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Inhibitory effect of 1-tetradecanol on *Helicobacter pylori*-induced production of interleukin-8 and vascular endothelial growth factor in gastric epithelial cells

GREEN KIM[1,2*], JAE-EUN KIM[3*], MIN-JUNG KANG[1], AH-RA JANG[1], YOUNG RAN KIM[4], SUNOH KIM[5,6], KYU-TAE CHANG[2], JUNG JOO HONG[2] and JONG-HWAN PARK[1]

[1]Laboratory Animal Medicine, College of Veterinary Medicine, Chonnam National University, Gwangju 61186; [2]National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungbuk 28116; [3]Jeonnam Biopharmaceutical Research Center, Hwasun 58141; [4]College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 61186; [5]B&Tech Co., Ltd., R&D Center, Gwangju 61239; [6]Division of Food and Nutrition, Chonnam National University, Gwangju 61186, Republic of Korea

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Abstract. *Helicobacter pylori* (*H. pylori*) infection activates pro-inflammatory mediators, including interleukin (IL)-8 and vascular endothelial growth factor (VEGF) in gastric epithelial cells. 1-Tetradecanol (1-TD) has been purified from *Dendropanax morbifera* Leveille; its physiological activities are poorly understood. The present study assessed whether 1-TD has an effect on *H. pylori*-mediated inflammation in AGS gastric epithelial cells. 1-TD reduced IL-8 production by AGS cells in response to *H. pylori* in a significant and dose-dependent manner, as measured by ELISA. Western blot analysis demonstrated that 1-TD also suppressed the activation of nuclear factor-κB, and two mitogen activated protein kinase species (p38 and extracellular signal-regulated kinase 1/2), but not c-Jun N-terminal kinase in *H. pylori*-infected AGS cells. As predicted, VEGF expression and hypoxia inducible factor-1α stabilization induced by *H. pylori* in AGS cells were inhibited by 1-TD. In addition, 1-TD directly inhibited the growth of *H. pylori* in a dose-dependent manner, as investigated by measuring the optical density. These findings indicated that 1-TD may be a potential preventive or therapeutic agent for *H. pylori*-induced gastric inflammation.

Correspondence to: Dr Jung Joo Hong, National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, 30 Yeongudanji-ro, Ochang-eup, Cheongju, Chungbuk 28116, Republic of Korea
E-mail: hong75@kribb.re.kr

Professor Jong-Hwan Park, Laboratory Animal Medicine, College of Veterinary Medicine, Chonnam National University, 77 Yongbong-ro, Buk-gu, Gwangju 61186, Republic of Korea
E-mail: jonpark@jnu.ac.kr

*Contributed equally

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Introduction

Dendropanax morbifera Leveille (*D. morbifera*) is a member of the Araliaceae family. It is a subtropical broad-leaved evergreen tree that has been used in traditional medicine for the treatment of headache, infectious disease, skin diseases, and neurological disorders (1,2). The plant contains several components that exhibit various pharmacological effects. One of these is the triterpenoid compound Oleifolioside A, that inhibits nitric oxide (NO) and prostaglandin E2 (PGE2) through the downregulation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages (3), and induces caspase-independent cell death in HeLa human cervical carcinoma cells (4). Ethanol extract of *D. morbifera* induces apoptosis of human leukemia U937 cells through the caspase dependent pathway (1). Anti-cancer activities of methanol extracts have been reported in hepatocarcinoma, colon adenocarcinoma, biliary tract cancer and human osteosarcoma cells (5). Anti-inflammatory responses by *D. morbifera* extracts involve the suppression of NF-κB dependent pathways in LPS-stimulated macrophages (6) and microglia (7).

Helicobacter pylori (H. pylori) is a gram negative-bacterium that is commonly located in the stomach of individuals. In some people, *H. pylori* is a pathogen, causing gastric inflammatory diseases including gastritis, peptic ulcer, duodenal ulcer, and even mucosa-associated lymphatic tissue lymphoma (8,9). Production of important inflammatory mediators, such as interleukin (IL)-8 and vascular endothelial growth factor (VEGF), via NF-κB and MAPK signaling in gastric epithelial cells results in gastric inflammation (10) and tumor progression (11).

