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Immunomodulatory Activity of Oenothein B Isolated from Epilobium angustifolium

Igor A. Schepetkin,* Liliya N. Kirpotina,* Larissa Jakiw,* Andrei I. Khlebnikov,† Christie L. Blaskovich,* Mark A. Jutila,* and Mark T. Quinn2*

Epilobium angustifolium has been traditionally used to treat of a number of diseases; however, not much is known regarding its effect on innate immune cells. In this study, we report that extracts of E. angustifolium activated functional responses in neutrophils and monocyte/macrophages. Activity-guided fractionation, followed by mass spectroscopy and NMR analysis, resulted in the identification of oenothein B as the primary component responsible for phagocyte activation. Oenothein B, a dimeric hydrolysable tannin, dose-dependently induced a number of phagocyte functions in vitro, including intracellular Ca2+ flux, production of reactive oxygen species, chemotaxis, NF-κB activation, and proinflammatory cytokine production. Furthermore, oenothein B was active in vivo, inducing keratinocyte chemoattractant production and neutrophil recruitment to the peritoneum after intraperitoneal administration. Biological activity required the full oenothein B structure, as substructures of oenothein B (pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid) were all inactive. The ability of oenothein B to modulate phagocyte functions in vitro and in vivo suggests that this compound is responsible for at least part of the therapeutic properties of E. angustifolium extracts. The Journal of Immunology, 2009, 183: 0000 – 0000.

Enhancement of innate immunity by immunomodulators can increase host resistance to pathogens (1), and a number of innate immunomodulators have been identified, including cytokines (2), substances isolated from microorganisms and fungi (3), and substances isolated from plants (4, 5). However, many of these substances are high molecular mass carbohydrates (6) or lectins (7), and only a few plant-derived compounds with a significant amount of interest in identifying low molecular mass bioactive compounds present in Epilobium taxa (27). However, although polyphenols are known for their antioxidant activity, recent evidence indicates that the therapeutic effects of these compounds is not solely due to antioxidant properties and that they can directly modulate cellular responses (reviewed in Ref. 33). For example, it has been reported that oenothein B has antitumor activity and that this may be due to enhancement of the host-immune system via induction of IL-1β (27). However, little else has been reported on the effects of oenothein B on innate immunity. Thus, we evaluated the effects of E. angustifolium extracts on phagocyte function.

In this study, we report that E. angustifolium extracts can activate phagocyte functional responses. Furthermore, fractionation of the extracts indicated that the active component was oenothein B. Oenothein B activated monocyte/macrophages and neutrophils, resulting in increased intracellular Ca2+ flux, production of reactive oxygen species (ROS)3 and cytokines, and chemotaxis. Thus, part of the observed therapeutic effects of oenothein B and Epilobium extracts is due to modulation of innate immune function.

Materials and Methods

Reagents

Corilagin (1-O-galloyl-3,6-hexahydroxydiphenol-β-D-glucopyranose) was from Toronto Research Chemicals; 1,2,3,4,6-pentakis-O-galloyl-β-D-glucose

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3 Abbreviations used in this paper: ROS, reactive oxygen species; PGG, 1,2,3,4,6-pentakis-O-galloyl-β-D-glucose; iMLF, N-formyl-Met-Leu-Phe; NBT, nitro blue tetrazolium; LAL, Limulus amebocyte lysate; KC, keratinocyte chemoattractant; PI, fold increase.

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Combined extracts were concentrated, and any precipitates were removed by extracted with 80% methanol at room temperature for 3 days. The com-

A. Klein (Department of Plant Sciences and Plant Pathology, Montana

Baker. HBSS (pH 7.4), with and without Ca2

Escherichia coli

cerevisiae

were purchased from Sigma-Aldrich. IL-8 was purchasedPepro-

N

LH-20 column (2.8

fractions were stored at

Sephadex LH-20 column (2.5

DMSO. Bioactive subfraction S-3 was rechromatographed twice on a

biological activity, the crude extract and pooled fractions were dissolved in

Extract and isolation of active compound

Fully blossomed E. angustifolium were collected and identified by Robyn A. Klein (Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT). The dried plant material (400 \( \times \) g) was extracted with 80% methanol at room temperature for 3 days. The com-

bined extracts were concentrated, and any precipitates were removed by filtration through a 0.22-\( \mu \)m filter. The filtrate was lyophilized to obtain the crude extract or subjected to concentration and fractionation on a Sephadex LH-20 column (2.8 \( \times \) 33 cm) using 80% methanol as the eluent. The rele-

vant fractions were pooled and evaporated to dryness. For analysis of biological activity, the crude extract and pooled fractions were dissolved in DMSO. Bioactive subfraction S-3 was rechromatographed twice on a Sephadex LH-20 column (2.5 \( \times \) 25 cm) using 80% methanol as the mobile phase, resulting in 180 mg of the final product. All lyophilized extracts and fractions were stored at

Compound identification

For NMR analysis, samples (5 mg) were dissolved in 0.5 ml D2O or acetone-\( d_2 \), with 2% (v/v) D2O and 1 H spectrum were recorded on a Bruker DRX-600 spectrometer (Bruker) at 20°C using 3-(trimethylsilyl)-propionic-2,2,3,3,3-d_4 acid sodium salt as an internal reference. For 13C-NMR, spectra were recorded on a Bruker DRX-500 spectrometer at 20°C.

Mass spectrometry experiments were performed using a Bruker Mic-

rotof high resolution time of flight mass spectrometer. Ionization was achieved using standard electrospray conditions, and data were acquired in positive-ion mode with nitrogen as the nebulizing gas. 200°C, drying gas flow rate 3 L/min, nebulizer pressure 0.2 bar, and capi-

illary voltage was 4000 V. Each sample was dissolved in HPLC-grade methanol and directly infused into the mass spectrometer. The full mass scan ranged between (m/z) 100 and 1700 Da.

Purity of isolated oenothein B was determined by reverse-phase HPLC on an automated HPLC system (Shimadzu) with a Phenomenex Jupiter C18 500A column (5 \( \mu \)m, 250 \( \times \) 4.6 mm) eluted with acetonitrile/water (16/84, v/v) containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min and detection at 270 nm, at 30°C over 15 min (34). Purity was >95% based on reversed-phase HPLC and mass spectroscopic analysis.

