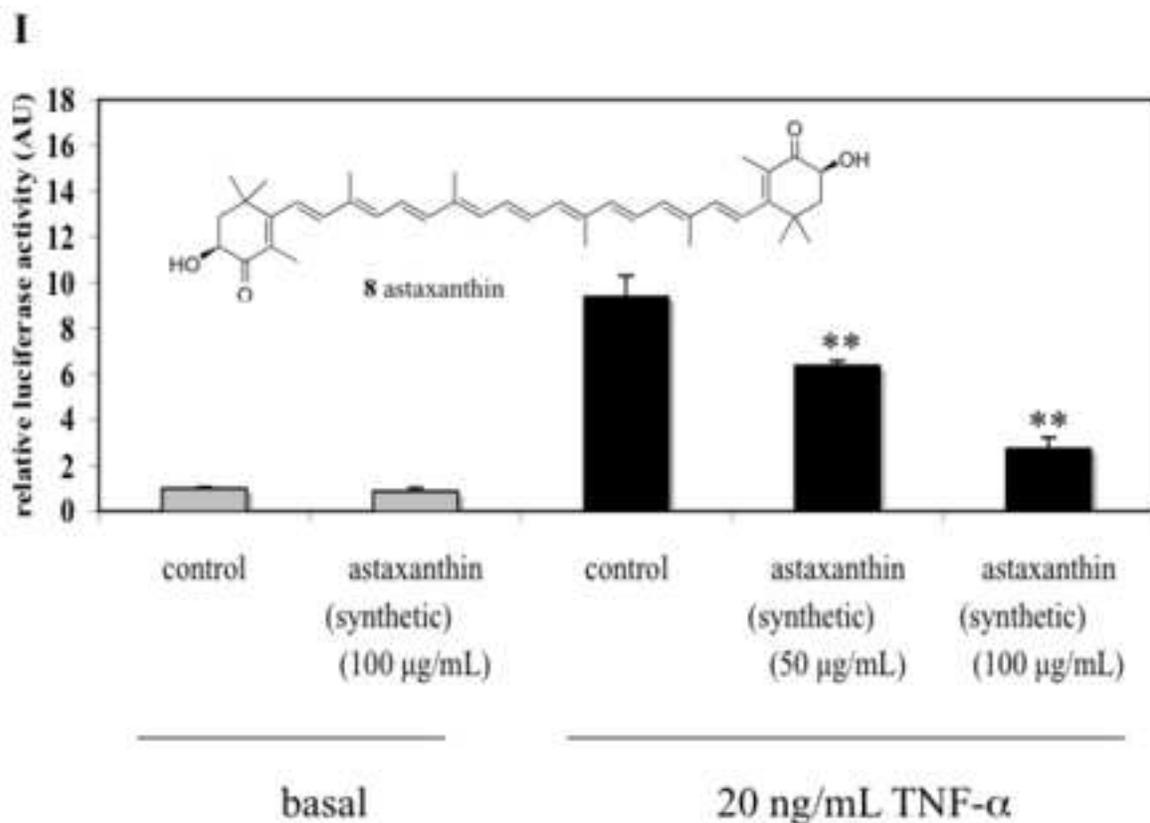


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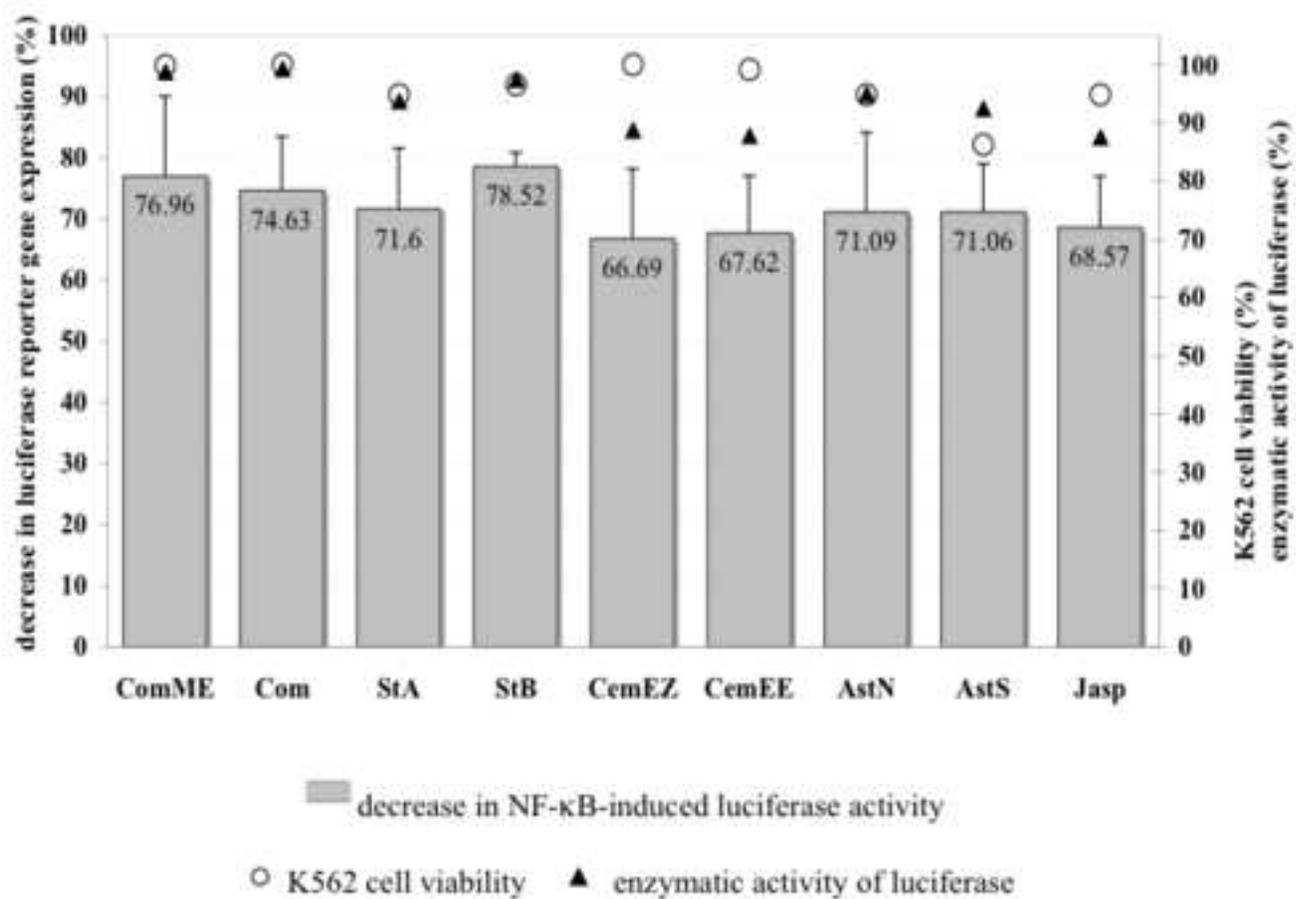