The present study aimed to investigate whether 1-tetradecanol (1-TD), which was recently isolated from the

n-hexane fraction of *D. morbifera,* has anti-inflammatory activity in *H. pylori*-infected gastric epithelial cells.

Materials and methods

Preparation of 1-TD. Water extract of *Dendropanax morbifera* leaves was prepared at 100˚C for 4 h. The extracted solution was filtered, concentrated with an evaporator under a vacuum, and freeze-dried. The preparation was suspended in water and successively divided with n-hexane, chloroform, ethyl acetate and n-butanol (3x500 ml). Tetradecanol used in this study was isolated from the n-hexane fraction and the purity was confirmed to be >99% as described previously (12).

H. pylori strain and culture conditions. H. pylori strain 26695 (American Type Culture Collection, Manassas, VA, USA) was cultured on Brucella broth (BD Biosciences, Franklin Lakes, NJ, USA) containing 10% fetal bovine serum (FBS; Corning Incorporated, Corning, NY, USA) and antibiotic supplement in a micro-aerobic environment. The bacteria were grown to an optical density at 600 nm (OD600) of 0.6 measured using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments, Inc., Winooski, VT, USA), which corresponded to ~10^9 colony-forming units (CFU)/ml and were diluted to the desired concentrations (13).

Cell culture and treatment. The AGS (KCLB; 21739) and MKN45 (KCLB; 80103) human gastric epithelial cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 medium (Welgene, Inc., Daegu, Korea) supplemented with 10% FBS and 1X penicillin/streptomycin (100 U/ml) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. Briefly, to determine the secretion of VEGF and IL-8, AGS and MKN45 cells (1x10^5 cells/well in a 48-well plate) were infected with *H. pylori* 26695 at the indicated multiplicity of infection (MOI; 50) in the absence or presence various quantities of 1-TD (30-300 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h in a humidified incubator at 37˚C in an atmosphere of 5% CO_2. To measure the levels of hypoxia-inducible factor-1α (HIF-1α), AGS cells (1x10^6 cells/well in a 6-well plate) were infected with *H. pylori* 26695 at MOI 50 with or without 1-TD (300 μM) for 6 h.

Determination of IL-8 and VEGF. The culture supernatants of *H. pylori*-infected AGS and MKN45 cells were collected for ELISA for IL-8 and VEGF. Commercial Duoset ELISA kits (DY208 for IL-8 and DY293B for VEGF; R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's protocol.

MTT assay. An MTT assay was performed to determine the cytotoxicity of 1-TD on gastric epithelial cell lines. The cells (1x10^5 cells/well in a 48-well plate) were treated to different concentrations of 1-TD (30, 100 and 300 μM) for 24 h. Each well was incubated with MTT (4 mg/ml; Sigma-Aldrich; Merck KGaA) in RPMI-1640 medium (Welgene, Inc.) for 4 h at 37˚C. After 4 h, the MTT solution was removed and replaced with 200 μl of dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). The plates were agitated for 5 min to dissolve the formazan crystals. The OD were determined at a wavelength of 570 nm using an ELISA plate reader (BioTek Instruments, Inc.).