Endotoxin assays

A Limulus amebocyte lysate (LAL) assay kit (Cambrex) was used to evaluate isolated oenothein B for possible endotoxin contamination, according to the manufacturer’s protocol. In brief, the LAL was reconstituted in 250 \( \mu \)l of isolated oenothein B dissolved in endotoxin-free 0.05 M phosphate buffer (pH 7.2). Each vial was incubated at 37°C for 1 h. At the end of the incubation period, each vial was inverted 180° to estimate gel forma-

 tion compared with control (endotoxin-free buffer).

To further evaluate the possible role of endotoxin, isolated oenothein B dissolved in elution buffer (0.05 M phosphate buffer (pH 7.2) containing 0.5 M NaCl) was applied to a column containing Detoxi-Gel Endotoxin Removal Resin (Biorad). The concentration of oenothein B in the eluted sample was adjusted to match that of the untreated fraction, as determined by UV spectroscopy at 265 nm, and both treated and untreated samples were analyzed for biological activity.

Cell culture

Human monocyte TFP-1Blue cells obtained from InvivoGen were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 \( \mu \)g/ml streptomycin, 100 \( \mu \)M penicillin, 100 \( \mu \)g/ml Zeocin, and 10 \( \mu \)g/ml blastidin S. TFP-1Blue cells are stably transfected with a secreted em-

bryonic alkaline phosphatase gene that is under the control of a promoter inducible by NF-\( \kappa \)B.

Human leukemia HL-60 cells were cultured in RPMI 1640 supple-

mented with 10% (v/v) heat inactivated FCS, 10 m\( \mu \)M HEPES, 100 \( \mu \)g/ml streptomycin, and 100 \( \mu \)M penicillin. HL-60 cells were differentiated to macrophage-like cells by treatment with 10 nM PMA for 3 days (35). All cultured cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Cell number and viability were assessed microscopically using trypan blue exclusion.

Isolation of murine bone marrow leukocytes and neutrophils

All animal use was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Montana State Un-

iversity. Murine bone marrow cells were flushed from tibias and femurs of BALB/c mice with HBSS using a syringe with 27-gauge needle. The cells were dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of RBC, as described previously (36). Leukocyte pellets prepared as described above were resuspended in 3 ml of 45% Percoll solution and layered on top of a Percoll gradient consisting of 2 ml each of 50, 55, 62, and 81% Percoll solutions in a conical 15-ml polypropylene tube. The gradient was centrifuged at 1600 \( \times \) g for 30 min at 10°C, and the cell band located between the 61 and 81% Percoll layers was collected. The cells were washed, layered on top of 3 ml of Histopaque 1119, and centrifuged at 1600 \( \times \) g for 30 min at 10°C to remove contaminating RBC. The pu-

rified neutrophils were collected, washed, and resuspended in HBSS .

Isolation of human neutrophils and mononuclear cells

Blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils and mononuclear cells were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of RBC, as described previously (38). Isolated neutro-

phils were washed twice and resuspended in HBSS . Neutrophil prepara-

tions were routinely >95% pure, as determined by light microscopy, and >98% viable, as determined by trypan blue exclusion. PBMC were iso-

lated from blood using dextran sedimentation and Histopaque 1077 gradi-

ent separation (39).

Analysis of phagocyte ROS production

ROS production was determined by monitoring t-012-ECL, which repre-

sents a sensitive and reliable method for detecting superoxide anion (\( \mathrm{O}_2^- \)) produc-

tion in vitro (40). In brief, phagocytes were aliquoted into wells (10000 cells/well) of 96-well flat-bottom white microplates, and test extracts or lyophilized fractions diluted in DMSO were added (final DMSO concentra-

tion of 1%). After preincubation at 37°C for the indicated times, an equal volume of PMA (0.05% BSA in HBSS) was added to each well, the plates were centrifuged, the medium was removed, and fresh HBSS supplemented with 40 \( \mu \)M t-012 and 8 \( \mu \)g/ml HRP was added. In some experiments, the plates were read directly without replacing the medium to evaluate PMA-

or zymosan-stimulated ROS production in the presence of the oenothein B or total E. angustifolium extract. Luminescence was monitored for 60 min (2-min intervals) at 37°C using a Fluoroscan Ascent FL microplate reader (Thermocolor). The curve of light intensity (in relative luminescence units) was plotted against time, and the area under the curve was calculated as total luminescence.

Xanthine/xanthine oxidase system

\( \mathrm{O}_2^- \) was generated in an enzymatic system consisting of 500 \( \mu \)M xanthine, 500 \( \mu \)M NBT, 3.75 \( \mu \)M U/xanthine oxidase, and 0.1 M phosphate buffer (pH 7.5), and \( \mathrm{O}_2^- \) production was determined by monitoring reduction of NBT to bluish monooxygenase dye at 560 nm in the presence or absence of oenothein B. The reactions were monitored with a SpectraMax Plus mi-

croplate spectrophotometer at 27°C. To evaluate whether oenothein B af-

fected the generation of \( \mathrm{O}_2^- \) by direct interaction with xanthine oxidase,
enzyme activity was evaluated by spectrophotometric measurement of uric acid formation from xanthine at 295 nm (41). The reaction mixture contained 500 μM xanthine, 5 μL/mL xanthine oxidase, and 0.1 M phosphate buffer (pH 7.5), and the reaction was monitored in the presence or absence of oenothein B at 27°C.

Ca2+ mobilization assay
Changes in intracellular Ca2+ were measured with a Flexstation II scanning fluorometer using a FLIPR 3 Calcium Assay Kit (Molecular Devices). Human and murine neutrophils or HL-60 cells, suspended in HBSS containing 10 mM HEPES, were loaded with FLIPR Calcium 3 dye following the manufacturer’s protocol. After dye loading, Ca2+ was added to the cell suspension (2.25 mM final), and cells were aliquotted into the wells of a flat-bottom black microplate (2 × 10^5 cells/well). The compound source plate contained dilutions of E. angustifolium extract or test compounds in HBSS or DMSO. Changes in fluorescence were monitored (λex = 485 nm, λem = 525 nm) every 5 s for 120 s at room temperature after automated addition of compounds to the wells. Maximum change in fluorescence, expressed in arbitrary units over baseline signal observed in cells treated with vehicle (HBSS or DMSO), was used to determine agonist response. Curve fitting and calculation of median effective concentration values (EC50) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software).

