N-Glycidyl D-Tryptophan Ether-Based Ointment with Anti-Infective, Anti-Inflammatory, and Wound-Healing Properties

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Supplementary file

Materials and Methods

Semi-quantitative RT-PCR analysis. The expression of different pro-inflammatory cytokine molecules was analyzed by semi-quantitative RT-PCR using a RT-PCR kit from BD Clontech as described earlier [1-2]. Briefly, total RNA was isolated from polymer treated or untreated cells by using the RNA isolation kit (Qiagen) and then digested with DNase to remove contaminating genomic DNA. Then, 1 μg of DNase-digested RNA was reverse transcribed using oligo $(dT)_{12–18}$ as primer and MMLV reverse transcriptase (BD Clontech) in a 20-μl reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium Taq DNA polymerase and gene specific primers. The following primers were used to amplify mouse pro-inflammatory cytokines: IL-1B: sense: 5′-ATG GCA ACT GTT CCT GAA CTC AAC T -3′; antisense: 5′-CAG GAC AGG TAT AGA TTC TTT CCT TT -3′; IL-6: sense: 5′-TGG AGT CAC AGA AGG AGT GGC TAA G -3’; antisense: 5’-TCT GAC CAC AGT GAG GAA TGT CCA C -3’; TNF-α: sense: 5′-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3′; antisense: 5′-GTATGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3′; CD11B: sense: 5′-

CAG ATC AAC AAT GTG ACC GTA TGG G -3′; antisense: 5′- CAT CAT GTC CTT GTA CTG CCG CTT G-3′; GAPDH: sense: 5′-GGT GAA GGT CGG TGT GAA CG-3′, antisense: 5′-TTG GCT CCA CCC TTC AAG TG-3′. Amplified products were electrophoresed on 1.8% agarose gels and visualized by ethidium bromide staining. GAPDH was used as control to ascertain that an equivalent amount of cDNA was synthesized from RNA and used during PCR for different samples.

Real-time PCR analysis. It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems, Foster City, CA) as described earlier [3-5]. All primers and FAM-labeled probes for IL-1B, IL-6, TNF-α, CD11B and GAPDH were obtained from Applied Biosystems. The mRNA expression of all the genes was normalized to the label of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software.

Assay of IL-1β, TNF-α and IL-6 promoter-driven reporter activity

RAW cells plated at 50–60% confluence in 12-well plates were cotransfected with 0.25 μg of pIL-1β-Luc, pIL-6-Luc, pIL-TNF-α-Luc and 25 ng of pRL-TK (a plasmid encoding Renilla luciferase, used as transfection efficiency control; Promega) using Lipofectamine Plus (Invitrogen). After 24 h of transfection, cells were treated with different concentrations of Tryptophan and Tryptophan polymer for 2 h prior to the addition of LPS under serum free condition for 6 h. Firefly and Renilla luciferase activities were analyzed in cell extracts using the Dual Luciferase kit (Promega) in a TD-20/20 Luminometer (Turner Designs) as described earlier [6-8]. Relative luciferase activity of cell extracts was typically represented as the ratio of firefly luciferase value:Renilla luciferase value $\times 10^{-3}$.

Antioxidant properties

DPPH free radicals scavenging activity assay

Determination of the free radical scavenging activity of both tryptophan and tryptophan polymer was carried out using a modified quantitative DPPH (1, 1-diphenyl-2-picrylhydrazyl; Sigma Aldrich, St. Louis, MO, USA) assay [9]. Various concentrations of tryptophan and tryptophan polymer were prepared (100, 50, 25, and 10 $\mu g.ml^{-1}$). Gallic acid was used as a positive control at concentrations of 100, 50, 25, and 10 $\mu g.ml^{-1}$. Blank samples were run using 1 ml methanol in place of the sample. One ml of 0.2 mM DPPH in methanol was added to 1 ml of the test solution, or standard, plus 1 ml of methanol for dilution and allowed to stand at room temperature in a dark chamber for 30 min. The change in color from deep violet to light yellow was then measured at 517 nm using UV–Vis spectrophotometer (ThermoFisher scientific, USA). Inhibition of free radical in percent (I %) was calculated according to the equation:

$$I\ \% = [(A_0 - A_1)/A_0] \times 100$$

A_0 being the absorbance of the control reaction (containing all reagents except for the extract) and A_1 the absorbance of the extract. Measurements were carried out in triplicates.