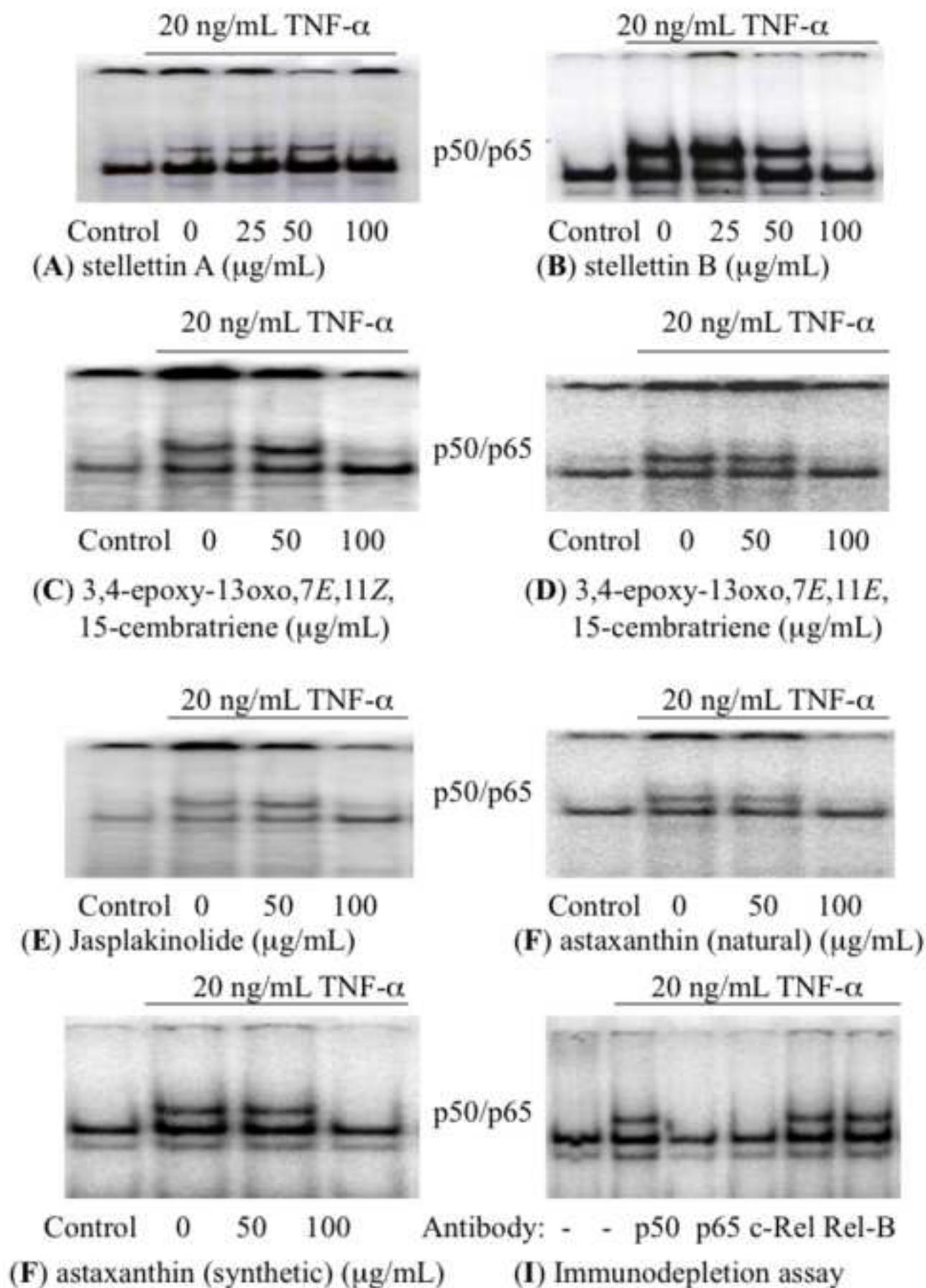
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