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Title:

Cinnamomum osmophloeum extracts inhibit growth of *Helicobacter pylori* and postinfectious interleukin-8 expression in human gastric epithelial cells

Short title:

In vitro effects of *Cinnamomum osmophloeum* extracts

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ABSTRACT

Essential oils from leaves of Taiwan's indigenous cinnamon, *Cinnamomum osmophloeum* extracts (CEs), contain similar constituents to those of commercial cinnamons from dried barks of other *Cinnamomum* species. However, cinnamon bark extracts showed controversial *in vitro* and *in vivo* results in their antibacterial activities against *H. pylori*. Whether CEs inhibit *H. pylori* growth and inflammation of *H. pylori*-infected gastric epithelium is unknown. In this study, we used 4-h water distillation to obtain CEs, which comprised >95% *trans*-cinnamaldehyde and other 4 minor compounds that were confirmed by GC-MS and contained 1305.5 μg/ml of *trans*-cinnamaldehyde that was quantified by HPLC analysis. The minimal inhibitory concentration of *trans*-cinnamaldehyde in CEs against *H. pylori* was 40.8 μg/ml in 48-h co-cultures. We subsequently performed 2 cinnamon extract assays: 3-h treatment of CEs containing 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde significantly inhibited IL-8 mRNA and protein expression in the 1-hour *H. pylori*-infected cells, whereas 6-h treatment of CEs containing 21 μg/ml of *trans*-cinnamaldehyde, but not CEs containing 10.5 μg/ml of *trans*-cinnamaldehyde, substantially downregulated IL-8 mRNA expression and these two CEs strongly suppressed IL-8 protein secretion in the 2-h *H. pylori*-infected cells. In the 2-h IL-1β-treated cells, both CEs and *t*-SSs containing 21 μg/ml and 10.5 μg/ml of *trans*-cinnamaldehyde for 6-h markedly downregulated IL-8 mRNA expression; CEs inhibited IL-8 protein production more

potently than *t*-SSs when both contained the same concentration of *trans*-cinnamaldehyde at 21 μg/ml. In conclusion, CEs concentration-dependently inhibit *H. pylori* growth and postinfectiously inhibit IL-8 mRNA and protein expression in *H. pylori*- and IL-1β-pretreated AGS cells. Moreover, CEs exhibited a stronger anti-inflammatory effect than *trans*-cinnamaldehyde, indicating that CEs can potentially be used to treat *H. pylori* infection.

Keywords:

Cinnamomum osmophloeum; *trans*-cinnamaldehyde; *Helicobacter pylori*; human gastric epithelium; minimal inhibitory concentration; interleukin-8.

Abbreviations:

Amoxici: Amoxicillin

CE: *Cinnamomum osmophloeum* extract

ELISA: Enzyme-linked immunosorbent assay

GC-MS: gas chromatography-mass spectrometry

HPLC: high-performance liquid chromatography

IL-8: interleukin-8

MIC: minimal inhibitory concentration

qRT-PCR: quantitative real-time PCR

TC : *trans*-cinnamaldehyde

Tetracy : Tetracycline

t-SS: *trans*-cinnamaldehyde standard solution

MAIN TEXT

1. Introduction

Cinnamon is a flavoring ingredient used in foods. Commercial cinnamons are sold in the markets and are obtained from the dried inner barks of many *Cinnamomum* species, such as Ceylon cinnamon and Cassia cinnamon.[1] *Cinnamomum*, a genus belonging to the *Lauraceae* family, comprises approximately 250 species distributed in Australia and Asia.[2] *C. osmophloeum*, an indigenous cinnamon species in Taiwan, is classified into 5 types based on the chemical composition of its leaf essential oils: *trans*-cinnamaldehyde, *trans*-cinnamaldehyde/cinnamyl acetate, T-cadinol/α-cadinol, camphor, and linalool.[3] The essential oils from leaves of Taiwan's indigenous cinnamon (*Cinnamomum osmophloeum* ct. cinnamaldehyde) have similar constituents to those from commercial bark cinnamons. All the tested indigenous cinnamon clones contain much low levels of the hepatotoxic and carcinogenic compound, coumarin, which is often seen in Cassia cinnamons.[1]

Helicobacter pylori (*H. pylori*) is the most common human pathogen that has infected at least 50% of the world population.[4] In developing countries, nearly 70% of the children are infected with *H. pylori* by the age of 15.[5] If left untreated, *H. pylori* infection is lifelong and may cause gastritis, peptic ulcer, mucosal-associated lymphoid tissue lymphoma, or gastric atrophy.[6] Although *H. pylori* infection can be eradicated by administering antimicrobials plus a proton-pump inhibitor or bismuth,[7] such therapies are associated with increasing antibiotic resistance, which is the major cause of treatment failure in *H. pylori* infection.[8] Therefore, second-line treatment strategies must be developed.