Chemotaxis assay
Human neutrophils were suspended in HBSS containing 2% (v/v) FBS (2 × 10^6 cells/ml), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe), as described previously (42). In brief, lower wells were loaded with 30 μL of HBSS containing 2% (v/v) FBS and E. angustifolium extract, the indicated concentrations of oenothein B vehicle control (DMSO or HBSS), or IL-8 and FMLP as positive controls. The number of migrated cells was determined by measuring ATP in lysates of transmigrated cells using a luminescence-based assay (CellTiter-Glo; Promega), and luminescence measurements were converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. The results are expressed as percentage of negative control and were calculated as follows: (number of cells migrating in response to test compounds/spontaneous cell migration in response to control medium) × 100. EC50 values were determined by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software).

Cytokine analysis
Cells were incubated for 24 h in culture medium supplemented with 3% (v/v) endotoxin-free FBS, with or without compounds or LPS as a positive control. Human PBMCs and THP-1 cells were plated in 96-well plates at a density of 2 × 10^5 cells/well. A human cytokine MultiAnalyte ELISArray Kit (SA Biosciences) was used to evaluate various cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF) in supernatants of PBMC or THP-1 cells. Human TNF-α or IL-6 ELISA kits (BD Biosciences) were used to confirm TNF-α or IL-6 levels in the cell supernatants. Cytokine concentrations were determined by extrapolation from cytokine standard curves, according to the manufacturer’s protocol.

Analysis of NF-kB activation
Activation of NF-kB was measured using an alkaline phosphatase reporter gene assay in THP1-Blue cells. Extracts, compounds, or LPS (100 ng/mL) were added, the cells (2 × 10^5 cells/well) were incubated for 24 h, and alkaline phosphatase activity was measured in cell supernatants using QUANTI-Blue mix (InvivoGen). Activation of NF-kB is reported as an absorbance at 655 nm and compared with positive control samples (LPS).

Cytotoxicity assay
Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega), according to the manufacturer’s protocol. Following treatment, the cells were allowed to equilibrate to room temperature for 30 min, substrate was added, and the samples were analyzed with a Fluoroscan Ascent FL.

In vivo analysis
Oenothein B was dissolved in saline and injected i.p. into 8–12 wk old female BALB/c mice. At 4 h postinjection, the mice were euthanized, their peripheral cavities were washed with 5 mL of HBSS, and the fluid was collected and centrifuged at 500g for 5 min. The resulting cell preparation was subjected to flow cytometric analysis using standard techniques. In brief, peritoneal cells were stained with FITC-conjugated RB6–CS5 (anti-GR-1, pan neutrophil stain), allophycocyanin-conjugated anti-CD45.2 (pan-leukocyte stain; disregards RBC, epithelial cells, and fibroblasts), and PE-conjugated anti-CD11b (stains macrophages and neutrophils, bright on highly activated cells). All incubation and wash buffers contained 2% horse serum to minimize background staining of the cell preparations. The samples were analyzed on a FACSCalibur equipped with a high-throughput sampler (BD Biosciences). Viable leukocytes were gated based on forward-scatter/side-scatter and positive staining for CD45. Of the viable CD45+ leukocytes, the percentage of neutrophils (identified by distinctive light scatter and staining of GR1bright/CD11b+alg) was determined.

In some experiments, serum samples were collected, and keratinocyte chemoattractant (KC) levels were quantified using a Mouse KC QuantiBrite ELISA kit (R&D Systems). Assay plates were read at 450 nm using a Molecular Devices VERSAMax microplate reader. KC levels were determined by extrapolation from recombinant KC standard curves, according to the manufacturer’s protocol.

Statistical analysis
Statistical analysis was performed using Prism 5. The data were analyzed by one way ANOVA, followed by Tukey’s Multiple Comparison Test, with the exception of the data from in vivo experiments, which were analyzed by Student’s t test. Statistically significant differences (p < 0.05) were compared with the appropriate controls are indicated.

Results
Effect of E. angustifolium extract on ROS production and NF-kB activity
To evaluate the effects of E. angustifolium extracts on phagocyte functional responses, we analyzed the effects of methanol extracts from this plant on phagocyte ROS production and NF-kB activation. As shown in Fig. 1, extracts from E. angustifolium dose-dependently activated ROS production in murine bone marrow leukocytes and induced NF-kB in human THP-1 monocytes.

Identification of the phagocyte-activating component in E. angustifolium extract
Based on these results, we fractionated the extract in efforts to identify the active component in this crude mixture. Concentrated methanol extract from E. angustifolium was fractionated by preparative Sephadex LH-20 chromatography and sixty 10-ml fractions were collected (Fig. 2A). These fractions were pooled into three subfractions, designated as S-1 to S-3, and activity of these subfractions was evaluated in the NF-kB reporter assay. Only subfraction S-3 activated NF-kB in this assay (Fig. 2B).

The pooled subfraction S-3 was rechromatographed twice more on a Sephadex LH-20 to obtain the final sample, which was concentrated to dryness and analyzed by HPLC, mass spectroscopy, and 1H- and 13C-NMR (supplemental Table S1). Based on comparison of mass spectroscopy and NMR data with those in the literature (34, 43–45), we found that the active component present in subfraction S-3 isolated from E. angustifolium was oenothein B (M = 1568; structure of the compound is shown in supplemental Figure S1).

As shown in supplemental Figure S2, the mass spectrum was characterized by the presence of monosodium (M plus Na) at m/z 1592.1 (1 m/z unit separation between isotopic peaks) and doubly charged disodium (M plus 2Na)+ at m/z 807.1 (0.5 m/z unit separation between peaks) adducts of oenothein B. The prominent series at m/z 1068.7 is apparently a noncovalent dimeric aggregate of the monosodium and disodium adducts. It should be noted that time-of-flight mass spectroscopy of polyphenols, including ellagitannins, tends to favor association with sodium ions because naturally occurring Na+ ions are abundant in these samples (46, 47).