Gene-specific LA-QPCR assays

Another part of the above treated cells were harvested for genomic DNA extraction using the QIAGEN Genomic-tip 20/G kit (Qiagen) as per the manufacturer's directions. This kit is particularly useful, as it minimizes DNA oxidation during the isolation step and has been previously used for LA-QPCR assays [10-12]. After quantitation by Pico Green (Molecular Probes) in a 96-well black bottomed plate, the genomic DNA was digested with the *E. coli* enzymes Fpg (NEB) and Nei (NEB), which are able to remove a variety of oxidized purine and pyrimidine bases and to induce strand breaks at the sites of unrepaired oxidized base by

cleaving the phosphodiester bond with their associated AP lyase activity, facilitating efficient detection of DNA damage by inhibiting Taq polymerase to surpass the lesion site, thereby decreasing the amplification efficiency. *E. coli* enzymes are chosen for the purpose of inducing strand breaks because of their robust activity to remove oxidized bases on double stranded DNA whereas Nei homologs in mammalian cells, like NEIL2 prefers lesions in single-stranded DNA over duplex DNA and interacts with a number of transcription factors, *in-vivo,* including RNA polymerase II, which help to open up the DNA strands and thus, to remove bases in a transcription-coupled repair pathway. Gene-specific LA-QPCR assays [29] for measuring DNA damage were performed using Long Amp Taq DNA Polymerase (New England Biolabs) to amplify a 10.4 kb region of the HPRT or 12.2 kb of the POLB gene in human genomic DNA using the following primers: 5′-TGG GAT TAC ACG TGT GAA CCA ACC-3′ and 5′-GCT CTA CCC TCT CCT CTA CCG TCC-3′ for HPRT and 5′-CAT GTC ACC ACT GGA CTC TGA AC-3′ and 5′-CCT GGA GTA GGA ACA AA ATT GCT-3′ for POLB. Preliminary assays were carried out to ensure the linearity of PCR amplification with respect to the number of cycles and DNA concentration. The final PCR reaction condition was standardized at 94 ^{0}C-30 sec^{-1} cycle; 94 ^{0}C-30 sec, 58 ^{0}C 30 sec, 65 ^{0}C 10 min for 25 cycles; 65^{0}C 10 min^{-1} cycle and 15 ng of DNA template was used for each reaction. Since amplification of a small region would be independent of DNA damage, a small DNA fragment was also amplified for normalization of long amplicons using the following primers: 5′-TGC TCG AGATGT GAT GAA GG-3′ and 5′-CTG CAT TGT TTT GCC AGT GT-3′ for HPRT; 5′-AGT GGG CTG GAT GTA ACCTG-3′ and 5′-CCA GTA GAT GTG CTG CCA GA-3′ for POLB. The amplified products were then visualized on gels and quantitated with ImageJ automated digitizing system on the basis of three replicate gels in each case.

Quorum sensing inhibitory properties

The inhibition of QS-mediated violacein production in *C. violaceum,* by polymer was studied by the agar well assay. Molten nutrient agar and cells of *C. violaceum* MTCC 2656 was mixed and plated on petridishes. A well of 5mm diameter was made at the centre of the agar plate,100 µg of polymer was added and incubated at 37°C for 16 h. Clear zone of inhibition of bacterial growth surrounding the well appeared confirmed the inhibition of quorum sensing.

Burning procedure

The method of burning was like the techniques reported by earlier authors with minor modification [13]. Total thirty number of rats were employed in the investigation. During selection of the rat, both C-reactive protein (CRP) and cytokine level were measured and confirmed that mice are not immuno-compromised by any infection. Ketamine (100 µg/g) and xylazine (10 µg/g) were injected intraperitoneally to produce general anesthesia and lidocaine (5 µg/g) was injected subcutaneously to produce local anesthetic. To manage pain before and after wound formation. First of the region of the bodies of animals, where burning is intended, the fur/hairs were shaved using sterile scissor and razor, on the dorsal surface. A fire protective cloth having a window of 1-2 inch was held tightly over the shaved region on the back of the anaesthetized animal followed by spreading of 0.2 ml of 95% ethanol in the window space. The spread ethanol was lit up with a matchstick and left to burn for 15 s. Instantaneously, after the burn, 0.5 ml of physiological saline Solution (0.85% NaCl) was applied to intraperitoneal to recover from the burn shock.