The inflammation of *H. pylori*-infected gastric mucosa is reflected by the increased levels of interleukin (IL)-8 mRNA and protein expression. The gastric

mucosa infected by *H. pylori* produces proinflammatory cytokines, and especially IL-8, which is a potent chemokine that attracts neutrophils and mononuclear cells.[9] The IL-8 levels correlate not only with the density of *H. pylori* in gastric antrum and corpus but also with cellular infiltration in gastric antrum.[9] Additionally, *H. pylori* induced IL-8 mRNA and protein synthesis with respective peaks at 2–4 h and 24 h postinfection in 3 gastric cell lines, but not in nongastric epithelial cell lines.[10]

The effects of cinnamon extracts on *H. pylori*-infected human gastric epithelium remain obscure. Alternative non-antibiotic therapies such as phytotherapy have shown promising antimicrobial activities against *H. pylori*, but such therapies await rigorous scientific validation.[11] Cinnamon has been used as a seasoning and as herbal medicine for centuries, and evidence is available for cinnamon's anti-inflammatory, antimicrobial, antioxidant, antitumor, and immunomodulatory effects.[12] However, previous studies examining the effects of cinnamon extracts on *H. pylori* infection have reported inconsistent results between *in vitro* studies and clinical trials. Extracts of *Cinnamonum cassia* (*C. cassia*) stem bark inhibited *in vitro* growth and urease activity, respectively, of *H. pylori*.[13] Extracts of *Cinnamomum verum* (*C. verum*) bark showed *in vitro* bactericidal activity against *H. pylori*.[14] Ethanolic extracts of *C. cassia* Blume and its several constituents, including coumarin, eugenol, and *trans*-cinnamaldehyde, showed potent anti-*H. pylori*, antioxidant, and acid-neutralizing activities.[15] By contrast, a clinical trial demonstrated ineffective eradication of *H. pylori* after treatment with *C. cassia* extracts.[16]

Essential oils from *Cinnamomum* species are extracted using bark or young branches. However, essential oils of *Cinnamomum osmophloeum* (*C. osmophloeum*),[17] an indigenous cinnamon tree that grows at an altitude of 400–1500 m in Taiwan,[18] can be extracted from leaves with chemical constituents similar to those of the widely

used *C. cassia* bark oils.[19] Among the various chemical compounds present in the essential oils of two *C. osmophloeum* clones, *trans*-cinnamaldehyde was one of the main components which, relative to other constituents, exhibited the strongest antibacterial activity against 9 different bacterial strains.[19] However, whether *C. osmophloeum* extracts (CEs) can inhibit *H. pylori* growth and inflammation of *H. pylori*-infected gastric epithelium is unknown.

In this study, we used 4-h water distillation to extract essential oils from dried leaves of *C. osmophloeum* and analyzed the extracts using gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) to determine the major component. We subsequently conducted an *in vitro* inhibition assay with 48-h *H. pylori* cultures to measure the minimal inhibitory concentration (MIC) of *trans*-cinnamaldehyde in CEs for suppressing *H. pylori* growth. Finally, we established an *in vitro* model using human gastric epithelial AGS cells, which were exposed to *H. pylori* or IL-1β and subsequently treated with CEs or *trans*-cinnamaldehyde standard solutions (*t*-SSs) at various concentrations. IL-8 was selected as a marker of inflammation and IL-8 mRNA and protein expression levels were quantified to determine how CEs and *t*-SSs modulate, post-exposure, *H. pylori*- or IL-1β-induced inflammation of AGS cells.

2. Materials and methods

2.1. Water distillation of *C. osmophloeum*

Fifty grams of the dry leaves of indigenous cinnamon (*C. osmophloeum*, Wenberli Biotech Corporation, 349, Section 2, Zhongzheng Road, Fenglin Town, Hualien County 982, Taiwan) were ground into powder, mixed with 500 ml of double-distilled water, and boiled for 4 hours. Finally, 400 ml of cinnamon essential oils were

extracted. The CEs that passed through 0.22-μm-pore filters were stored at 4 °C for following experiments.

2.2. GC-MS analysis of *C. osmophloeum* extracts

The CEs were analyzed in duplicate by a Perkin Elmer TurboMass (ion source 110 °C, 70 eV) instrument, equipped with an Elite-5MS capillary column (length 30 m · I.D. 0.32 mm, film thickness 0.25 μm). The oven temperature was held at 80°C for 3 min, increased to 280 °C at a rate of 10 °C/min, and held for 5 min. Injector temperature was maintained at 250 °C. The components of CEs were identified by comparison with standards using the TurboMass 5.4.2 GC/MS Software. The relative abundance of compounds was compared by integrating the peak areas of the spectrograms.

2.3. HPLC analysis of *C. osmophloeum* extracts

The 0.01%, 0.05%, 0.20%, 0.40%, and 0.50% dilutions of *t*-SSs (239968, purity ≥99%, 1.05 g/ml; Sigma-Aldrich), *i.e.* 105 μg/ml, 525 μg/ml, 2100 μg/ml, 4200 μg/ml, and 5250 μg/ml of *trans*-cinnamaldehyde were dissolved in 95% ethanol. Then the correlation curve and the regression equation among the 5 concentrations of *trans*-cinnamaldehyde in the *t*-SSs and their peak areas in HPLC chromatograms were determined by using HPLC as described previously,[18] with a flow rate of 1.0 ml/min during the mobile phase. The retention times of the 5 dilutions of *t*-SSs and the CE were acquired using the PEAK-ABC Chromatography Data System (ChromTech, Taiwan) and compared. Subsequently, the peak area value of *trans*-cinnamaldehyde of the CE in the HPLC cromatogram was applied to the regression equation that was

derived from the 5 dilutions of *t*-SSs. Finally, the concentration of *trans*-cinnamaldehyde in the CE was calculated.

