Secondary Metabolites from Fungal Endophytes of Echinacea purpurea Suppress Cytokine Secretion by Macrophage-Type Cells

By: Amninder Kaur, Martina Oberhofer, Monika Juzumaite, Huzefa A. Raja, Travis V. Gulledge, Diana Kao, Stanley H. Faeth, Scott M. Laster, Nicholas H. Oberlies, and Nadja B. Cech

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Abstract:

Botanical extracts of *Echinacea purpurea* have been widely used for the treatment of upper respiratory infections. We sought to chemically examine fungal endophytes inhabiting *E. purpurea*, and to identify compounds produced by these endophytes with in vitro cytokine-suppressive activity. Twelve isolates from surface sterilized seeds of *E. purpurea* were subjected to fractionation and major components were isolated. Sixteen secondary metabolites belonging to different structural classes were identified from these isolates based on NMR and mass spectrometry data. The compounds were tested for their influence on cytokine secretion by murine macrophage-type cells. Alternariol (**1**), O-prenylporriolide (**4**), porritoxin (**10**) *β*-zearalenol (**13**), and (*S*)-zearalenone (**14**) inhibited production of TNF-α from RAW 264.7 macrophages stimulated with LPS in the absence of any significant cytotoxicity. This is the first report of a cytokine-suppressive effect for **4**. The results of this study are particularly interesting given that they show the presence of compounds with cytokine-suppressive activity in endophytes from a botanical used to treat inflammation. Future investigations into the role of fungal endophytes in the biological activity of *E. purpurea* dietary supplements may be warranted.

Keywords: Echinacea purpurea | Asteraceae | fungal endophytes | TNF-α | inflammation | seeds

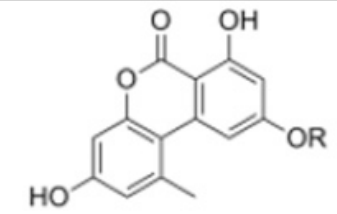
Article:

Medicinal plants retain their popularity as an important part of health care worldwide. The *Echinacea*genus has been employed for treating rhinosinusitis, the common cold, upper respiratory infections, and other inflammatory conditions [1–4]. While debates over therapeutic effects of *Echinacea* abound in the literature, preparations of this multi-million dollar herb

consistently rank among the best-selling botanicals in the United States [5], and are also very popular in Europe [6]. Some clinical studies have shown faster recovery from cold episodes on consumption of *Echinacea* preparations, while others suggest no useful impact [3].

The purported health benefits of *Echinacea* preparations are generally believed to occur via action on the immune system. In support of this, a number of *in vitro* studies have shown various immunomodulatory effects of *Echinacea* (reviewed in [7]). However, these studies have also shown confounding and often conflicting results. *Echinacea* extracts contain an array of different chemical constituents, including alkylamides, phenylpropanoids, polysaccharides, and volatile oils [3], which have different biological effects. Moreover, the levels of these constituents in a particular *Echinacea* product can vary depending on the species, plant parts, and/or geographical origin of plants used, and the method of processing [3]. Such variability may be one explanation for the inconsistencies reported for both clinical and *in vitro* studies of *Echinacea* preparations. Furthermore, the scientific community that studies botanical medicines has become increasingly aware of the potential importance of endophytes (microbes that reside in internal living tissues of plants) in altering or influencing the chemical composition of botanicals. Endophytes live in close association with plants, and their populations can differ depending on seasonal changes and geographic conditions [8, 9]. Hence, it has been proposed that the biological activity of a particular botanical could vary due to differences in endophyte profile. Along these lines, there is evidence that bacterial endophytes can alter the *in vitro* immunomodulatory activity of *Echinacea* and other botanicals [10, 11], and it has been shown that several bacterial endophytes from *Echinacea* possess immunomodulatory activity [12]. Several recent studies have demonstrated that *Echinacea* plants also harbor fungal endophytes [13, 14]. However, the potential immunomodulatory effects of these fungal endophytes or their constituents have yet to be evaluated. Our goals with this project were to culture and characterize fungal endophytes from *E. purpurea* seeds, isolate secondary metabolites from these endophytes, and evaluate their activity *in vitro* against RAW 264.7 murine macrophage-type cells. Our study focused on fungal endophytes isolated from *Echinacea purpurea* (L.) Moench (Asteraceae), which is the species most commonly cultivated for medicinal use in the United States.