External Infection in the burn-wound site

Infection of the animals (that have undergone burn) was done by inoculating the burn area with the bacterial strain, *Pseudomonas aeruginosa* HW01. The strain was isolated from the hospital wastewater following methods described earlier [14]. Overnight grown bacterial cells in Luria broth (Himedia, India) were centrifuged to obtain the cell pellet. The cell pellet was suspended in sterile PBS, centrifuged and re-suspended. After another round of washing in PBS, cell pellet was finally diluted in the same buffer to obtain a cell density of 10^9 cells. ml^{-1}. A measured volume of 200 μL cell suspension was used to infect single burn zone in each animal. Sterile cotton gauze (single layer) was placed over the burn-area to aid effective absorption of the inoculum for a period of 1h before. any other application. All *in-vivo* experiments were performed following the approval from the ethical committee and necessary guidelines were followed while conducting the experiments.

Scratch Assay for Wound Healing Activity

Human keratinocytes (HaCaT) cells were cultured in DMEM-F12 medium in 35-mm petri dishes on lysine-coated coverslips (18 mm 18 mm) in a 37°C incubator with 5% CO_2. A confluent population attached to a coverslip was scratched *in vitro* with a sterile pipette tip. PBS was used to wash the coverslips on the plates twice (pH 7.4). In DMEM-F12 complete media, the polymer was added to a plate and for control, a coverslip without polymer was used. The wound images were photographed and observed under a phase contrast microscope (10, Zeiss Axio Observer Z1, Carl Zeiss, Germany) at various time points (0, 8, and 16 h). To calculate the fraction of wound healing, the wound width was measured using Axiovision software (Version 4.7.2, Carl Zeiss, and Germany).

References

[1] M. Jana, K. Pahan, Induction of lymphotoxin-alpha by interleukin-12 p40 homodimer, the so-called biologically inactive molecule, but not IL-12 p70, Immunology 127,312-25, 2009..

[2] M. Jana, K. Pahan, Redox regulation of cytokine-mediated inhibition of myelin gene expression in human primary oligodendrocytes, Free Radical Biology and Medicine 39,823-31,2005..

[3] A. R. Fatema, Z. Ahmad, M. A. Iskandar, J. Tuomilehto, F. Al-Mulla, R. Ahmad, TNF-α induces a pro-inflammatory phenotypic shift in monocytes through ACSL1: relevance to metabolic inflammation, Cellular Physiology and Biochemistry 52,397-407,2019..

[4] R. Karol, D. T. Shea, D. B. McKim, B. F. Reader, J. F. Sheridan, Imipramine attenuates neuroinflammatory signaling and reverses stress-induced social avoidance, Brain, Behavior, and Immunity 46,212-220,2015..

[5] L. Gang, G. Fan, G. Guo, W. Kang, D. Wang, B. Xu, J. Zhao, FK506 attenuates the inflammation in rat spinal cord injury by inhibiting the activation of NF-κB in microglia cells, *Cell. Mol. Neurobiol* 37,843-855, 2017..

[6] M. Jana, K. Pahan, Induction of lymphotoxin-alpha by interleukin-12 p40 homodimer, the so-called biologically inactive molecule, but not IL-12 p70, *Immunology* 12 127312-325, 2009.

[7] M. Jana, J. A. Anderson, R. N. Saha, X. Liu, K. Pahan, Regulation of inducible nitric oxide synthase in proinflammatory cytokine-stimulated human primary astrocytes, Free Radical Biology & Medicine 38, 655-364, 2005.

[8] K. Pahan, M. Jana, X. Liu, B. S. Taylor, C. Wood, S. M. Fischer, Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric-oxide synthase in human astrocytes. J Biological Chemistry 277, 45984-45991, 2002..

[9] H. Muthukumar, M. Matheswaran, *Amaranthus spinosus* leaf extract mediated FeO nanoparticles: Physicochemical traits, Photocatalytic and Antioxidant activity, ACS Sustainable Chemistry & Engineering 3,3149-3156,2015..

[10] C. H. Jang, J. H. Choi, M. S. Byun, D. M. Jue, Chloroquine inhibits production of TNF-α, IL-1β and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes, *Rheumatology* 45,703-710,2006..

[11] Y. J. Chung, H. R. Zhou, J. J. Pestka, Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-α expression by deoxynivalenol (vomitoxin), Toxicology and Applied Pharmacology 193,188-201,2003..

[12] I. Holder, M. Jogan, Antigenic components of normal and burned mouse skin. Cellular and Molecular Life Sciences, 26, 1363-1365,1970.

[13] D. D. Stieritz, I. A. Holder, Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: description of a burned mouse model. J Infect. Dis. 131, 688-691, 1975.

[14] S. Mukherjee, R. Chakraborty, Incidence of class 1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from the River Torsa in India, Research in Microbiology 157 (2006) 220-226.