4 The online version of this article contains supplementary material.
The $^1$H-NMR spectrum of our sample in D$_2$O contained six $^1$H-singlets and two $^2$H-singlets in the aromatic region (supplementary Table S1), which is in agreement with the presence of two galloyl and two valoneoyl moieties in oenothein B. Two glucopyranose residues gave well-resolved signals of sugar protons, with characteristic coupling similar to that of the oenothein B spectrum in acetone (44). Anomeric proton doublets appeared at $^1$H$_254$ 4.47 (J 9 Hz) and 5.47 (J 3.4 Hz), indicating that the anomeric hydroxyls of both glucose residues were nonacylated. Although an equilibrium occurs between the $^1$H$_251$- and $^1$H$_252$-forms of each of the glucopyranose rings, the $^1$H-NMR spectrum showed that the $^1$H$_251$-form dominates one ring, whereas the $^1$H$_252$-form dominates the other glucopyranose moiety. This is in contrast to oenothein F, an isomer of oenothein B, where a mixture of anomeric forms for both rings results in a more complex $^1$H-NMR spectrum (43). Because previous NMR analysis of oenothein B has been performed in acetone-d$_6$, we obtained additional $^1$H-NMR spectra of our sample in acedone-d$_6$ with 2% (v/v) D$_2$O. Although some of the signals were overlapped by the broad singlet of residual water protons, many signals characteristic of oenothein B were observed. For example, resonances of $^1$H$_251$-glucose H-1 at $^1$H$_254$ 6.17 (d, J 3 Hz) and of $^1$H$_252$-glucose H-5 at $^1$H$_254$ 4.11 (dd, J 1 5, J 2 10 Hz) are very similar to the corresponding signals of oenothein B reported previously [$^1$H$_254$ 6.18 and $^1$H$_254$ 4.12, respectively, in acetone-d$_6$–D$_2$O (43); $^1$H$_254$ 6.24 and $^1$H$_254$ 4.14, respectively, in acetone-d$_6$ (44)]. These values are quite distinct from any glucose chemical shifts in the $^1$H-NMR spectrum of oenothein D (43). Eight singlets of galloyl and valoneoyl aromatic rings in the spectrum of our isolated sample were also very close (rms deviation of 0.07 ppm) to the corresponding signals of oenothein B in acetone-d$_6$ (44). Note that chemical shifts of aromatic moieties differ significantly between oenothein isomers, and galloyl and valoneoyl NMR signals for oenothein D and oenothein F are in quite different positions than for oenothein B (43). Taken together, our data demonstrate that the primary phagocyte-activating component of *E. angustifolium* extract is oenothein B.

**Effect of oenothein B on ROS production**

In studies described above, the *E. angustifolium* extract and subfractions were removed from the cells and replaced with fresh...
medium before analysis of ROS production by treated phagocytes. We used this approach to avoid antioxidant effects of potential plant-derived compounds, as it is known that *E. angustifolium* extract contains flavonoids, such as myricetin, kaempferol, and quercetin, which have antioxidant activity (45, 48, 49). To directly evaluate this issue, we analyzed potential scavenging effects of crude *E. angustifolium* extract or oenothein B on ROS produced by zymosan- and PMA-stimulated murine bone marrow leukocytes and human neutrophils. We found that the ROS signal was dose-dependently decreased when crude extract (5–20 µg/ml) or oenothein B were present. Indeed, the ROS signal was completely lost when oenothein B was present in the assay at concentrations >2–3 µM (Fig. 3, A and B). IC₅₀ values were 50 and 90 nM oenothein B for scavenging ROS produced by PMA-stimulated human neutrophils and murine bone marrow leukocytes, respectively, and 110 and 505 nM oenothein B for scavenging ROS produced by zymosan-stimulated human neutrophils and murine bone marrow leukocytes, respectively. Because ROS are generated extracellularly by PMA-stimulated cells, whereas zymosan-stimulated cells generate intracellular ROS, which then can diffuse out of the cell, it is not surprising that oenothein B had lower IC₅₀ values for scavenging ROS in the PMA-stimulated cell system.

To verify oenothein B was an effective ROS scavenger, we analyzed scavenging activity in an enzymatic, O₂⁻-generating system. As shown in Fig. 3C, oenothein B (1–50 µM) effectively scavenged O₂⁻ in a xanthine/xanthine oxidase assay. To confirm the effect of oenothein B was due to ROS scavenging and not direct inhibition of xanthine oxidase itself, we measured xanthine oxidase-dependent production of uric acid from xanthine and found no effect of oenothein B over the entire concentration range tested (Fig. 3C). Thus, it is clear that, in addition to its phagocyte-activating properties, oenothein B is an effective scavenger of phagocyte-derived ROS, which is consistent with previous reports demonstrating oenothein B has antioxidant properties (26).

To eliminate or decrease antioxidant effects of the compounds/extract under investigation, the medium containing test samples was removed and replaced with fresh medium before subsequent analysis of ROS production. As shown in Fig. 4A, the kinetics of murine bone marrow leukocyte ROS production induced by *E. angustifolium* extract and isolated oenothein B were similar, and ROS production was induced in a time-dependent manner by oenothein B (Fig. 4B). Likewise, purified murine neutrophils dose-dependently generated ROS in response to oenothein B (Fig. 4C). At concentrations of 10–50 µM oenothein B, the response plateaued, which may be due to competing antioxidant activity of compounds remaining even after medium replacement. SOD (50 U/ml) completely (>95%) inhibited ROS production in oenothein B-stimulated neutrophils (data not shown), indicating the response was primarily due to NADPH oxidase-generated O₂⁻.

To evaluate the role of oenothein B structural components on phagocyte activation, we evaluated ROS production by phagocytes treated with substructures of oenothein B, including pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, and two related tannins, PGG and corilagin (structures of the compounds are shown in supplemental Figure S3). Only oenothein B and PGG activated ROS production over the concentration range tested (10–100 µM). Fig. 5 shows the response to these compounds at selected concentrations, as compared with that induced by 25 µM oenothein B.

**Effect of *E. angustifolium* extract and purified oenothein B on phagocyte Ca²⁺ mobilization and chemotaxis**

The ability of *E. angustifolium* extract and oenothein B to induce an intracellular Ca²⁺ flux in neutrophils was examined. Crude *E. angustifolium* extract induced a dose-dependent increase in [Ca²⁺], in human neutrophils (Fig. 6A), with an EC₅₀ of 53 µg/ml. Likewise, oenothein B induced a rapid and dose-dependent Ca²⁺ flux in human (Fig. 6A) and murine neutrophils (Fig. 6B), with EC₅₀ values of 25.5 and 18.3 µM, respectively. The peak levels of intracellular Ca²⁺ were reached within 60 s of exposure to oenothein B and then decreased. Note however, that [Ca²⁺] were still higher than the basal levels at 3 min postexposure, which is...
similar to the response observed in cells treated with fMLF (Fig. 6). If cells were treated in the absence of extracellular Ca\(^{2+}\), no Ca\(^{2+}\) flux was observed, suggesting that oenothein B treatment induced influx of extracellular Ca\(^{2+}\) (data not shown).