Twelve fungal isolates from surface sterilized seeds of *E. purpurea* (Table 1) were subjected to natural products chemistry. From these, 16 known fungal secondary metabolites (Table 2 and Figure 1) were identified. These compounds were identified by comparison of their NMR and mass spectrometry data with literature (Table S1).



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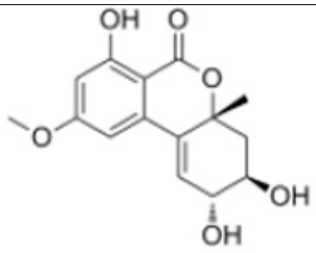

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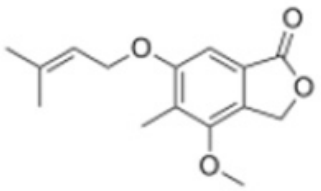

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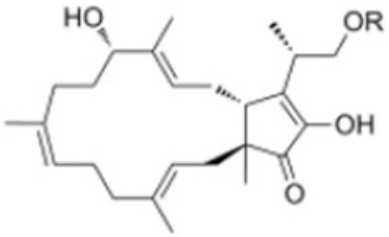

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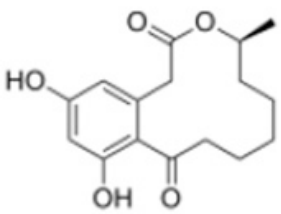

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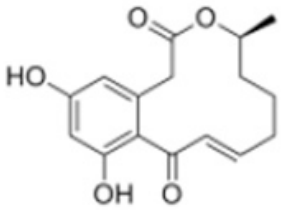

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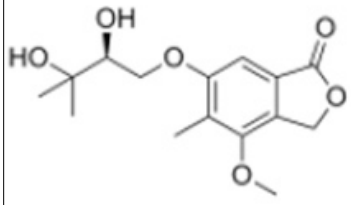

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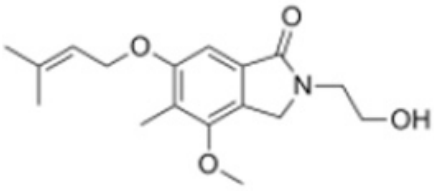

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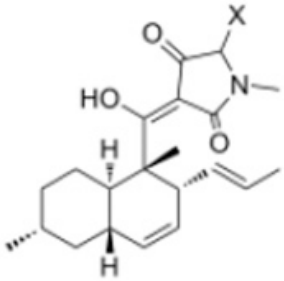

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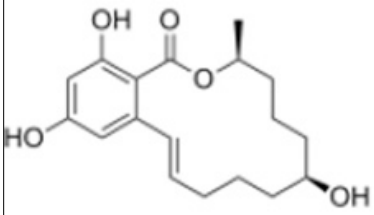
12: X= ▬CH$_2$OH



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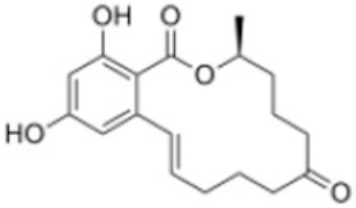

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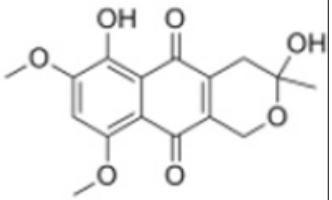