Western blotting. AGS cells were seeded into 35-mm dishes and incubated for 24 h. Cells infected with *H. pylori* 26695 (MOI 50) were then pre-treated with 1-TD (300 μM) for 2 h. After 0, 15, 30 or 60 min of treatment, cells were lysed in a buffer containing 1% Nonidet-P40 supplemented with protease inhibitor (complete Mini EDTA-free; Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich; Merck KGaA) and 2 mM dithiothreitol. The extracted protein concentration was examined by a Protein Assay kit (500-0006; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Lysates (30 μg) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes by electro-blotting. The membranes were blocked with blocking buffer [5% skimmed milk in PBS-Tween (0.05% Tween-20)] and incubated at room temperature for 1 h. The membranes were probed with primary antibodies against regular and phosphorylated (p) forms of c-jun N-terminal kinase (JNK; cat. no. 9252; 1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA), p38 (cat. no. sc 101759; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), extracellular signal-regulated kinase (ERK; cat. no. sc 7383; 1:1,000; Santa Cruz Biotechnology, Inc.), inhibitor of NF-κB kinase subunit α (IκB-α; 1:1,000; cat. no. 9242S; Cell Signaling Technology, Inc.), p-NF-κB p65 (1:1,000; cat. no. 3033S; Cell Signaling Technology, Inc.) and HIF-1α (1:1,000, cat. no. 610958; BD Transduction Laboratories; BD Biosciences), followed by an incubation at 4˚C overnight. A primary antibody against β-actin (cat. no. sc-130656; 1:1,000; Sigma-Aldrich; Merck KGaA) was used to verify equal loading of protein samples. After immunoblotting with corresponding goat anti-rabbit (cat. no. sc-2301; 1:4,000; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (cat. no. sc-2031; 1:2,000; Santa Cruz Biotechnology, Inc.) secondary antibodies for 2 h at room temperature, signals were detected with a SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Images of the blots were captured on a CP-BU new film (Agfa Gevaert N.V., Mortsel, Belgium).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene expression of VEGF was evaluated by RT-qPCR in AGS cells infected with *H. pylori* strain 26695 (MOI 50) in the absence or presence of 1-TD (300 μM) for 12 h. Total RNA was isolated from cultured cells using the easy-BLUE™ Total RNA Extraction kit (Intron Biotechnology, Inc., Seongnam, Korea). cDNA was synthesized from 0.1 μg RNA using the ReverTra Ace® qPCR RT Master Mix kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. qPCR was performed using the Qiagen SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Primers were: VEGF forward, 5'-CCTTGCTGCTCTACCTCCAC-3' and reverse, 5'-TGG TGATGTTGGACTCCTCA-3'; and GAPDH forward, 5'-CGA CTTCAACAGCAACTCCCACTCTTCC-3' and reverse, 5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3'. qPCR was performed by using a two-step cycle of 95˚C for 10 sec followed by 58˚C for 45 sec for 40 cycles in a Roter-GeenQ Real-time PCR system (Qiagen). Gene expression was quantified using the comparative Ct method, normalizing to GAPDH mRNA (14).

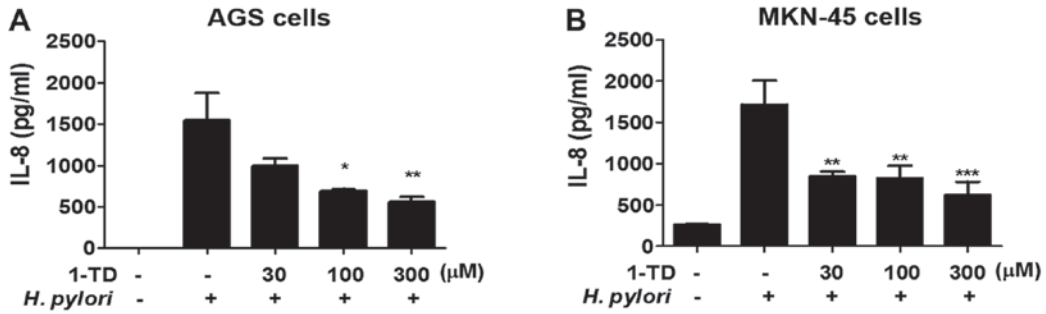

Figure 1. 1-TD inhibits *H. pylori*-induced IL-8 secretion in gastric epithelial cells. (A) AGS and (B) MKN45 cells were incubated with or without various doses of 1-TD and *H. pylori* (multiplicity of infection 50) for 24 h; IL-8 secretion in the culture supernatant was measured via ELISA. Data are presented as the mean ± standard deviation of three separate experiments. 1-TD treated groups were compared with the untreated one and the statistical significance was shown as *P<0.05, **P<0.01, ***P<0.001. *H. pylori, Helicobacter pylori*; 1-TD, 1-tetradecanol; IL-8, interleukin-8.