Treatment of PMA-differentiated human HL-60 with oenothein B resulted in an intracellular Ca\(^{2+}\) flux, whereas no significant changes in \([\text{Ca}^{2+}]_i\) were observed in undifferentiated HL-60 cells (Fig. 7, upper panel). In comparison, the Ca\(^{2+}\) ionophore ionomycin increased \([\text{Ca}^{2+}]_i\) in both nondifferentiated and differentiated HL-60 cells (Fig. 7, lower panel). These findings suggest that oenothein B does not act as an ion channel and that phagocyte activation by this compound depends on phagocyte differentiation and/or level of receptor expression.

Similar to the results observed for ROS production, pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, and corilagin failed to induce an intracellular Ca\(^{2+}\) flux in human and murine neutrophils, while PGG treatment resulted in increased \([\text{Ca}^{2+}]_i\), although with a higher EC\(_{50}\) (31.3 \(\mu\)M) than oenothein B.

Previous reports indicated that Ca\(^{2+}\) mobilization is associated with chemotactic activity of various agents (50). Thus, we examined the effect of E. angustifolium extract and oenothein B on human neutrophil chemotaxis. Both the extract (data not shown) and purified oenothein B exhibited neutrophil chemotactic activity and dose-dependently induced neutrophil migration, with EC\(_{50}\) values of 46 \(\mu\)g/ml and 11.8 nM, respectively (Fig. 8). Note, however, that the magnitude of this response was generally lower than that induced by the positive controls, fMLF and IL-8, which had EC\(_{50}\) values of \(~1\) and 12 nM, respectively (Fig. 8).

**Effect of oenothein B on phagocyte NF-\(\kappa\)B activity**

To evaluate possible signaling pathways involved in the immunomodulatory activity of oenothein B, we used a transcription factor-based bioassay for NF-\(\kappa\)B activation in human THP-1 monocytes. NF-\(\kappa\)B transcription reporter activity was time-dependently induced by oenothein B (25 \(\mu\)M) and LPS (100 ng/ml), but with slightly different kinetics for these stimuli (Fig. 9A).
Statistically significant activation of the NF-κB reporter was observed at 12 h in LPS-treated cells, whereas NF-κB reporter activity induced by oenothein B only reached statistical significance at 18 h. Note however, that the response does appear to be increasing in oenothein B-treated cells at 12 h, and that the level of NF-κB reporter activity induced by oenothein B is equal to or even slightly higher than that induced by LPS at 18 h. Thus, it is likely that the level of NF-κB reporter activity reaches statistical significance somewhere between 12 and 18 h in oenothein B-treated cells (Fig. 9A), suggesting that oenothein B induces NF-κB with similar or slightly delayed kinetics compared with LPS. As shown in Fig. 9B, the response of THP-1 monocytes to oenothein B was dose-dependent, and the level of NF-κB reporter activity induced by oenothein B at concentrations of 25–50 μM far exceeded that induced by 100 ng/ml LPS. Importantly, these concentrations of oenothein B exhibited little toxicity in THP1-Blue cells over the 24 h assay period (Fig. 9B). Finally, treatment of cells with both oenothein B and LPS together led to significantly enhanced responses at 12, 18, and 24 h, suggesting there was an additive or possibly synergistic effect of these two agents in activating NF-κB and again supporting their similar kinetics of activation (Fig. 9A).

Pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, carilagin, and PGG were also evaluated for activity in the NF-κB alkaline phosphatase reporter assay. All of these compounds, including PGG, failed to induce NF-κB reporter activity when tested in the concentration range of 10–100 μM. Supplemental Figure S4 shows the response to these compounds at selected concentrations, as compared with that induced by 25 μM oenothein B.

NF-κB activation by was not due to endotoxin contamination in our samples, as analysis of oenothein B for endotoxin using a LAL assay showed that the isolated compound contained <0.625 ng/mg endotoxin, which is considered to be insignificant for various bioactive products (51, 52). Because it is possible that oenothein B could inhibit a pathway in the LAL coagulation cascade, for example by binding to one of the protein factors, we also pretreated oenothein B by elution through a column of endotoxin-removing gel. We found that the pretreated oenothein B (oenothin B-ER) was just as active as untreated sample (supplemental Fig. S4), again confirming the absence of endotoxin contamination.

**Effect of E. angustifolium extract and purified oenothein B on phagocyte cytokine production**

Previous studies showed that oenothein B stimulated release of IL-1β from murine and human macrophages (28); however, the effect of this compound on other cytokines has not been evaluated. To address this issue, condition medium from THP-1Blue monocytes and human PBMCs was analyzed using a cytokine ELISA semiquantitative array. Among the 12 cytokines analyzed, five were consistently induced in PBMCs (>10-fold) by 10 μM oenothein B, as compared with control cells. These included IFN-γ (fold increase (FI) = 11), IL-1β (FI = 13), GM-CSF (FI = 15), TNF-α (FI = 31), and IL-6 (FI = 34) (Fig. 10A). IL-8 production was inconclusive because of high background production by PBMCs (data...
not shown), a problem which has also been documented previously (e.g., Ref. 53). Human monocytic THP-1Blue cells, treated with 25 μM oenothein B produced high levels of TNF-α (FI = 94), and IL-8 (FI = 98) (Fig. 10A).

To quantify dose-dependent effects of E. angustifolium extract and oenothein B on cytokine production, levels of TNF-α and IL-6 were determined by ELISA of supernatants from treated cells. Untreated cells produced negligible amounts of TNF-α and IL-6, whereas incubation of THP-1Blue monocytes and PBMCs with crude extract (Fig. 10, B and D) or purified oenothein B (Fig. 10, C and E) enhanced TNF-α and IL-6 production in a dose-dependent manner. Note, however, that pretreatment of oenothein B with endotoxin-removing gel no effect on its activity, indicating that the induction of TNF-α and IL-6 production was not due to endotoxin contamination (data not shown).