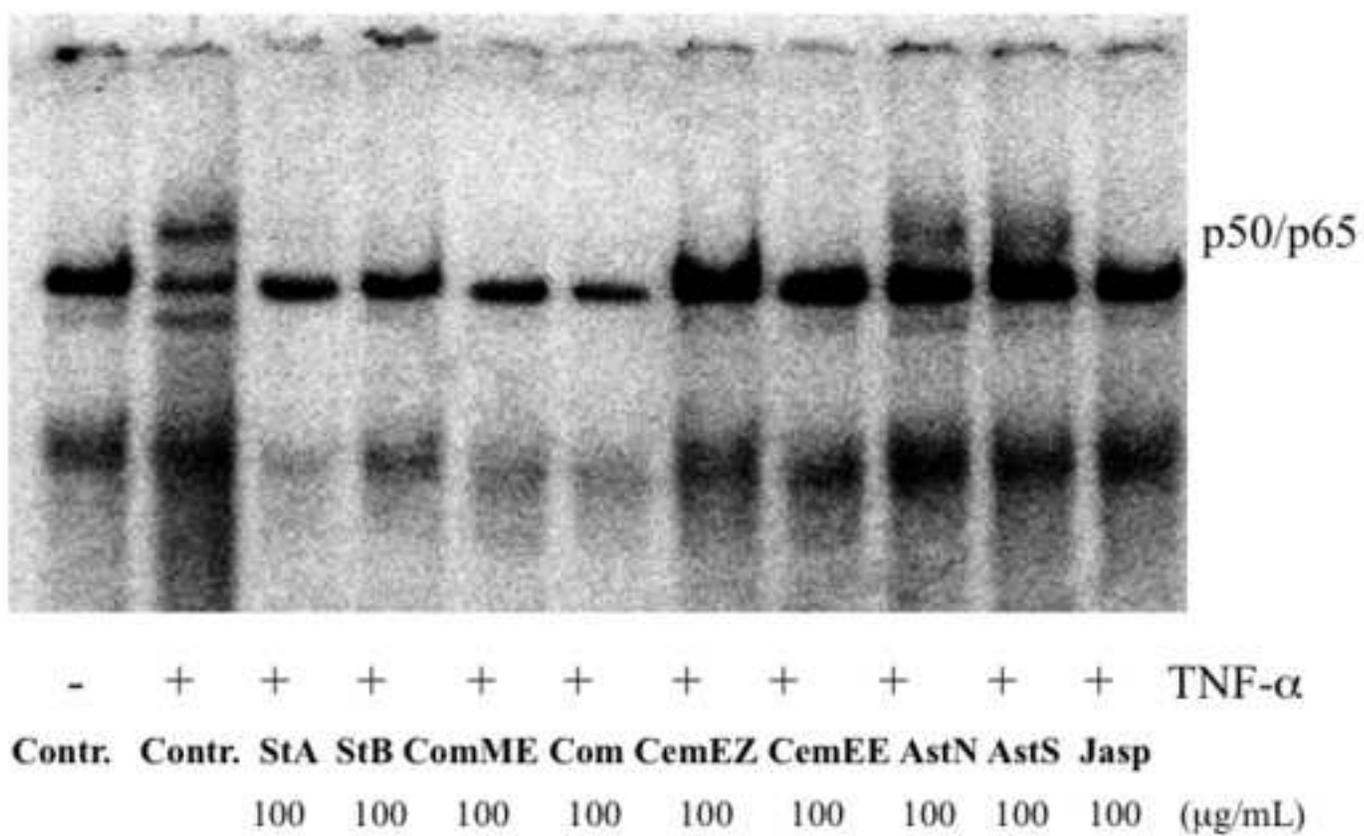