Anti-bacterial activity. For anti-bacterial testing, 50 μl bacterial suspension (1x10^{9} CFU/ml) was added to 2 ml Brucella broth containing various doses of 1-TD. After 6 or 12 h of incubation at 37˚C under microaerobic conditions, bacterial growth was determined by measuring the OD_{600} of the culture broth with an ELISA reader (Epoch; BioTek Instruments, Inc.). The experiment was repeated in triplicate.

Statistical analysis. Data are expressed as the mean ± standard error. Statistical significances were analyzed by one-way analysis of variance with a Bonferroni post hoc test, and analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.01 was considered to indicate a statistically significant difference.

Results

1-TD suppresses IL-8 production by H. pylori-infected gastric epithelial cells. To understand the effects of 1-TD on the *H. pylori*-induced inflammatory immune response in gastric epithelial cells, IL-8 production in AGS cells co-incubated with *H. pylori* (MOI 50) and 1-TD at concentrations of 30, 100 and 300 μM was analyzed. Following an incubation for 24 h, levels of IL-8 significantly decreased in a dose-dependent manner in *H. pylori*-infected AGS cells that were co-treated with 1-TD, compared with *H. pylori*-infected AGS cells that were not co-treated with 1-TD (Fig. 1A). Similar inhibition in another gastric epithelial cell line (MKN45) was also demonstrated (Fig. 1B). In addition, 1-TD was not cytotoxic in either gastric epithelial cell lines at 30, 100 and 300 μM using MTT assay (data not shown), suggesting that 1-TD is a candidate agent to suppress *H. pylori*-induced inflammation in gastric epithelial cells.

1-TD inhibits the activation of NF-κB, ERK1/2 and p38 in H. pylori-infected gastric epithelial cells. The activation of NF-κB and MAPK signaling is critical for the production of IL-8 in *H. pylori*-infected gastric epithelial cells (11). Therefore, the present study examined the impact of 1-TD treatment on their expression in *H. pylori*-infected AGS cells using western blotting. *H. pylori* induced decrease of IκB-α at 30 min after infection; however, this process was delayed by 1-TD (Fig. 2). Notably, 1-TD decreased the phosphorylation levels of p65, ERK1/2 and p38, whereas it had no influence

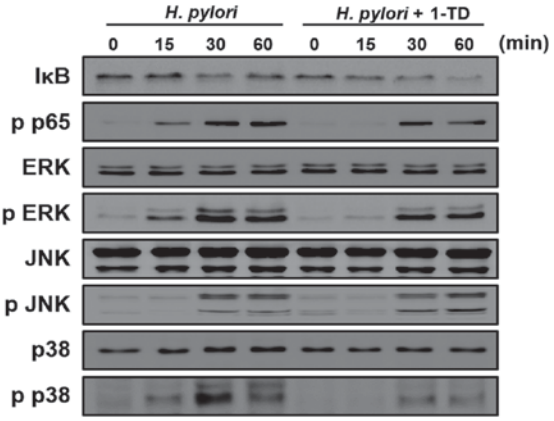

Figure 2. 1-TD inhibits the activation of NF-κB, ERK and p38 induced by *H. pylori* in infected gastric epithelial cells. AGS cells were pre-treated with 300 μM 1-TD or a solvent control for 6 h and then infected with *H. pylori* (multiplicity of infection 50). The levels of phosphorylated and total forms of IκB-α, p65 and mitogen activated protein kinases (p38, ERK1/2 and JNK) were assessed via western blot analysis. ERK1/2, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p, phosphorylated; *H. pylori, Helicobacter pylori*; IκB-α, inhibitor of NF-κB kinase subunit α; 1-TD, 1-tetradecanol.

on the level of p-JNK (Fig. 2). These results indicated that 1-TD potentially suppresses NF-κB and MAPK-dependent inflammation in *H. pylori*-infected gastric epithelial cells.