**In vivo analysis of oenothein B**

To evaluate the effect of oenothein B in vivo, mice were treated by intraperitoneal injection of oenothein B, and neutrophil recruitment to the peritoneum was evaluated. As shown in Fig. 11, oenothein B significantly induced neutrophil recruitment (~10-fold increase over saline controls). Because the mice were analyzed 4-h postinjection, neutrophils were the primary phagocyte recruited. As expected, little change in monocyte/macrophage levels was observed in this short period of time (data not shown). The primary neutrophil chemotactic agent at inflammatory sites is IL-8 (KC in mice). Thus, we evaluated KC levels in mice treated with oenothein B and found that oenothein B dose-dependently induced significant levels of serum KC, which correlated with the observed recruitment of neutrophils into the peritoneum (Fig. 12). Thus, these studies verify our in vitro experiments and confirm that this oenothein B is active in vivo.

**Discussion**

Extracts from E. angustifolium have been reported to be beneficial for treating a variety of medical problems, such as gastrointestinal, and prostate diseases, and to improve the healing of wounds (11). However, little is known regarding the effects of E. angustifolium on innate immune functions. In this study, we demonstrate that E. angustifolium extracts can induce or enhance phagocyte functional responses and that the active principle in these extracts is oenothein B. Because oenothein B is a major component of Epilobium (24), we propose that the effects of oenothein B on innate immune function likely contribute to the therapeutic efficacy of Epilobium extracts.

Oenothein B was first isolated from Oenothera erythrosepala (Onagraceae) (54) and was subsequently found in Eucalyptus, Eugenia species, and Lythraceae species (31). Previous studies on oenothein B have shown that it exhibits significant antioxidant (26), antitumor (27, 28, 30, 55), antibacterial (56), and antiviral

**FIGURE 8.** Human neutrophil chemotactic response to oenothein B. Neutrophil chemotaxis in response to the indicated concentrations of oenothein B, fMLF, and IL-8 was analyzed in chemotaxis chambers, as described. The data are presented as the number of migrated cells (mean ± SD; n = 3). A representative experiment from three independent experiments is shown.

**FIGURE 9.** Effect of oenothein B on NF-κB activity and cell viability in THP1-Blue monocytes. A, THP1-Blue monocytes (2 × 10⁵ cells/well) were incubated for the indicated times with control medium, 25 μM oenothein B, 100 ng/ml LPS, or 25 μM oenothein B plus 100 ng/ml LPS. Alkaline phosphatase release was analyzed spectrophotometrically in the cell supernatant. Statistically significant differences (*, p < 0.05; **, p < 0.01; ***, p < 0.001) vs medium control are indicated. B, THP1-Blue monocytes (2 × 10⁵ cells/well) were incubated for 24 h with the indicated concentrations of oenothein B or LPS, and alkaline phosphatase release was analyzed spectrophotometrically in the cell supernatant. Cell viability was also determined using a CellTiter-Glo Luminescent Cell Viability Assay Kit. For both panels, the data are presented as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments.
activities, although the mechanisms involved are not well defined. Most studies on oenothein B have focused on its antitumor activity, and it has been shown to inhibit poly-(ADP-ribose) glycohydrolase, 5α-reductase, and aromatase (24, 57, 58), and also to induce a neutral endopeptidase in prostate cancer cells (34). Miyamoto et al. (27) reported that oenothein B had potent antitumor activity upon intraperitoneal administration to mice before tumor inoculation and suggested that this may be due to enhancement of the host-immune system via macrophage activation. However, essentially nothing else has been reported on the effects of oenothein B on innate immunity. We show in this study that oenothein B activates phagocytic cells, including monocyte/macrophages and neutrophils, resulting in increased intracellular $Ca^{2+}$, production of ROS and cytokines, and chemotactic activity. Additionally, we demonstrated that oenothein B induced IL-8 production and neutrophil recruitment to the peritoneum of treated mice. Given the critical role played by phagocytes in innate immunity against pathogens and their contribution to tumor cell destruction (reviewed in Ref. 59), our data support the possibility that at least part of the observed therapeutic effects of oenothein B and *Epilobium* extracts in general are due to enhancement of innate immune responses.

In addition to enhancement of innate immune function, oenothein B also was found to scavenge ROS generated by activated phagocytes or by an enzymatic system, which confirms previous reports on the antioxidant activity of this compound determined using a radical scavenging assay (26, 45). Thus, oenothein B is able to stimulate local innate immunity but may also protect tissues from excessive ROS production. Although the antioxidant activity of polyphenols has been assumed to be the primary therapeutic property, recent studies indicate that many polyphenols directly impact cellular signaling events, which is independent of their antioxidant activity (e.g., see Ref. 33). Because antioxidant capacity is often diminished or even lost during absorption in vivo, the primary therapeutic properties of oenothein B and other polyphenols may indeed be due to direct modulation of cellular activity, such as the modulation of innate immune functions shown in this study.

FIGURE 10. Effect of *E. angustifolium* extract and oenothein B on cytokine production by human THP-1Blue monocytes and PBMCs. A, THP-1Blue cells and PBMCs were incubated for 24 h with 25 μM oenothein B, and production of cytokines in the supernatants was evaluated using a MultiAnalyte ELISAArray kit. Cytokine expression is shown as an OD ($A_{550}$-$A_{570}$) ratio normalized to background. B–E, THP-1Blue cells and PBMCs were incubated for 24 h with the indicated concentrations *E. angustifolium* extract (B and D) or oenothein B (C and E). Cell-free supernatants were collected, and secreted TNF-α (B and C) and IL-6 (D and E) were quantified by ELISA. For B–E, the data are presented as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments.
The nonspecific nature of immunomodulators makes them attractive because they can be used to treat a broad-spectrum of infections and are not susceptible to antibiotic resistance (60). In general, immunomodulators mimic the natural mechanisms used by pathogens to stimulate innate immunity and thus are potentially beneficial in preventing infection (60). Thus, the balance between therapeutic and proinflammatory properties is important to consider when evaluating immunomodulators, and the goal is to enhance or prime local host defense without inducing excessive or systemic inflammation. This balance is dependent on pharmacodynamic and pharmacokinetic properties of the compound and must be determined empirically. For example, CpG DNA, an immunomodulator with therapeutic promise, induces a range of phagocyte inflammatory responses via TLR9, which leads to beneficial Th1-type responses (60, 61). Conversely, excessive activation of TLR9 can contribute to detrimental inflammatory states (e.g., Ref. 62). Likewise, we suggest that therapeutic concentrations of oenothein B could prime or enhance innate immune cells without inducing adverse inflammatory responses, which is supported by our in vivo experiments. In contrast, our in vitro data suggest that treatment with high concentrations of oenothein B could lead to excessive inflammation if sufficient local concentrations were achieved. Thus, further analyses are clearly needed to further evaluate the specific pharmacological properties of oenothein B in vivo.