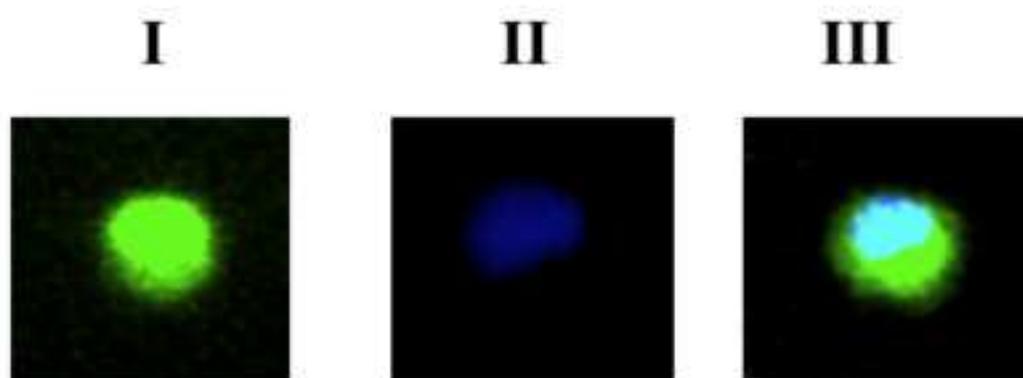


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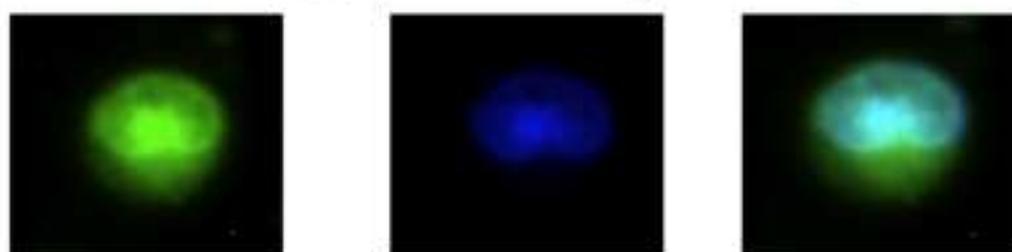