1-TD inhibits H. pylori-induced VEGF production in gastric epithelial cells. VEGF production is regulated via NF-κB, ERK and p38 MAPK in the gastric mucosa (11,15). Therefore, the present study examined the effect of 1-TD on secretion and mRNA expression of VEGF in *H. pylori*-infected AGS cells. The ELISA results demonstrated that the protein expression level of VEGF was significantly decreased in AGS cells co-treated with *H. pylori* (MOI 50) and 30, 100, and 300 μM of 1-TD after 24 h incubation in a dose-dependent manner (Fig. 3A). Additionally, the mRNA expression of VEGF tended to be lower in *H. pylori*-infected AGS cells co-treated with 1-TD over time, compared with those in the absence of 1-TD (Fig. 3B). The difference reached statistical significance only at 9 h (Fig. 3B). These results indicated that 1-TD inhibited protein synthesis and transcription of *H. pylori*-induced VEGF in gastric epithelial cells.

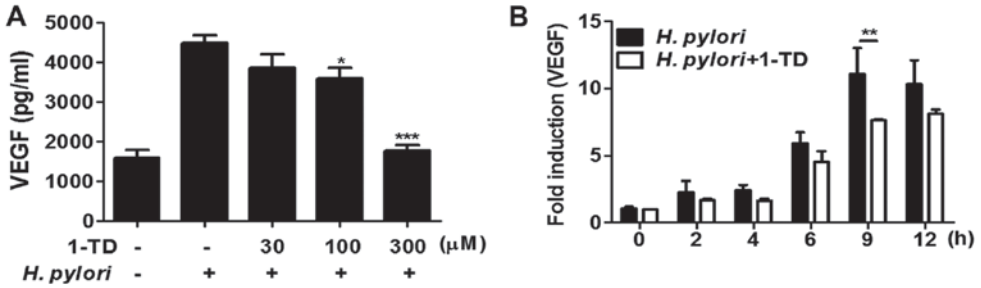

Figure 3. 1-TD inhibits *H. pylori*-induced VEGF secretion in gastric epithelial cells. (A) AGS cells were incubated with or without various concentrations of 1-TD and *H. pylori* at a MOI 50 for 24 h. VEGF secretion in the culture supernatants was measured via ELISA. (B) AGS cells were infected with *H. pylori* (MOI 50) in the absence or presence of 1-TD (300 μM) for indicated times. The expression levels of VEGF mRNA were measured using reverse transcription-quantitative polymerase chain reaction and the fold decrease (arbitrary unit) relative to untreated control cells was calculated. Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs. *H. pylori* group. MOI, multiplicity of infection; VEGF, vascular endothelial growth factor; *H. pylori, Helicobacter pylori*; 1-TD, 1-tetradecanol.