Initiation of intracellular Ca^{2+} flux is one of the earliest events associated with phagocyte receptor activation and plays a central role in receptor-mediated intracellular signaling events (50). Furthermore, Ca^{2+} mobilization is required for ROS production in phagocytes, mainly through the activation of NADPH oxidase (e.g., Ref. 63). We found that oenothein B induced a transient elevation of [Ca^{2+}]_{i} in neutrophils, indicating this compound is a phagocyte agonist; however, compounds structurally related to the building blocks of oenothein B (pyrocatechol, gallic acid, pyrogallol, and 3,4-dihydroxybenzoic acid) had did not induce Ca^{2+} mobilization or other phagocyte responses. In contrast, one of the two tannins tested (PGG) did induce a Ca^{2+} and ROS production in neutrophils. Although various tannins and other compounds with galloyl groups, including galloylpunicalagin, woodfordin, and cotton-derived tannins, have been reported previously to activate Ca^{2+} flux in phagocytes (64–67), this is the first report evaluating the effects of oenothein B on phagocyte Ca^{2+} mobilization, ROS production, and chemotaxis.

A number of ellagitannins and other tannins have been shown to activate phagocyte functions, including cytokine production and phagocytosis. For example, the Ellagitannin geraniin, which is isolated from Geranium funbergii, has been reported to induce macrophage phagocytosis (68). Likewise, cuphphin D1, tellimagrandins I and II, rugosin A, casuarictin, coriariin, agrimoniin, and PGG have been shown to induce IL-1β and/or TNF-α production by human peripheral mononuclear cells and macrophages in vitro (28, 69–71). We also found that oenothein B induced IL-1β and TNF-α production by human monocyte/macrophages, but also provide the novel finding that IFN-γ, GM-CSF, IL-6, and IL-8 are associated with phagocyte receptor activation and plays a central role in receptor-mediated intracellular signaling events (50).
also induced in human monocyte/macrophages. Additionally, we found that intraperitoneal administration of oenothein B induced a significant level of KC in treated mice and that KC induction directly correlated with neutrophil influx into the peritoneum, demon-
strating that cytokine induction defined in this report is relevant in vivo. Thus, the ability of oenothein B to induce these cytokines may also play an important role in the microbicidal, viricidal, and antitumor effects of this compound. For example, IL-1β is capable of upregulating the activity of tumoricidal NK cells and inducing antitumor reactivity in the regional lymph nodes spleen (reviewed in Ref. 72). Among the proinflammatory cytokines, IL-6 is one of the most important mediators of fever and the acute-phase response (73). TNF-α has direct in vitro and in vivo cytotatic and cytolfidal effects and, together with IL-6, is also considered as a major immune and inflammatory mediator (73). One of the most prominent characteristics of TNF-α is its ability to cause apoptosis of tumor cells, resulting in tumor necrosis (74). TNF-α also plays a pivotal role in host defense and can act on macrophages in an autocrine manner to enhance various functional responses and in-
duce the expression of a number of other immunoregulatory and inflammatory mediators (75).

NF-κB is activated in response to stimulation by inflammatory agents, including LPS, and activation of NF-κB is an essential step in inducing proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, receptors, and inhibitors of apop-
tosis (reviewed in Ref. 76). Treatment of phagocytes with oenothein B resulted in the activation of NF-κB. In addition, treatment of cells with both oenothein B and LPS resulted in an even greater NF-κB response, suggesting a synergistic effect. Therefore, the ability to activate phagocyte NF-κB signaling provides further ev-
idence that oenothein B possesses immunomodulatory properties. The synergistic effect of oenothein B and LPS is consistent with studies of Feldman (25), who suggested that dimeric tannins could mimic the lipid A moiety of LPS. In contrast with our data, Chen et al. (77) reported that oenothein B modestly inhibited LPS-in-
duced NF-κB activity in Bcl-2-overexpressing murine RAW264.7 macrophages. Although the reasons for this difference are not clear, it is possible that this may be due to differences in the cell lines used. Alternatively, NF-κB activation by oenothein B could be indirect and mediated by cytokines induced during the assay, as NF-κB reporter activity only began to increase at after 12 h incu-
bation with oenothein B and was not significant until 18 h. Thus, further studies are now in progress to evaluate this issue and de-
termine which receptor(s) and intercellular pathway(s) are in NF-κB activation and expression of various cytokines induced by oenothein B.

Oenothein B and a related tannin, PGG, induced ROS produc-
tion and Ca2+ mobilization in phagocytes, whereas, the mono-
ermic polyphenols pyrocatechol, gallic acid, pyrogallol, and 3,4-
dihydroxybenzoic acid were inactive. On the other hand, only oenothein B, which has a dimeric macrocyclic structure (see sup-
plemenal Fig. S1), induced NF-κB activity. In addition, HL-60 cells responded to oenothein B only after differentiation. These findings suggest oenothein B may be activating phagocytes through a specific receptor or cellular target, which is yet to be identified. Tannins bind to a wide range of targets, including phospholipids, carbohydrates, and proteins (78–80). For exam-
ple, Teng et al. (81) reported that the ellagitannin rugosin E acti-
ated platelet Ca2+ flux by acting as an ADP receptor agonist. Thus, it is possible that oenothein B could be interacting with a number of extracellular membrane targets, such as receptors or lipid rafts involved in regulating phagocyte activation. Neverthe-
less, further work is necessary to identify the target of oenothein B.