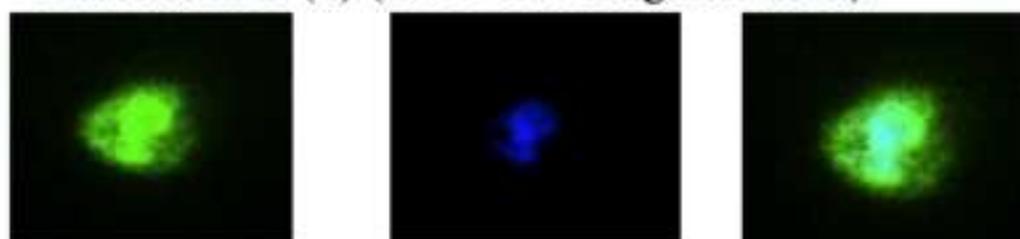




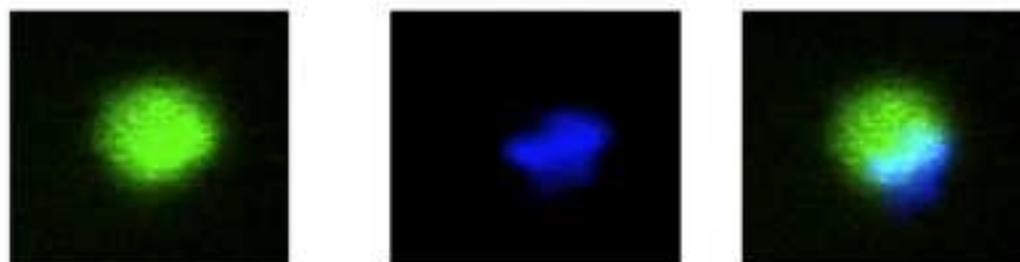
A Stellettin A (3) (1000-fold magnification)



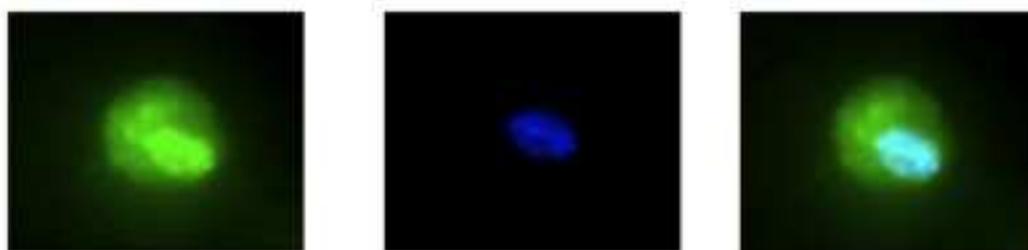
B Stellettin B (4) (1000-fold magnification)



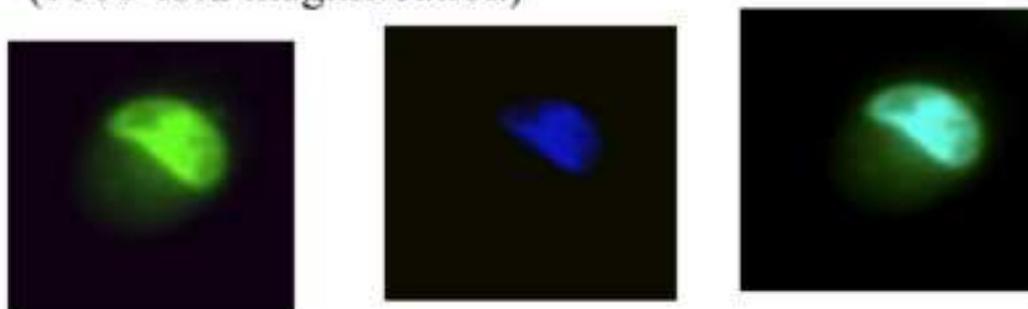
**C 6-methoxy-comaparvin-5-methyl ether (1)
(1000-fold magnification)**



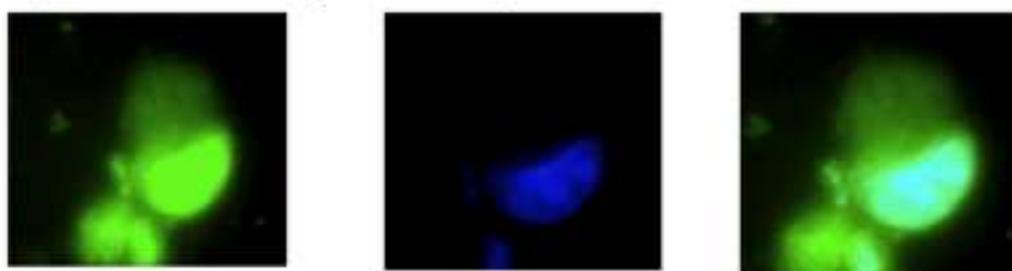
D 6-methoxy-comaparvin (2) (1000-fold magnification)