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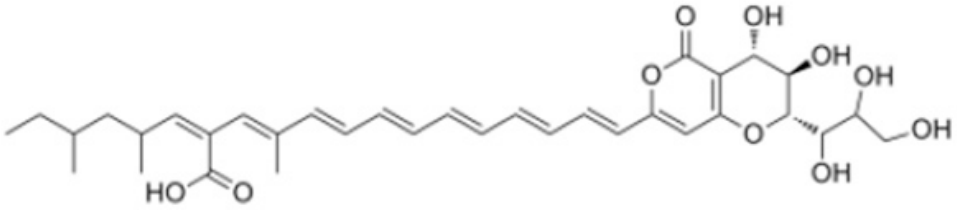

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Figure 1. Secondary metabolites isolated from fungal endophytes of *Echinacea purpurea*.

Table 1. Fungal endophytes isolated from surface sterilized *Echinacea purpurea* seed tissues. Twelve different isolates belonging to seven different operational taxonomic units (OTUs) were investigated in this study. The ITS region was sequenced and used for identification with GenBank BLAST search. All sequences are deposited at GenBank (accession numbers below). The strain numbers are internal references to the fungal library at the University of North Carolina at Greensboro.

Strains	Fungal taxon	GenBank accession number
G327	*Epicoccum nigrum*	KR094439
G328	*Fusarium equiseti*	KR094440
G330	*Phaeospheria* sp.	KR094442
G332	*Lewia infectoria*	KR094444
G335	*Lewia infectoria*	KR094447
G336	*Tilletiopsis pallescens*	KR094448
G389	*Fusarium equiseti*	KR094458
G390	*Epicoccum nigrum*	KR094459
G391	*Lewia infectoria*	KR094460
G393	*Alternaria* sp.	KR094462
G405	*Fusarium oxysporum*	KR094464
G406	*Lewia infectoria*	KR094465

Table 2. Secondary metabolites isolated from fungal endophytes. All endophytes were isolated from surface sterilized *Echinacea purpurea* seeds.

Compound #	Secondary Metabolite (References)	Isolate Number
1	Alternariol [31]	G330
1		G332
1		G336
1		G406
2	9-o-Methyl-alternariol [31]	G332
3	Altenuene [32]	G332
4	O-Prenylporriolide [33]	G330
5	Terpestacin [34]	G330
6	Fusaproliferin [35]	G330
7	(S)-Curvularin [36]	G330
8	(S)-10,11-Dehydrocurvularin [36]	G330
9	Porritoxinol [37]	G330
10	Porritoxin [37]	G330
11	Equisetin [38]	G328
11		G330
11		G335
11		G389
12	5'-Epi-equisetin [39]	G330

12		G389
13	β-Zearalenol [40]	G389
14	(S)-Zearalenone [40]	G389
15	8-O-Methyl-fusarubin [41]	G405
16	Orevactaene [42]	G327
		G390
		G391
		G393

The 16 compounds (Table 2) were tested for cytotoxicity against RAW 264.7 cells using rhodamine 123, a dye that is retained by cells with intact mitochondria and lost by cells undergoing either apoptosis or necrosis [15]. A range of concentrations was tested, from 0.0025–25 µg/mL, although effects were only noted at 2.5 and 25 µg/mL (Figure 2A). (*S*)-10, 11-Dehydrocurvularin (**8**) and 8-O-methyl-fusarubin (**15**) caused substantial dose-dependent cell death, while lower but significant levels of cytotoxicity were observed with porritoxin (**10**), equisetin (**11**) and 5'-epi-equisetin (**12**). Two of the resorcylic acid lactones, β-zearalenol (**13**) and (*S*)-zearalenone (**14**), have been reported to be cytotoxic towards RAW 264.7 cells [16, 17]. Although we did not observe this, it is possible that it could be observed at higher doses or longer incubation times.