2.4. Bacterial strain and culture condition

A human isolated strain of *H. pylori* (BCRC 17219 Taiwan, originally from ATCC 700392) was used in this study. *H. pylori* was recovered from Microbank® (Pro-Lab Diagnostics) stocks, stored at –80 °C, and plated onto blood agar plates (BAP, A01-05, Creative Media Products), which were then incubated in closed jars for 5–6 d at 37 °C in a micro-aerophilic atmosphere created using AnaeroPack-MicroAero (MGC, Japan). Several colonies of *H. pylori* were grown in 10 ml of tryptic soy broth (TSB, BD Difco™) supplemented with 10% of fetal bovine serum (FBS, SAFC Biosciences) in the micro-aerophilic atmosphere at 37 °C for 2 d as inoculums to infect AGS cells. These bacterial cultures were quantified for their bacterial concentrations after serial dilution, plating out, 5–6 d cultures on BAPs, and CFU counting. Their equivalent optical density values at 600 nm (OD_{600}) were determined, and a correlation curve was generated to determine the equation for adjusting bacterial concentrations in assays.

2.5. AGS cell culture

AGS cells (BCRC 60102 Taiwan, originally from ATCC CRL-1739), a cell line of human gastric adenocarcinoma, were seeded at a density of 1×10^5 cells/well into 12-well plates and incubated in Ham's F-12K medium (21127, Gibco®) supplemented with 10% FBS (SAFC Biosciences) in 5% CO_2 at 37 °C for 2–3 d to obtain a confluent density of 3×10^5 cells/well for cinnamon extract assays. The medium was replaced with Ham's F-12K medium without FBS 1 hour before assays.

2.6. Antibacterial susceptibility test against *H. pylori*

To determine the lowest concentration of CE that can inhibit *H. pylori* growth, the CEs were serially diluted in TSB supplemented with 10% FBS to generate 10 concentrations of CEs with equivalent concentrations of *trans*-cinnamaldehyde ranging from 1305.5 μg/ml (original CE) to 2.5 μg/ml (0.1953% CE), and then 900 μl of each of the CE solutions was mixed with 100 μl of 2-d-old *H. pylori* cultures grown in the same broth. The same test was also conducted for 4 selected comparable concentrations (2.5 μg/ml, 20.4 μg/ml, 163.2 μg/ml, 1305.5 μg/ml) of *trans*-cinnamaldehyde prepared from *t*-SSs. Concurrently, we also prepared *H. pylori* cultures in only the broth as a non-CE control (*H. pylori*-only or HP control), in the broth without *H. pylori* and CE as a non-treatment control, and in broths containing amoxicillin (A8523; Sigma-Aldrich) and tetracycline (T7660; Sigma-Aldrich) as positive controls as previously described.[20] These samples were incubated micro-aerophilically at 37 °C for 48 h. Their OD_{600} values were measured using a spectrophotometer (DeNovix DS-11+).

2.7. Cinnamon extract assays in *H. pylori*- and IL-1β-pretreated AGS cells

To investigate whether CE and its major constituent *trans*-cinnamaldehyde inhibited IL-8 expression at gene and protein levels in *H. pylori*-infected and/or IL-1β-treated AGS cells, 2 separate assays were conducted using different treatment protocols. The cytotoxic effect of the various treatments on AGS cells was excluded by measuring lactate dehydrogenase (LDH) release using the LDH Cytotoxicity Detection Kit (Clontech).

In the first cinnamon extract assay, AGS cells in each well were either infected

with 2-d-old cultures of *H. pylori* (MOI = 50) or not infected (non-treatment control) for 1 h. After washing twice with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich), the infected cells were incubated in a medium with CE containing 0.63 μg/ml, 6.3 μg/ml, and 63 μg/ml of *trans*-cinnamaldehyde (equivalent to 0.00006%, 0.0006%, and 0.006% *t*-SS) or no CE (*H. pylori* only control) for 3 h. The cells were subsequently lysed in TRIzol Reagent (Invitrogen) at the end of 4-hour incubation for RNA extraction. Concomitantly, an identical set of the above *H. pylori*-pretreated and CE-pretreated cells were incubated for an additional 20 h in fresh Ham's F-12K medium without FBS. Then the 20-h-cultured media were collected at the end of 24-h incubation for IL-8 ELISAs.

In the second cinnamon extract assay, the durations of treatments with *H. pylori*, IL-1β, and CEs were doubled. AGS cells in each well were treated either with 2-d-old cultures of *H. pylori* (MOI = 50) or with 10 ng/ml of recombinant IL-1β (R&D Systems), or left untreated (non-treatment control) for 2 h. After washing twice with DPBS, the treated cells were incubated in a medium with CE containing 10.5 μg/ml and 21 μg/ml of *trans*-cinnamaldehyde or their comparable *t*-SSs at 2 dilutions (0.001% and 0.002%) or in a medium without CE or the *t*-SS (*H. pylori*/IL-1β only controls) for 6 h. The cells were subsequently lysed in TRIzol at the end of 8-hour incubation for RNA extraction. Concomitantly, an identical set of the above *H. pylori*/IL-1β-pretreated and CE/*t*-SS-pretreated cells were incubated for an additional 16 h in fresh Ham's F-12K medium without FBS. Then the 16-h-cultured media were collected at the end of 24-h incubation for IL-8 ELISAs.