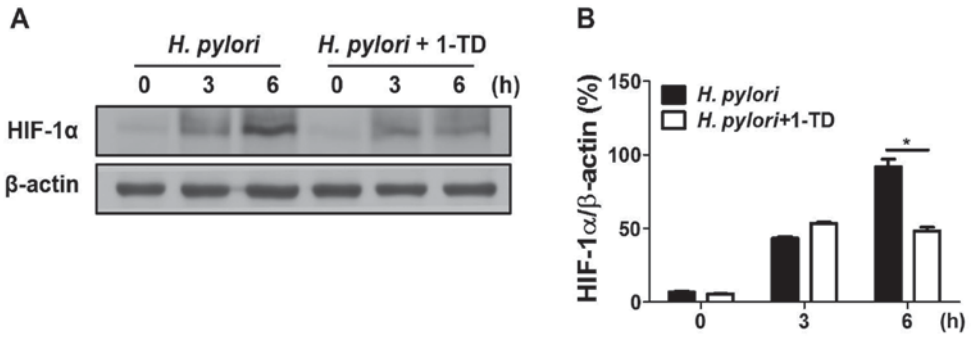

Figure 4. 1-TD inhibits *H. pylori*-nduced HIF-1α stabilization in gastric epithelial cells. (A) AGS cells were pretreated with or without 300 μM of 1-TD or a solvent control for 6 h and then cultured with *H. pylori* (multiplicity of infection 50) for indicated times. The protein levels of HIF-1α were measured by western blotting. β-actin was used as a protein loading control for band density normalization. (B) HIF-1α levels were quantified based on the band density. Data are presented as the mean ± standard deviation of three separate experiments. 1-TD treated group was compared with the untreated one at each time point (0, 3, 6 h). The statistical significance was shown as *P<0.05. *H. pylori, Helicobacter pylori*; 1-TD, 1-tetradecanol; HIF-1α, hypoxia inducible factor-1α.

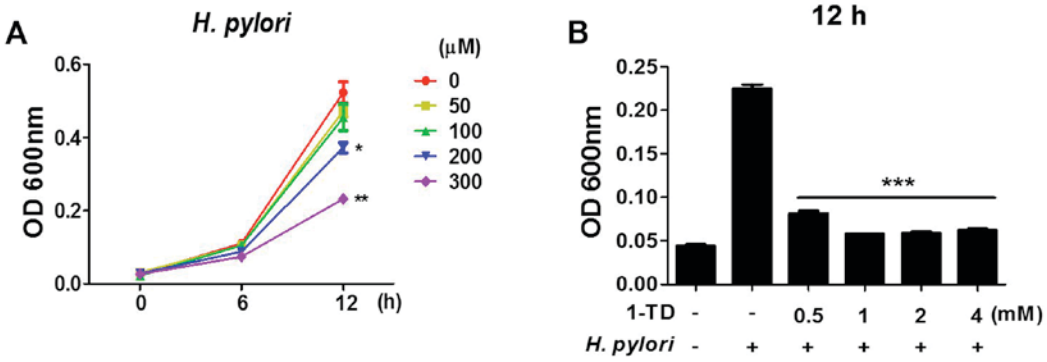

Figure 5. 1-TD exhibits anti-bacterial activity against *H. pylori*. (A) and (B) growth curves of *H. pylori*, incubated with various doses of 1-TD, in Brucella broth at 37˚C under microaerobic conditions for (A) 0, 6 and 12 h, and (B) 12 h. Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05 and **P<0.01 vs. 0 μM. 1-TD treated groups were compared with the untreated one and the statistical significance was shown as ***P<0.001. OD, optical density; *H. pylori, Helicobacter pylori*; 1-TD, 1-tetradecanol.

1-TD inhibits H. pylori-induced HIF-1α stabilization in gastric epithelial cells. To determine whether 1-TD affects *H. pylori*-mediated HIF-1α stabilization in AGS cells, western blot analysis was performed to measure the expression of HIF-1α. 1-TD treatment inhibited *H. pylori*-induced HIF-1α protein expression levels at 6 h, compared with that at 0 and 3 h (Figs. 4A and B), suggesting that 1-TD has an inhibitory effect on *H. pylori*-induced VEGF production through HIF-1α stabilization.

1-TD affects anti-bacterial activity against H. pylori. To assess whether 1-TD has any direct effect on the viability of *H. pylori*, bacterial growth curves in the presence of 1-TD (0, 50, 100, 200 and 300 μM) for 12 h were assessed. It was

demonstrated that there was a dose-dependent reduction in the growth of *H. pylori* at high doses (200 and 300 μM), while no effect was observed at lower doses (Fig. 5A). To determine a minimal inhibitory concentration (MIC), the bacterial growth was determined in the presence of much higher doses of 1-TD (>500 mM). More than 1 mg of 1-TD completely inhibited the growth of *H. pylori* (Fig. 5B), indicating that the MIC of 1-TD on *H. pylori* is between 0.5 and 1 mg. These data suggested a direct inhibitory effect of 1-TD on the growth of *H. pylori*.