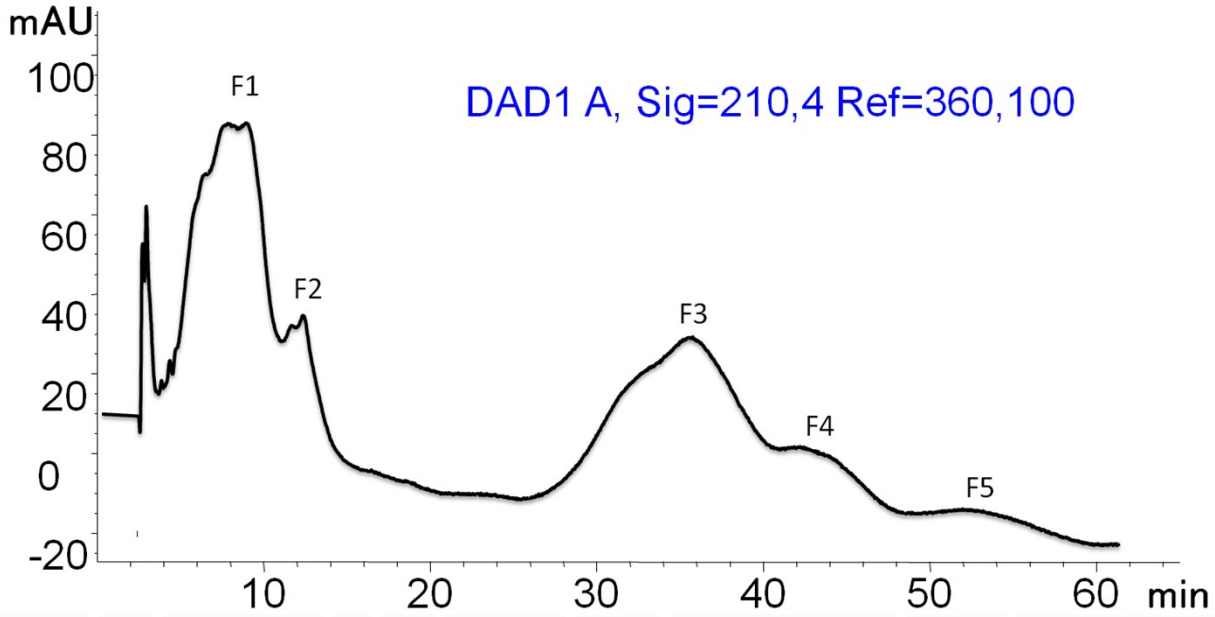

Figure S1: Separation of the product using reversed-phase HPLC. Chromatogram profile of product showed five peaks (F1-F5) and among these fractions F3 showed antibacterial activity.

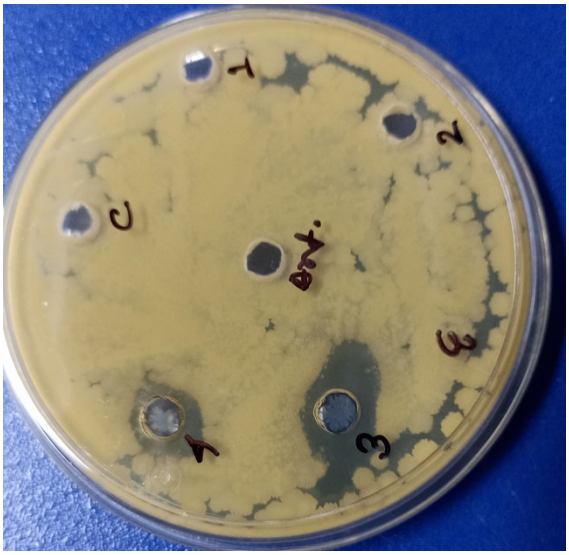

Figure S2: Antibacterial activity of the purified fractions against *S. epidermidis.*

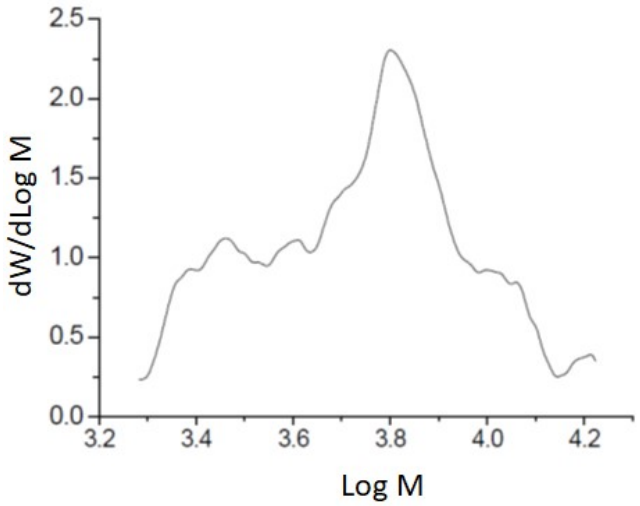

Figure S3: Gel permeation chromatography (GPC) chromatogram of synthesized D-tryptophan ether polymer.