2.8. RNA extraction and qRT-PCR assays for measuring IL-8 mRNA expression

Total RNA from the treated AGS cells was isolated using TRIzol Reagent (Invitrogen)

according to the manufacturer's instructions. Both the quantity and quality of the isolated RNA were confirmed by the absorbance at 260 nm (A_{260}) and the A_{260}/A_{280} ratios of 1.6–1.8. A template cDNA was reverse transcribed from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) in a 20-μl reaction according to the manufacturer's protocol.

Oligonucleotide primers specific to IL-8 (forward 5'-TCTCAGCCCTCTTCAAAAACTTC-3', reverse 5'-ATGACTTCCAAGCTGGCCGTGGC-3') were designed as described.[21] Primers for 2 housekeeping genes, β-actin (forward 5'- GGATGCAGAAGGAGATCACTG-3', reverse 5'-CGATCCACACGGAGTACTTG-3')[22] and hypoxanthine phosphoribosyl-transferase 1 (HPRT1, forward 5'-TGACACTGGCAAAACAATGCA-3', reverse 5'-GGTCCTTTTCACCAGCAAGCT-3') were also designed as previously described. [23] Using the StepOne Real-Time PCR System (Applied Biosystems), 5 μl of the template cDNA was amplified in a 20-μl reaction containing 0.25 μM of each primer and 10 μl of 2× Fast SYBR Green Master Mix (Applied Biosystems) with 40 cycles of enzyme activation at 95 °C for 20 s, denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s (IL-8) or 60 s (β-actin and HPRT1), and extension at 72 °C for 25 s. Specificity of the PCR products was confirmed by melting-curve analysis and agarose-gel electrophoresis. Reactions for each gene were performed in triplicate, and for each sample in duplicate. The geometric means of the 2 housekeeping genes' Ct values were used to normalize the differences in total cDNA between samples. The expression levels of IL-8 mRNA in samples exposed to various conditions were calculated relative to the matched non-*H. pylori*-treated controls using the $2^{-\Delta\Delta Ct}$ method,[24] expressed as fold change relative to non-*H. pylori* control.

2.9. ELISA measurements of IL-8 protein

In the 2 cinnamon extract assays, the harvested media were centrifuged at 13300 × *g* for 5 min and the supernatants were collected for performing IL-8 ELISAs in triplicate according to the manufacturer's instructions (Human IL-8 ELISA Development Kit, PreproTec EC). The levels of IL-8 secreted into the medium under various experimental conditions were measured using a Varioskan Flash Multimode Reader (Thermo Scientific); amounts of IL-8 (pg/ml) were calculated by Microsoft Office Excel 2007 using the standard curves generated from serially diluted standard solutions.

2.10. Statistical analysis

Each experimental condition was performed in duplicate within the wells, and each experiment was repeated 3 times. The quantitative data were expressed as mean ± standard error of the mean (SEM) of triplicate measurements. Statistical analyses were performed with the Student's *t* test using SPSS 17.0. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Preparation and analysis of *Cinnamomum osmophloeum* extracts

Cinnamon essential oil was extracted from dry leaves of *C. osmophloeum* using water distillation. First, the GC-MS analysis showed that CE was comprised of predominantly *trans*-cinnamaldehyde (>95%) and other 4 minor compounds including benzaldehyde, 3-phenylpropionaldehyde, cinnamaldehyde, and eugenol (Fig. 1A). Second, the correlation curve and the regression equation were obtained among 5

concentrations of the *t*-SSs and their peak area values in HPLC chromatograms (Fig. 1B & 1C). Subsequently, the HPLC analysis revealed that the retention time of CE was 10.162 min, which was the same as those of *t*-SSs at 5 concentrations, and the area of the CE peak was 6597664 arbitrary units (Fig. 1C). After calculating the peak area of the CE in the regression equation, we identified the concentration of *trans*-cinnamaldehyde in the CE as 1305.5 μg/ml, which was equivalent to that in 0.124% *t*-SS.

In this study, we confirmed that the predominent constituent of CE was *trans*-cinnamaldehyde. Our HPLC analysis showed that CE and *t*-SSs shared the same peak position with a retention time of 10.162 min. Therefore, the indigenous cinnamon we used can be categorized as the *trans*-cinnamaldehyde type; this type of *C. osmophloeum* killed mosquito larvae most effectively out of 11 chemical compounds found in CEs.[3] In contrast to the cinnamaldehyde obtained from the barks of *C. zeylanicum* and *C. cassia*, 2 ESCOP-approved medicinal herbs of the genus *Cinnamomum*,[12] we extracted >95% of *trans*-cinnamaldehyde from the dry leaves of the selected clone of *C. osmophloeum* using 4-h water distillation. This indigenous cinnamon contains about 80% of cinnamaldehyde and 0.4-2.7% of eugenol.[1] Using the proposed method, we successfully extracted essential oils from this high-*trans*-cinnamaldehyde, no-coumarin, and eugenol-containing clone of *C. osmophloeum*. This combination of *trans*-cinnamaldehyde and eugenol can provide our *C. osmophloeum* extracts a better shelf life than regular commercial cinnamons, and no coumarin in our essential oils reduces the risks of developing hepatotoxicity and carcinogenicity.[1] Therefore, leaves of this indigenous cinnamon can be an accessible source for a safer cinnamon substitute and can reduce deforestation.