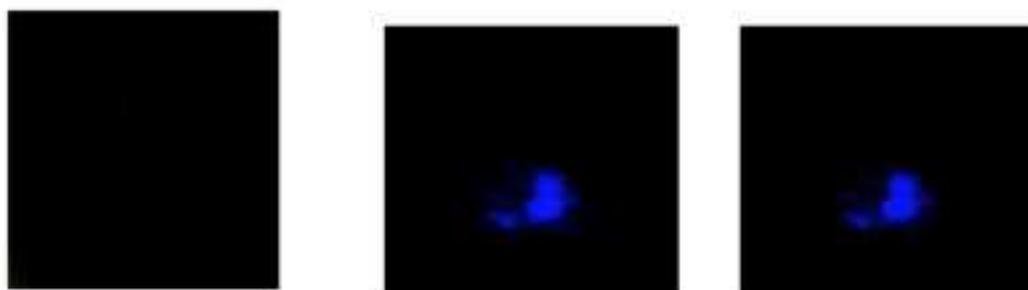
E 3,4-epoxy-13-oxo-7*E*,11*Z*,15-cembratriene (**6**)
(1000-fold magnification)



F 3,4-epoxy-13-oxo-7*E*,11*E*,15-cembratriene (**7**)
(1000-fold magnification)



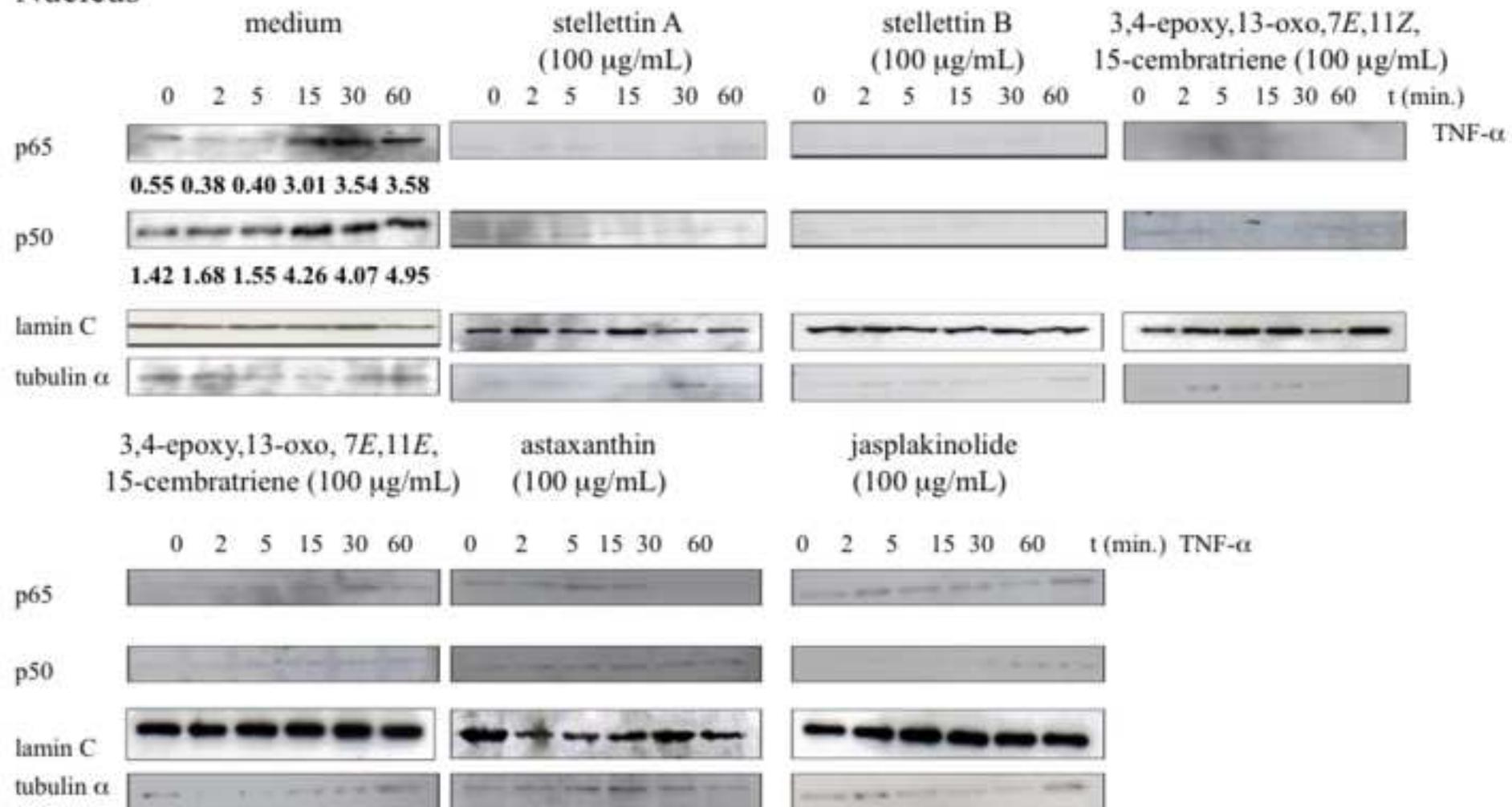
G jasplakinolide (**9**) (1000-fold magnification)