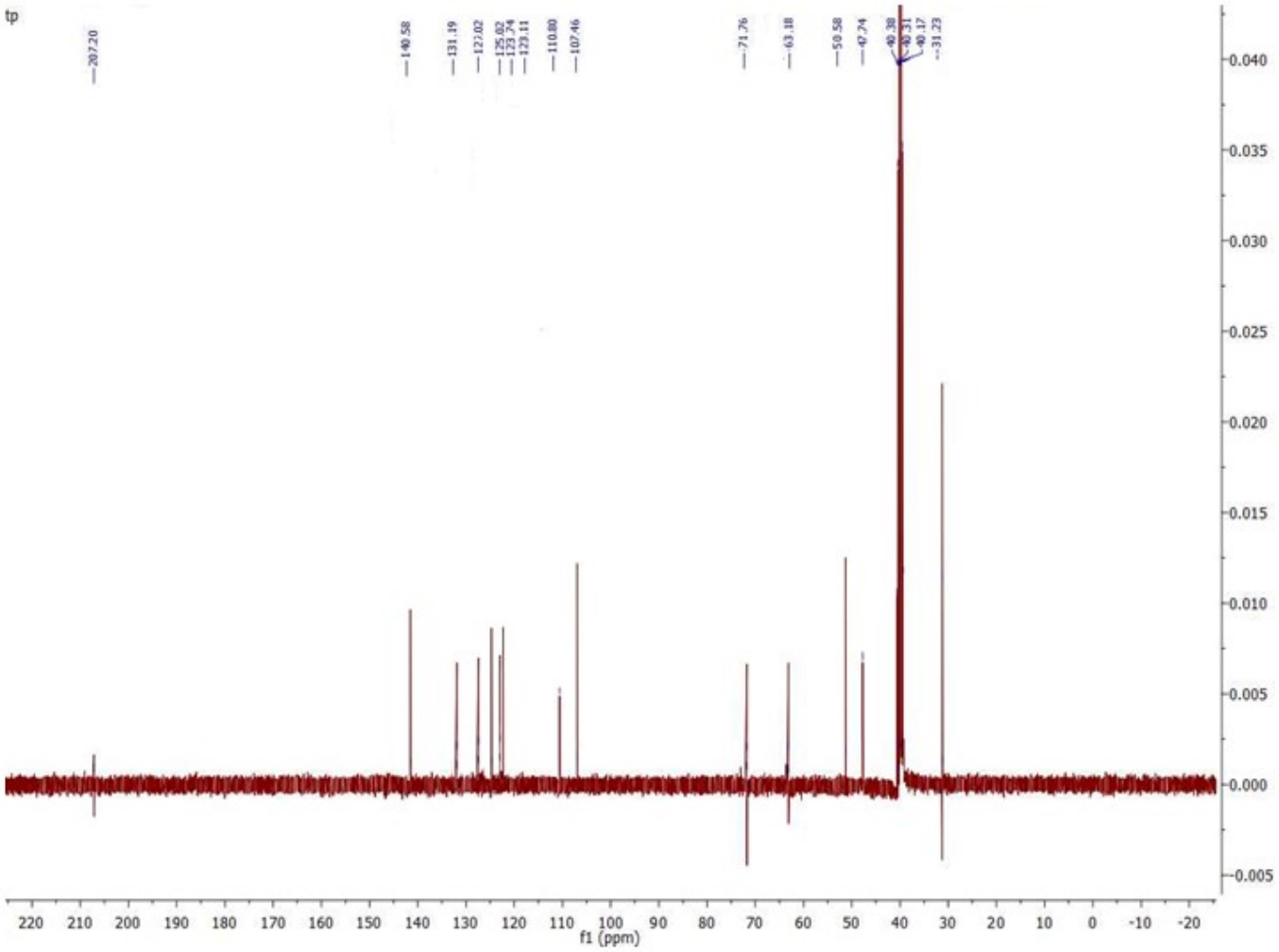

Figure S4: ^{13}C NMR(400 MHz,$CDCl_3$). δ:
1.2,47.7,50.6,63.2,71.8,107.5,110.8,123.1,123.8,125.0,127.0,131.2,140.6,207.2;