3.2. Antibacterial activity of *trans*-cinnamaldehyde in CE against *H. pylori*

CEs at concentrations equal to or higher than 40.8 μg/ml of *trans*-cinnamaldehyde, the minimal inhibitory concentration (MIC), significantly inhibited 48-h growth of *H. pylori* (Fig. 2). Our results showed that *H. pylori* growth was more suppressed by the *t*-SSs than the corresponding CEs when their concentration of *trans*-cinnamaldehyde was increased to 163.2 μg/ml; 1305.5 μg/ml of *trans*-cinnamaldehyde in CE demonstrated a maximal antibacterial effect that was revealed by the reduced OD_{600} value lower than those of 1305.5 μg/ml of *trans*-cinnamaldehyde in *t*-SS, 0.03 μg/ml and 0.25 μg/ml of amoxicillin, and 0.125 μg/ml of tetracycline, and close to that of 0.25 μg/ml of tetracycline (Fig. 2).

To our knowledge, this is the first study to report that CEs can dose-dependently inhibit *in vitro* growth of *H. pylori*, although these essential oils have been shown to inhibit 9 other bacteria,[19] fungi,[25,26] termites,[27] and mosquito larvae.[3] We demonstrated that CEs containing *trans*-cinnamaldehyde at concentrations ranging between 40.8 μg/ml and 1305.5 μg/ml significantly suppressed *H. pylori* growth in 48-h-old cultures (Fig. 2). Consistent with our results, *C. cassia* bark extracts had an MIC of 50 μg/ml for 7 tested bacterial isolates, and the extracts inhibited *H. pylori* growth at a concentration range of common antibiotics.[13] *C. zeylanicum* extracts at a concentration of 361 μg/ml strongly inhibited the urease activity, which indirectly suggests anti-*H. pylori* activity.[28] However, 2-h treatment with 100 μg/ml of *C. cassia* extracts did not markedly affect *H. pylori* viability.[29] In our study, equal to or higher than 40.8 μg/ml of *trans*-cinnamaldehyde in CE potently inhibited *H. pylori* growth, which reached nearly the positive control levels in amoxicillin and tetracycline (Fig.

2). This suggests that *C. osmophloeum* has an outstanding antibacterial activity as *C. cassia* and *C. zeylanicum*. A previous report demonstrated that commercial preparations of cinnamaldehyde at 2 μg/ml substantially reduced the viability of *H. pylori* strains within 72 h and that the bacterial strains developed no resistance to cinnamaldehyde and eugenol at sub-inhibitory concentrations even after 10 passages,[30] suggesting a low risk of developing antimicrobial resistance for our *C. osmophloeum* extracts when used as an antimicrobial against *H. pylori*.

3.3. Post-exposue effects of CEs and *trans*-cinnamaldehyde on *H. pylori*- or IL-1β-induced IL-8 expression in AGS cells

CEs dose-dependently inhibited *H. pylori*-induced IL-8 mRNA and protein expression. In our first assay, CEs comprising 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde, but not CEs containing 0.63 μg/ml of *trans*-cinnamaldehyde, significantly downregulated IL-8 mRNA expression in *H. pylori*-infected AGS cells compared with the *H. pylori* control (Fig. 3). Consistent with these results on IL-8 mRNA expression, CEs comprising 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde significantly inhibited IL-8 protein production in *H. pylori*-infected AGS cells compared with the *H. pylori* control (Fig. 4). In our second assay, the concentrations of *trans*-cinnamaldehyde (10.5 μg/ml and 21 μg/ml) in CEs and *t*-SSs (0.001% and 0.002%) ranged between the aforementioned 2 effective concentrations 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde in CEs in the first assay, but were used for longer durations. Similar to the result of the first assay, CEs containing 21 μg/ml of *trans*-cinnamaldehyde significantly downregulated IL-8 mRNA expression in *H. pylori*-infected AGS cells compared with the *H. pylori* control, but CEs containing 10.5 μg/ml of *trans*-cinnamaldehyde did not (Fig. 5A). Moreover, CEs in both

concentrations of *trans*-cinnamaldehyde significantly suppressed IL-8 protein secretion from *H. pylori*-infected AGS cells compared with the *H. pylori* control, with their IL-8 levels close to that in the non-*H. pylori* control (Fig. 6A).

H. pylori-induced expression of IL-8 protein, but not mRNA, was inhibited by *t*-SSs. We found that *t*-SSs at the 2 tested concentrations did not significantly downregulate IL-8 mRNA expression in *H. pylori*-infected AGS cells, although lower concentrations of the *t*-SSs appeared to downregulate IL-8 mRNA more effectively than did higher concentrations (Fig. 5A). This trend is distinct from that of IL-8 downregulation by CEs. However, 0.002% *t*-SS (21 μg/ml of *trans*-cinnamaldehyde) significantly suppressed IL-8 protein production in *H. pylori*-infected AGS cells (Fig. 6A).

CEs and *t*-SSs downregulated IL-1β-induced IL-8 mRNA expression, but only CEs dose-dependently inhibited IL-1β-induced IL-8 protein secretion. Both CEs and *t*-SSs at both dilutions significantly downregulated IL-8 expression in IL-1β-treated AGS cells compared with the IL-1β control, and CEs exhibited stronger inhibitory effects than *t*-SSs (Fig. 5B). CEs at both dilutions significantly suppressed IL-8 protein secretion from IL-1β-pretreated AGS cells compared with the IL-1β control, with amounts of IL-8 secreted by the treated cells stepping down concentration-dependenly towards that of the non-IL-1β control (Fig. 6A).