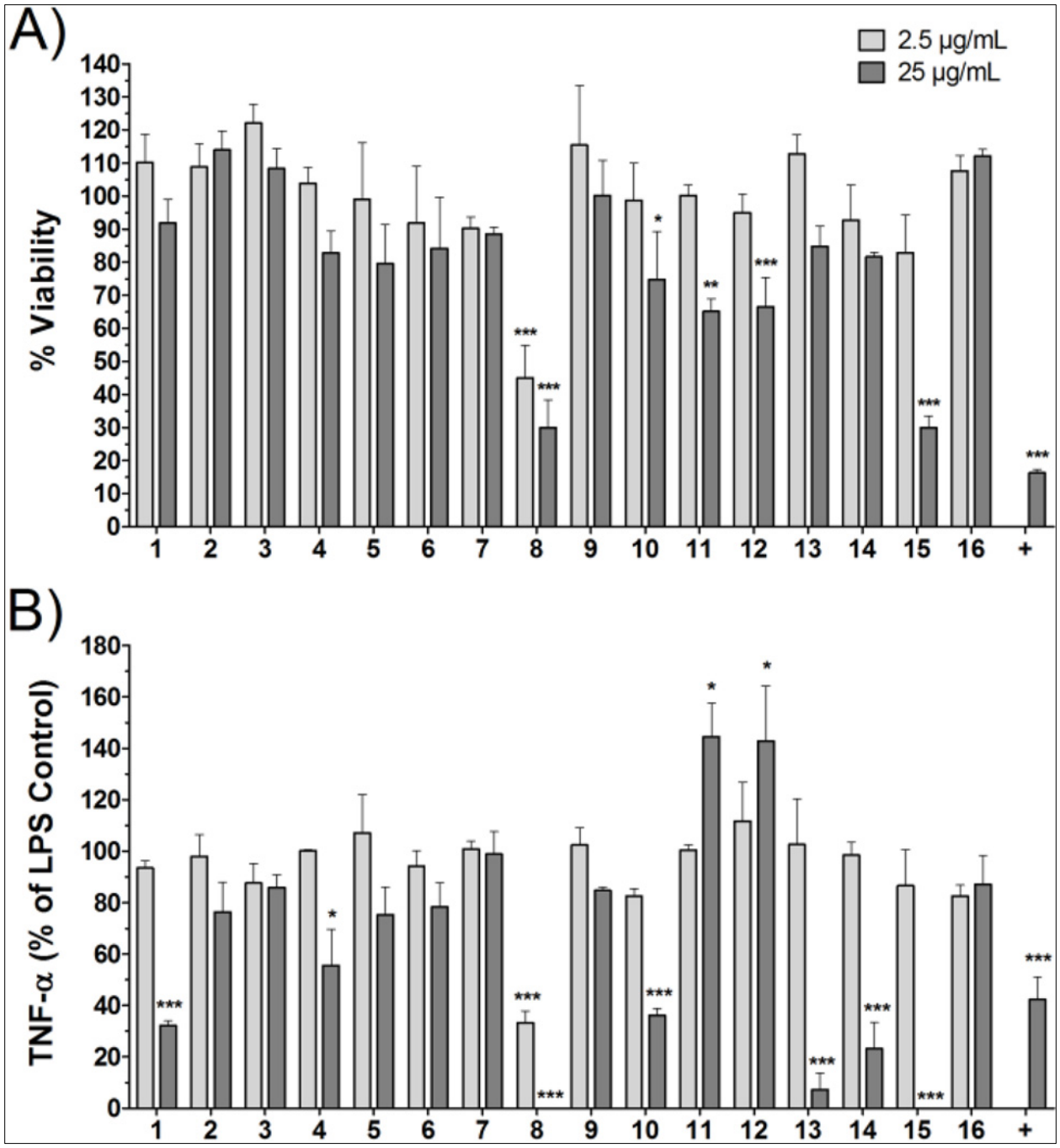

Figure 2. Influence of fungal metabolites on RAW 264.7 viability and secretion of TNF-α. Cells were treated with fungal compounds (numbered via Table 2) at 2.5 and 25 µg/mL alone (A) or in the presence of 10 ng/mL LPS (B). Cytotoxicity was measured using a rhodamine 123 assay (A). In B, culture supernatants were collected and assayed for TNF-α levels using ELISA. Values shown are means of two independent experiments, each of which included duplicate biological replicates (total of four biological replicates). Positive controls (+) include 100 µg/mL cycloheximide for cytotoxicity (A) [31] and dodeca-2E,4E-dienoic acid isobutylamide for inhibition of TNF-α production [30] (B). Data was analyzed using a one way ANOVA with

Tukey's post hoc test; *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$. Compound 2 was not soluble at 25 µg/mL and was tested at 1.25 and 12.5 µg/mL.

The isolated fungal metabolites (Table 2) were also tested at the same range of concentrations for their ability to affect both constitutive and LPS-induced production of TNF-α. Levels of constitutive TNF-α production from RAW 264.7 cells were low (50–100 pg/mL), and none of the compounds caused statistically significant enhancement or suppression (Figure S1). In contrast, in the presence of 10 ng/mL LPS, production of TNF-α was robust, with 6–8 ng accumulating in cultures in the different experiments. For purposes of comparison, these data are shown as percent of control (Figure 2B). Several compounds, **1, 4, 13**, and **14**, demonstrated pronounced inhibition of LPS-induced TNF-α production in the absence of significant toxicity. Compounds **4** and **9** are structurally related, but compound **4** was a more potent inhibitor of TNF-α production. This observation suggests that the diol moiety in **9** decreases potency. Compound **10** produced a weak cytotoxic effect and strongly inhibited LPS-induced production of TNF-α, while compounds **11** and **12** also produced weak cytotoxic effects but enhanced TNF-α production for LPS treated cells (Figure 2B). It is possible that these compounds produce an effect akin to activation-induced apoptosis, first acting in concert with LPS to activate the cells, which is followed by an apoptotic response [18].