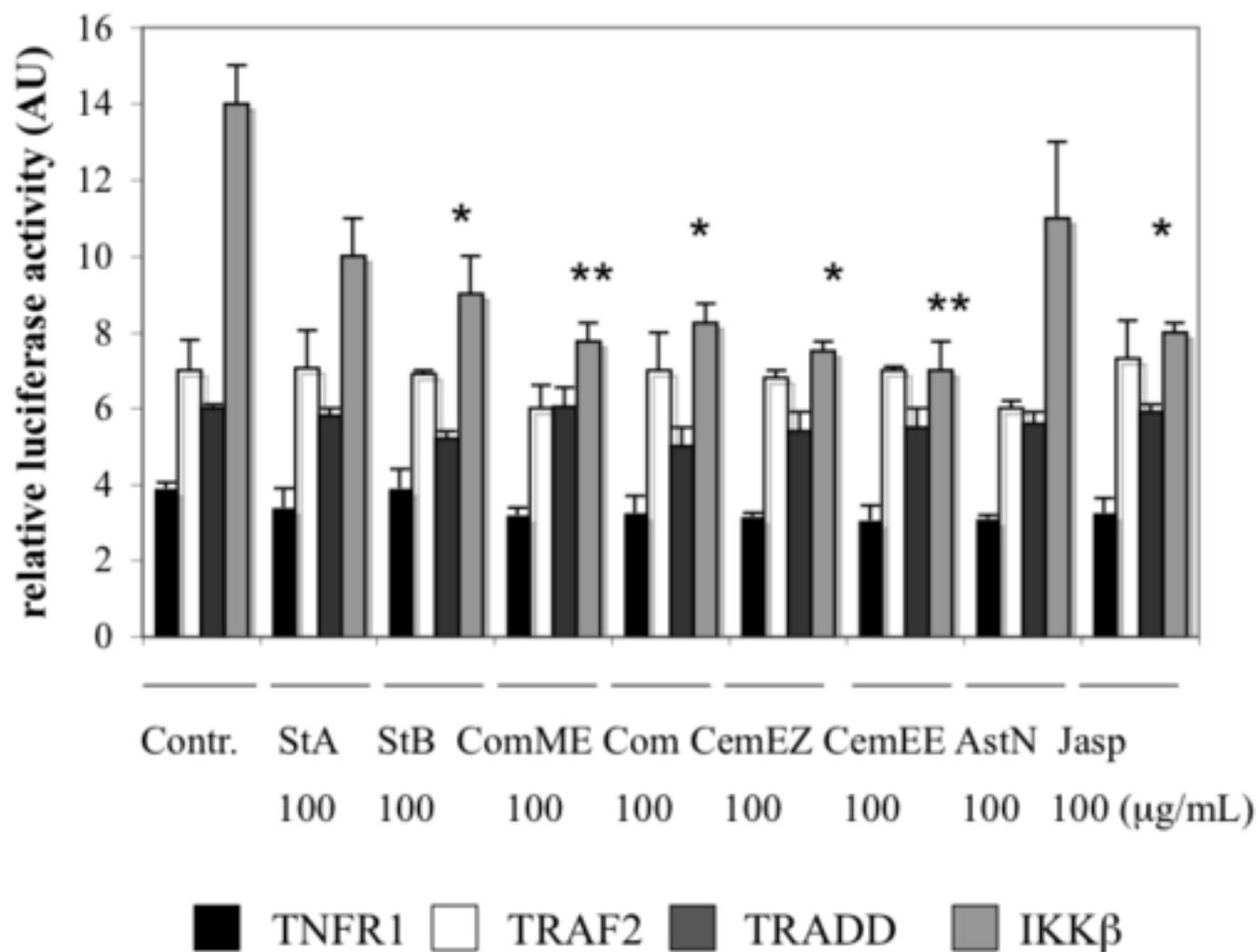
H background fluorescence (no treatment)
(1000-fold magnification)