So far, the postinfection therapeutic effects of cinnamon extracts on *H. pylori*-infected human gastric epitheium have not been investigated. A previous study demonstrated that preventive treatment of AGS cells with *C. cassia* extracts at 50 μg/ml and 100 μg/ml preceding a 4-h coculture with *H. pylori* markedly inhibited IL-8 secretion in a concentration-dependent manner.[29] Demonstrating a similar effective dosage range as

obtained with *C. cassia* noted above, our first assay showed that 3-h treatment with CEs comprising 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde potently downregulated IL-8 mRNA expression in the *H. pylori*-infected AGS cells, although the mRNA expression levels were still higher than that in the *H. pylori* control (Fig. 3). After an additional 20-h incubation, CEs containing 6.3 μg/ml and 63 μg/ml of *trans*-cinnamaldehyde reduced IL-8 protein secretion to levels almost the same as that in the non-*H. pylori* control (Fig. 4). We also observed similar disparities in mRNA expression and protein production after treatment with CEs comprising 10.5 μg/ml and 21 μg/ml of *trans*-cinnamaldehyde in our second assay (Figs. 5A and 6A). These results suggested that the inhibitory effect of CEs on host IL-8 protein production might be maximized by the suppression of IL-8 translation by CEs after the downregulation of IL-8 transcription.

3.4. Potential influences of minor components on anti-inflammatory effects of *trans*-cinnamaldehyde in CEs

CEs exhibited stronger inhibition on IL-8 expression than did *trans*-cinnamaldehyde alone. The *H. pylori*-induced IL-8 mRNA expression was more strongly inhibited by CE containing 21 μg/ml of *trans*-cinnamaldehyde than by 0.002% *t*-SS (21 μg/ml of *trans*-cinnamaldehyde) (Fig. 5A). Both CEs comprising 10.5 μg/ml and 21 μg/ml of *trans*-cinnamaldehyde more potently downregulated IL-1β-induced IL-8 expression than did 0.001% and 0.002% *t*-SSs with the same concentrations of *trans*-cinnamaldehyde, respectively (Fig. 5B). However, at protein levels, CE with 21 μg/ml of *trans*-cinnamaldehyde inhibited IL-1β-induced IL-8 production more potently than did 0.002% *t*-SSs (Fig. 6B).

CEs, but not *trans*-cinnamaldehyde, inhibited IL-8 production in human gastric

epithelium induced by IL-1β, although both CEs and *trans*-cinnamaldehyde downregulated IL-8 mRNA expression. Derived mainly from gastric epithelial cells and macrophages, IL-1β is substantially upregulated by *H. pylori* in both types of cells.[31] It stimulates significant IL-8 secretion in AGS cells.[32,33] IL-8 production by gastric epithelial cells and infiltration of the gastric mucosa by inflammatory cells are characteristic to the immunopathogenesis of *H. pylori* infection.[9] However, whether CEs and *trans*-cinnamaldehyde inhibit IL-8 expression in human gastric epithelium remained unclear. We demonstrated that IL-1β-induced IL-8 mRNA expression was downregulated by CEs containing 10.5 μg/ml and 21 μg/ml of *trans*-cinnamaldehyde, and *t*-SSs with the same concentrations of *trans*-cinnamaldehyde (Fig. 5B); however, IL-1β-induced IL-8 protein secretion was inhibited by CEs but not by *t*-SSs with the same concentrations of *trans*-cinnamaldehyde (Fig. 6). At a *trans*-cinnamaldehyde concentration of 21 μg/ml, CEs inhibited IL-1β-induced IL-8 mRNA and protein expression more strongly than did *t*-SSs (Figs. 5B and 6B). These results suggest that the minor components in CE other than *trans*-cinnamaldehyde play a key role in suppressing IL-8 expression in IL-1β-stimulated AGS cells, a synergistic effect that was particularly evident at the posttranscriptional level.

Trans-cinnamaldehyde might inhibit IL-8 secretion in the infected human gastric epithelium through its antibacterial activity against *H. pylori* rather than by downregulating IL-8 expression directly. We observed a discrepancy in the effect of *trans*-cinnamaldehyde on the transcription and translation of IL-8 (Figs. 5A and 6A). One possible explanation is that *trans*-cinnamaldehyde directly inhibited the growth of *H. pylori* colonized on the AGS cells during the 6-h infection period and indirectly reduced bacterial provocation of host cell inflammation. This possibility is supported by our results in the antibacterial susceptibility test of CEs and *t*-SSs against against *H.*

pylori, and a previous study showing the anti-*H. pylori* property of cinnamaldehyde with no detection of antimicrobial resistance.[30]

The minor components in CEs other than *trans*-cinnamaldehyde modulate the anti-inflammatory effect of *trans*-cinnamaldehyde on IL-8 responses in the inflamed human gastric epithelium. Similarly, a previous study using GC-MS also detected not only *trans*-cinnamaldehyde as the major compound but also 2 minor components in CEs, benzaldehyde and 3-phenylpropionaldehyde, which could not be identified using HPLC.[18] Our study showed that the original CE inhibited bacterial growth of *H. pylori* more significantly than the equivalent *trans*-cinnamaldehyde (Fig. 2), and CEs concentration-dependently inhibited IL-8 expression more strongly than did *t*-SSs containing the same concentrations of *trans*-cinnamaldehyde (Figs. 5 and 6). Despite their low percentages in CEs, benzaldehyde, 3-phenylpropionaldehyde, cinnaldehyde, and eugenol might synergize with the major compound *trans*-cinnamaldehyde in CEs for their antibacterial and anti-inflammatory effects on *H. pylori* infection.