Whereas studies on fungal endophytes present in other plant organs exist [13,19], this is the first to investigate endophytes present in *E. purpurea* seeds. The rationale is that fungi present in seed material may be transmitted vertically (from parent to progeny) and may be host-specific. However, it is also possible to find horizontally transmitted endophytes (originating from the environment) in seeds. Thus, the investigated fungi may be in therapeutic preparations, however, it is more common to employ root material for dietary supplements from this botanical [20]. Future studies of endophytes from other *E. purpurea* plant tissues might be of interest.

It is notable that *Echinacea purpurea* preparations are commonly used to treat inflammation, and the cytokine-suppressive activity observed for the fungal metabolites is consistent with an anti-inflammatory effect. Moreover, it is possible that the toxicity of several mycotoxins encountered in this study could influence the therapeutic properties of herbal preparations. However, the studied metabolites were produced by cultured endophytes. Whether they are present at biologically relevant concentrations to alter the toxicity or biological activity of *Echinacea* preparations is a question that would require further investigation.

In summary, a number of compounds with *in vitro* cytokine-suppressive activity were isolated from the fungal endophytes of *E. purpurea* seeds. Although no new compounds were encountered, this is the first report of cytokine suppression by compound **4**.

Experimental

General Instrumentation Techniques

Optical rotations were acquired on a Rudolph Research Autopol III polarimeter. NMR experiments used a JEOL ECS-400 (400 MHz for 1H and 100 MHz for 13C; JEOL Ltd., Tokyo, Japan). HRESIMS data were collected with an electrospray ionization source coupled to a Q-ToF