Discussion

Anti-oxidant, anti-complement, anti-plasmodium and anti-cancer activities have been associated with extracts from the leaves and stems of *D. morbifera* (5,16,17). However, limited data are available about the physiological effects of the compound 1-TD, isolated from the lower stem of *D. morbifera*. Only a few studies have examined its anti-inflammatory effects on cells such as microglial cells (6,7) and macrophages (3). Furthermore, IL-8, a member of the CXC chemokine family, is involved in inflammatory responses, leukocyte chemotaxis and cancer development (18,19). The high expression of IL-8 is markedly associated with the proliferation, invasion and migration of gastric epithelial cells (20,21). In the case of *H. pylori* infection, IL-8 production is directly increased in gastric epithelial cells (22,23). To the best of our knowledge, the present study was the first to report that 1-TD has potent inhibitory effects against *H. pylori*-induced inflammation. 1-TD significantly inhibited IL-8 production in AGS cells infected with *H. pylori*, suggesting that 1-TD may act as a potential agent to suppress *H. pylori*-induced inflammatory responses involved in IL-8 production.

No cytotoxic effects of 0-300 μM 1-TD on *H. pylori*-infected gastric epithelial cells were evident. In contrast, previous studies have demonstrated that oleifolioside A and B, ethanol and methanol extracts isolated from *D. morbifera* have cytotoxic effects on human leukemia cells (1), lung carcinoma cells (24), cervical carcinoma cells (4) and hepatocellular carcinoma cells (5). Therefore, the results of the present study suggested that the inhibition of *H. pylori*-induced inflammation in gastric epithelial cells is not due to cell toxicity. Furthermore, 1-TD restricted the growth of *H. pylori* in Brucella broth, indicating direct antibacterial activity, as was previously observed for other long-chain fatty alcohols (25). However, the mechanism through which 1-TD inhibits the bacterial growth is yet to be elucidated.

MAPKs (ERK1/2, p38 and JNK) regulate oxidative stress, gene expression, mitosis, metabolism and apoptosis (26,27). NF-κB and MAPK activation are required for transcription of IL-8 gene induced by *H. pylori*-infected gastric epithelial cells (28). In the present study, pre-treatment with 1-TD inhibited *H. pylori*-induced phosphorylation of p38 MAPK, ERK1/2, and NF-κB, suggesting that the inhibition of the levels of IL-8 induced by *H. pylori* was MAPK- and NF-κB-dependent. In contrast, the phosphorylation of JNK was not affected. A similar observation was previously reported in LPS-stimulated RAW 264.7 macrophages (3). The authors also reported the proinflammatory mediators involved in NF-κB, p38 MAPK and ERK1/2 signaling by oleifolioside A (3). The potential reduction of these mediators by 1-TD in *H. pylori*-infected epithelial cells was not elucidated in the present study.

During *H. pylori* infection, VEGF can be produced. This is important in vascular remodeling in gastric epithelial cells (29). Our previous study revealed that this production is associated with the activation of NF-κB and MAPK signaling, as well as HIF-1α stabilization resulting from the generation of reactive oxygen species induced by *H. pylori* (30,31). In the present study, 1-TD significantly decreased *H. pylori*-induced VEGF production and inhibited HIF-1α stabilization in AGS cells. These findings suggested that 1-TD may be a novel agent on the inhibition of inflammatory responses involved in angiogenesis in *H. pylori*-infected gastric epithelial cells.

In conclusion, 1-TD effectively inhibited the production of inflammatory mediators (IL-8 and VEGF) through the inhibition of p38 MAPK, ERK1/2, and NF-κB signaling in *H. pylori*-infected gastric epithelial cells. Moreover, 1-TD inhibited the production of VEG and HIF-1α stabilization induced by *H. pylori* in gastric epithelial cells. Taken together, these data suggested that 1-TD may serve as a novel anti-inflammatory agent for *H. pylori*-infected gastric epithelial cells. Further studies are warranted to evaluate the impact of this anti-inflammatory effect in an *in vivo* model.

Acknowledgements

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