As reported previously, oenothein B is a major component of Epilobium extracts and can be present at concentrations up to 14% (~9 μM in 100 μg/ml extract), although the level varies between species and is reported to be 4.5% in Epilobium angustifolium (24). However, it has not yet been determined whether these concentrations vary in different regions of the world or between different lots of plants. We found that Epilobium angustifolium extracts induced intracel-

ular Ca2+ flux, ROS production, chemotaxis, and cytokone production in qualitatively similar patterns as purified oenothein B, supporting the conclusion that oenothein B is indeed the active component in these extracts. However, it is also apparent that the extracts were more potent than oenothein B if we use the estimated concentration of 4.5% in our extracts (~3 μM oenothein B in 100 μg/ml extract). The reasons for this difference are not clear; how-
ever, this observation is supported by studies from Kiss et al. (34) who showed that E. angustifolium extract was ~10-fold more potent that pure oenothein B in inducing neutral endopeptidase activity in prostate cancer cells. One possibility is that other constituents in the extract may help to increase oenothein B bioavailability or stability. For example, the solubility of the active component in a crude extract can often be reduced when the component is purified. It is also possible that some reactive groups are altered during purification, thus affecting activity. These issues are currently under investigation.

Overall, our studies demonstrate that oenothein B activates a number of phagocyte functions, including Ca2+, NADPH oxidase activity, chemotaxis, and cytokone production. In addition, we es-

tablished that oenothein B can modulate phagocyte activity in vivo. The ability of this compound to modulate innate immune functions suggests that at least part of the reported effects of Épi-
lobeum extracts and purified oenothein B on wound healing and inhibition of tumor growth is through modulation of macrophage function. These studies suggest that oenothein B may serve as a promising lead for further therapeutic development.

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Disclosures

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Supplemental Material

for

Immunomodulatory Activity of Oenothein B Isolated from *Epilobium angustifolium*

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Supplemental Table S1. NMR Spectroscopy of Subfraction S-3

$^1$H NMR [500 MHz, D$_2$O]

$\beta$-glucose: $\delta$ 4.47 (d, $J$ 9 Hz, H-1), 5.09 (dd, $J_1$ 6.5, $J_2$ 9 Hz, H-2), 5.29 (t, $J$ 9.5 Hz, H-3), 4.89 (t, $J$ 10.5 Hz, H-4), 3.92 (dd, $J_1$ 5.5, $J_2$ 10.5 Hz, H-5), 4.45 (d, $J$ 12.5 Hz, H-6), 3.75 (d, $J$ 12.5 Hz, H-6); ring A: 6.41 (s, H-6); ring B: 6.28 (s, H-6’); ring C: 6.53 (s, H-6’); ring G: 6.95 (2H, s, H-2” and H-6”); $\alpha$-glucose: 5.47 (d, $J$ 3.4 Hz, H-1), 5.36 (dd, $J_1$ 3.4, $J_2$ 10.5 Hz, H-2), 5.60 (t, $J$ 10 Hz, H-3), 4.99 (t, $J$ 9 Hz, H-4), 4.53 (dd, $J_1$ 7, $J_2$ 9 Hz, H-5), 4.48 (d, $J$ 12 Hz, H-6), 3.73 (d, $J$ 12 Hz, H-6); ring A’: 6.15 (s, H-6’); ring B’: 6.15 (s, H-6’); ring C’: 6.42 (s, H-6’); ring G’: 6.57 (2H, s, H-2” and H-6’).

$^{13}$C NMR [400 MHz, D$_2$O]

$\beta$-glucose: $\delta$ 94.8 (C-1), 74.8 (C-2), 71.0 (C-3), 72.2 (C-4), 70.6 (C-5), 63.5 (C-6); ring A: 124.6 (C-1’), 116.3 (C-2’), 143.5 (C-3’), 136.1 (C-4’), 145.8 (C-5’), 106.5 (C-6’), 169.8 (CO); ring B: 125.0 (C-1’), 116.7 (C-2’), 146.4 (C-3’), 134.4 (C-4’), 148.0 (C-5’), 105.4 (C-6’), 169.5 (CO); ring C: 115.0 (C-1’), 139.7 (C-2’), 140.5 (C-3’), 140.8 (C-4’), 142.9 (C-5’), 113.7 (C-6’), 168.8 (CO); ring G: 119.2 (C-1’), 108.7 (C-2” and C-6”), 144.3 (C-3” and C-5”), 138.6 (C-4”), 166.3 (CO); $\alpha$-glucose: 89.5 (C-1), 74.3 (C-2), 69.9 (C-3), 69.5 (C-4), 67.4 (C-5), 63.2 (C-6); ring A’: 121.4 (C-1’), 115.1 (C-2’), 144.0 (C-3’), 134.8 (C-4’), 144.8 (C-5’), 106.9 (C-6’), 169.1 (CO); ring B’: 124.8 (C-1’), 116.7 (C-2’), 144.3 (C-3’), 135.9 (C-4’), 147.5 (C-5’), 106.5 (C-6’), 169.6 (CO); ring C’: 114.3 (C-1’), 137.5 (C-2’), 139.1 (C-3’), 141.6 (C-4’), 142.3 (C-5’), 109.8 (C-6’), 167.1 (CO); ring G’: 117.7 (C-1”), 110.3 (C-2” and C-6”), 144.4 (C-3” and C-5”), 138.6 (C-4”), 169.4 (CO).
Supplemental Figure S1. Chemical Structure of Oenothein B
Supplemental Figure S2. Mass Spectrum of Subfraction S-3

Mass spectrometry experiments were performed using a Bruker Microtof high resolution time of flight mass spectrometer (Bruker Daltonics, Inc., Billerica, MA).
Supplemental Figure S3. Structures of Related Compounds Under Investigation

- Pyrocatechol
- Pyrogallol
- Gallic acid
- 3,4-dihydroxybenzoic acid (protocatechuic acid)
- Corilagin
- 1,2,3,4,6-pentakis-O-galloyl-β-D-glucose (PGG)
Supplemental Figure S4. Effect of Oenothein B and Related Compounds on NF-κB Activity

THP1-Blue monocytes (2×10^5 cells/well) were incubated for 24 hr with 100 μM pyrocatechol, gallic acid, pyrogallol, and 3,4-dihydroxybenzoic acid (DHB); 50 μM corilagin and PGG; and 25 μM oenothein B (OB) and oenothein B pretreated with endotoxin-removing gel (OBr). Alkaline phosphatase release was analyzed spectrophotometrically in the cell supernatant. The data are presented as mean±S.D. of triplicate samples from one experiment that is representative of three independent experiments. Statistically significant differences (***P<0.001) versus buffer or DMSO controls are indicated.