4. Conclusion

To date, the only one clinical trial using 80 mg/d of *C. cassia* extracts for 4 wk in 23 patients has failed to effectively eradicate *H. pylori*, possibly due to the small sample number, varying degrees of *H. pylori* colonization, low dosage of the cinnamon extracts used, and early evaluation for *H. pylori* eradication.[16] Our study demonstrated that CEs containing primarily *trans*-cinnamaldehyde and 4 minor compounds at specific concentrations can inhibit *H. pylori* growth and, when applied post exposure, suppress *H. pylori*- and IL-1β-induced IL-8 responses in human gastric epithelium *in vitro*. Therefore, cinnamon species, appropriate dosages of extracts, and durations of treatment should be considered in future clinical trials. The CEs obtained from dry

leaves of the indigenous cinnamon in Taiwan exhibited a more potent antibacterial activity and a stronger anti-inflammatory effect than did *trans*-cinnamaldehyde alone, demonstrating the potential for using CE in the treatment of *H. pylori* infection.

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Conflict of interest

No conflict to disclose.

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FIGURE LEGENDS

Fig 1. GC-MS and HPLC analysis of CEs. (A) GC-MS spectrograms of CEs showed 5 peaks representing 5 components with their relative abundance and chemical structure formula (4.22 min: benzaldehyde, 7.51 min: 3-phenylpropionaldehyde, 8.44 min: *cis*-cinnamaldehyde, 9.27 min: *trans*-cinnamaldehyde, 10.36 min: eugenol). (B) The correlation curve and the regression equation among the 5 concentrations of *t*-SSs and their peak area values. (C) Merged HPLC chromatograms of *t*-SSs at 5 concentrations and of CE.

Fig. 2. Antibacterial susceptibility test of CEs against *H. pylori*. Bars represent the OD_{600} values of the *H. pylori* cultures incubated in TSB broth with 10% FBS for 48 h with 10 concentrations of *trans*-cinnamaldehyde (TC) in CEs, or 4 selected comparable concentrations of TC in *t*-SSs, or without CE (HP: *H. pylori* only). NT control indicates non-treated controls. The broths containing 2 concentrations of amoxicilln (Amoxici) and tetracycline (Tetracy) are positive controls. Significant differences between OD_{600} values of various groups and HP cultures, and between certain CEs and *t*-SSs/positive controls (brackets) are indicated by asterisks (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Fig. 3. IL-8 mRNA expression in AGS cells at the end of 4-h incubation, after initial 1-h infection with *H. pylori* (HP) followed by 3-h treatment with CEs at 3 dilutions. Significant differences between IL-8 mRNA expression levels in cells exposed to various conditions and HP-treated cells are indicated by asterisks (**$p < 0.01$, *$p < 0.05$).

Fig. 4. Secreted IL-8 levels in the 20-h-cultured media from AGS cells collected at the end of 24-h incubation, after initial 1-h infection with *H. pylori* (HP) followed by 3-h treatment with CEs at 3 dilutions. Significant differences between the IL-8 protein levels in cells exposed to various conditions and HP-treated cells are indicated by asterisks (**$p < 0.01$, *$p < 0.05$).

Fig. 5. IL-8 mRNA expression in AGS cells at the end of 8-h incubation, after initial 2-h exposure to *H. pylori* (HP) or IL-1β followed by 6-h treatment with CEs or *t*-SSs at 2 dilutions. Significant differences between IL-8 mRNA expression levels in cells exposed to various conditions of *trans*-cinnamaldehyde (TC) and HP/IL-1β-treated cells, and between CE- and *t*-SS-treated cells (brackets), are indicated by asterisks (**$p < 0.01$, *$p < 0.05$).

Fig. 6. Secreted IL-8 levels in the 16-h-cultured media from AGS cells collected at the end of 24-h incubation, after initial 2-h exposure to *H. pylori* (HP) or IL-1β followed by 6-h treatment with CEs or *t*-SSs at 2 dilutions. Significant differences between IL-8 levels in cells exposed to various conditions of *trans*-cinnamaldehyde (TC) and HP/IL-1β-treated cells, and between CE- and *t*-SS-treated cells (bracket), are indicated by asterisks (**$p < 0.01$, *$p < 0.05$).