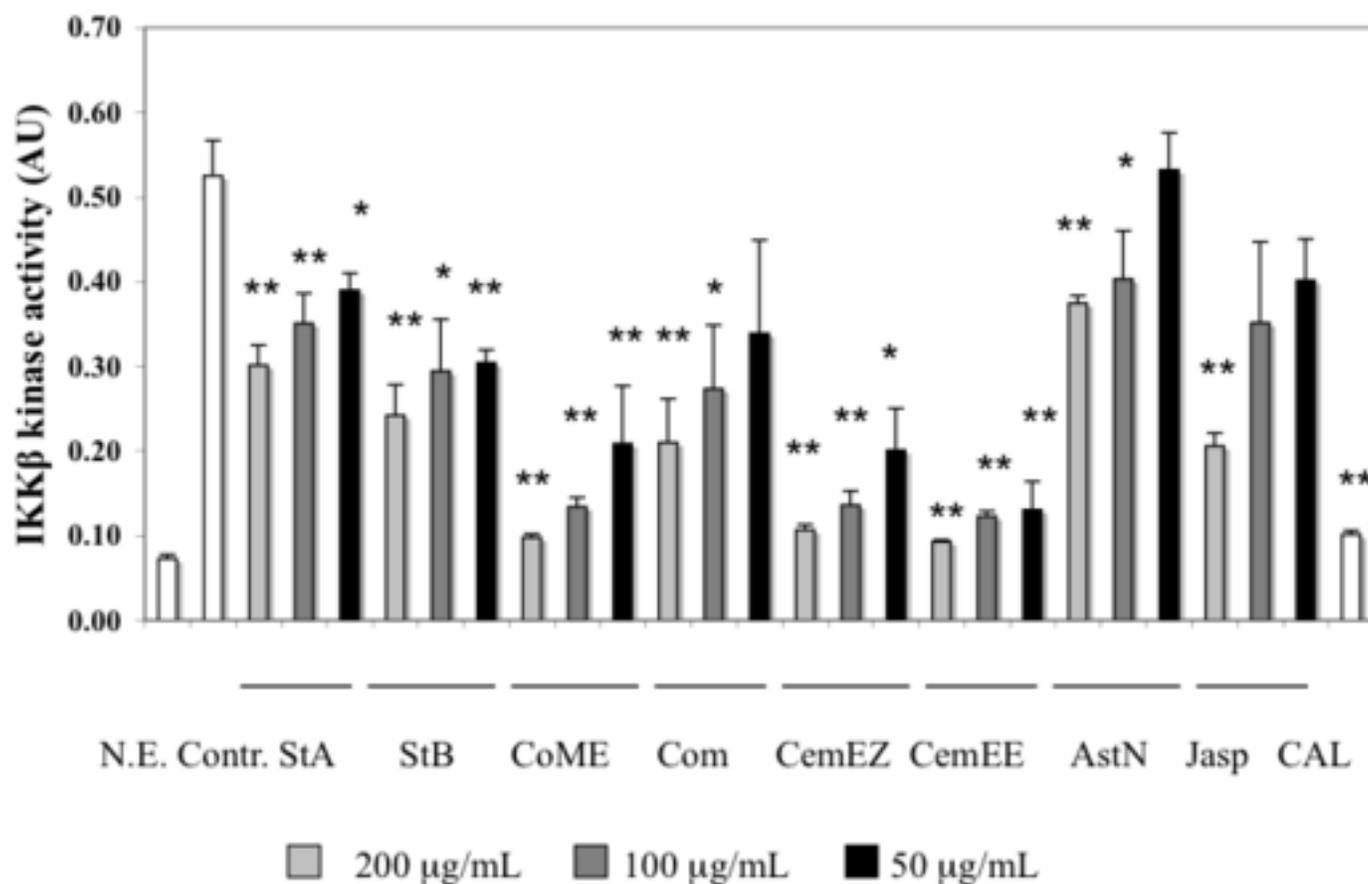


Nucleus



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C