Premier mass spectrometer (Waters Corp., Milford, MA, USA) or a LTQ Orbitrap XL system (Thermo Fisher Scientific, San Jose, CA, USA) in positive and/or negative ionization modes by direct injection or via an Acquity UPLC system (Waters Corp.). A CombiFlash Rf 200 system using a RediSep Rf Si-gel Gold column (both from Teledyne-Isco, Lincoln, NE, USA) was employed for normal-phase flash chromatography. HPLC was performed via a ProStar HPLC system with a 335 photodiode array detector (Varian Inc., Palo Alto, CA, USA). YMC ODS-A (Waters Corp.; 5µm; columns of dimensions 250 × 20 mm, 250 × 10 mm, and 150 × 4.6 mm) or Kinetex C18 (Phenomenex; 5µm; columns of dimensions 250 × 21.2 mm and 250 × 4.6 mm) were used for preparative, semi-preparative, and analytical HPLC, respectively. For UPLC analysis, a BEH C18 (Waters Corp.; 1.7 µm; 50 × 2.1 mm) column was used.

Endophyte Isolation

E. purpurea seeds (lot # 6784) were obtained from Horizon Herbs, LLC (Williams, OR, USA), and a voucher of an *E. purpurea* plant from this source is deposited at the University of North Carolina at Chapel Hill Herbarium (NCU583422). The seeds were surface sterilized [21] and placed on 1.5 % water agar Petri dishes. All fungi were isolated within 14 days of culture and three times single spore isolated to obtain pure cultures.

Endophyte Identification

Axenic cultures were harvested and ground under sterile conditions using liquid nitrogen. DNA amplification was performed combining the primers ITS1F/ITS1 and ITS4 [22, 23] under defined cycling conditions [24]. PCR products were purified with a QIAquick Purification Kit (QIAGEN Inc., Valencia, CA, USA) and sequenced (Eurofins MWG Operon LLC, Huntsville, AL, USA). The sequences were manually edited and identified by BLASTn search against authentic sequences in GenBank. Sequences from the entire ITS region (IT1-5.8-ITS2) were deposited at GenBank (Table 1).

Extraction and Isolation of Fungal Secondary Metabolites

Methods for preparing fungal inoculum for growth on rice, and procedures for isolation of secondary metabolites, have been detailed [24]. Key references for the characterization of all 16 compounds are provided as Supporting Information (Table 1S).

Cytotoxicity Assays and Measurement of TNF-α

Each compound was tested in duplicate experiments, performed on different days, over a range of concentrations (0.0025–25.0 µg/mL), with two biological replicates in each experiment (for a total of four biological replicates). Ethanol (1% final concentration) was used as the diluent for all but three compounds (**1, 2**, and **15**) where dimethylsulfoxide (DMSO) was used (0.2% final concentration). The compounds were tested for toxicity using the rhodamine 123 (Rho123) assay. Cells were treated overnight with each secondary metabolite in 96 well plates. After incubation, the supernatant was removed, and Rho123 was added in the buffer for 30 min (5 µM final concentration), removed, and cells washed with 1× PBS [28]. The fluorescence was measured using a BMG FLUOstar Galaxy microplate reader (492/538) (BMG LABTECH GmbH, Ortenberg, Germany). For TNF-α testing, cells were treated, supernatants harvested, and

then TNF-α measured using murine TNF-α ELISA kit purchased from eBioscience, Inc. (San Diego, CA). To stimulate TNF-α production, 10 ng/mL ultrapure LPS from *Salmonella minnesota* R595 (List Biological Laboratories, Inc. Campbell, CA, USA) was used. As a positive control for inhibition of TNF-α production, the compound dodeca-2*E*,4*E*-dienoic acid isobutylamide (ChromaDex, Irvine, CA, USA) was used at a concentration of 25 µg/mL. The cytokine-suppressive activity of this compound has been documented elsewhere [29].

Statistical Analysis

Experimental results were analyzed with a one way ANOVA with Tukey's post hoc test using Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Supplementary Material

Supporting Information

Acknowledgments

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Footnotes

Supplementary data: Structures of isolated compounds, high-resolution MS data, and effects of extracts on constitutive production of TNF-α are included as supplementary data.

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