1 FIGURES

2 **Fig 1.**

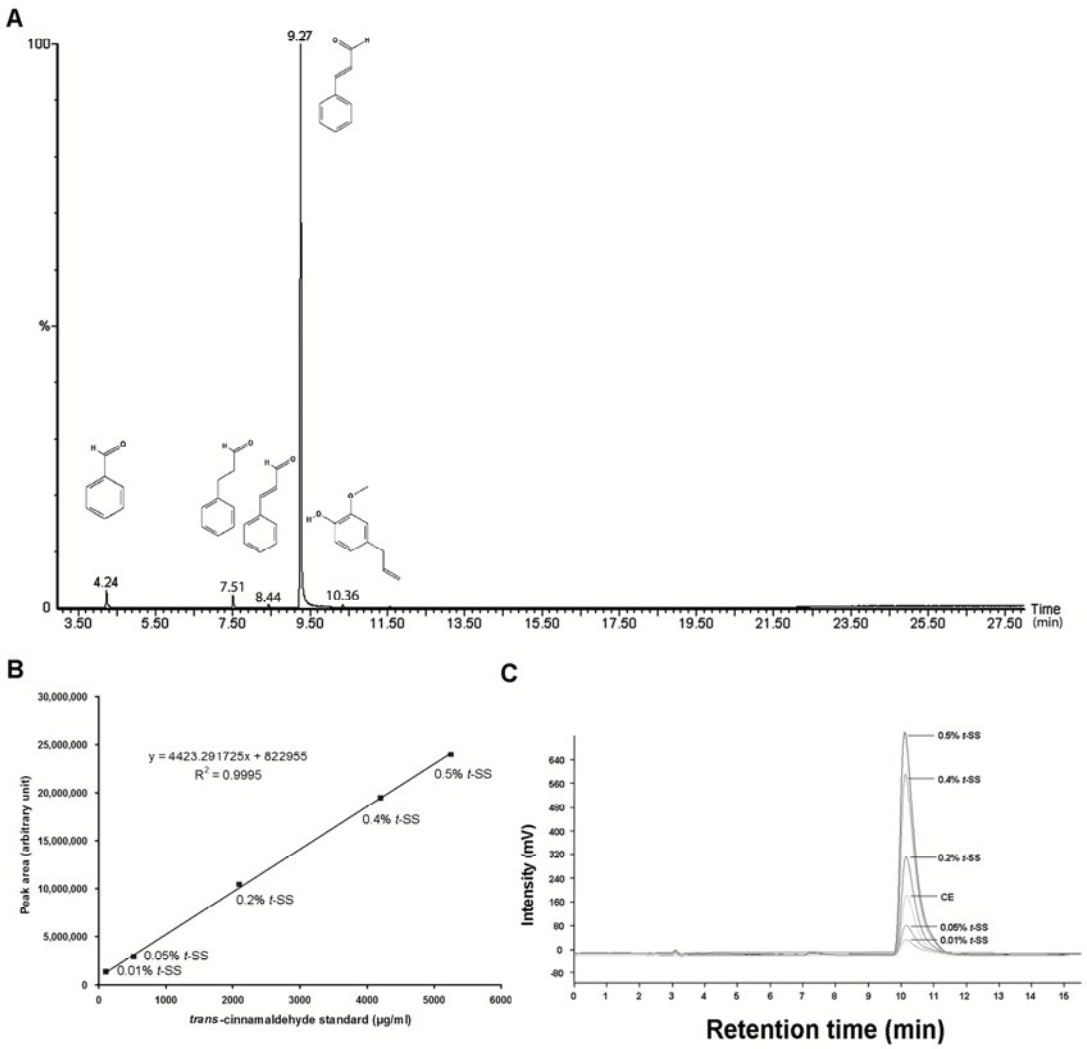

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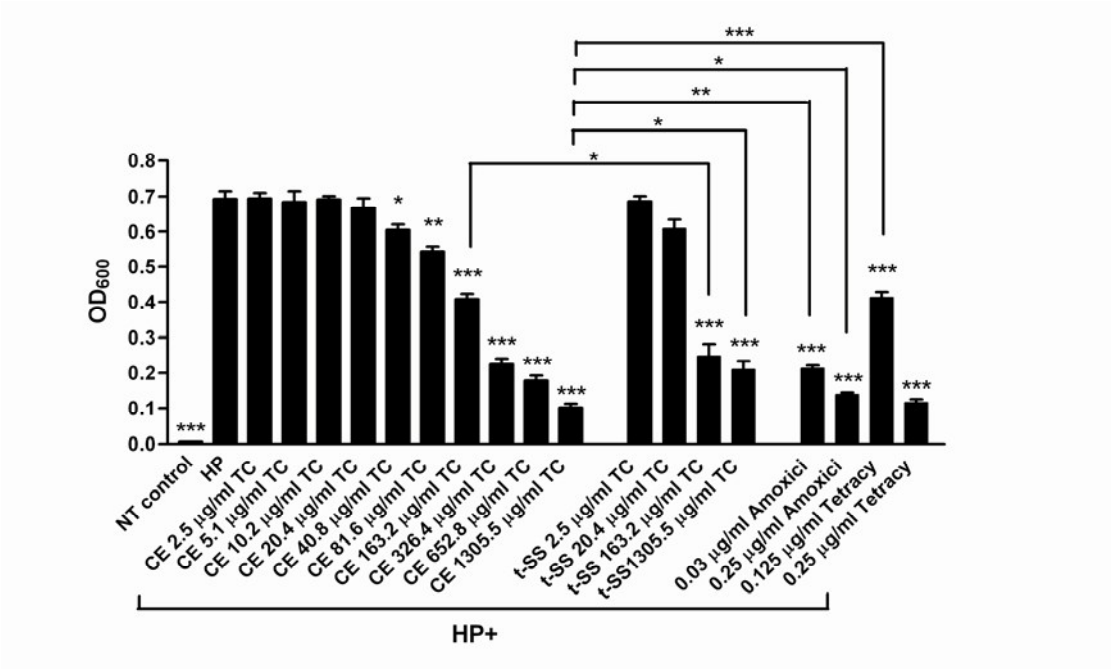
Fig 2.



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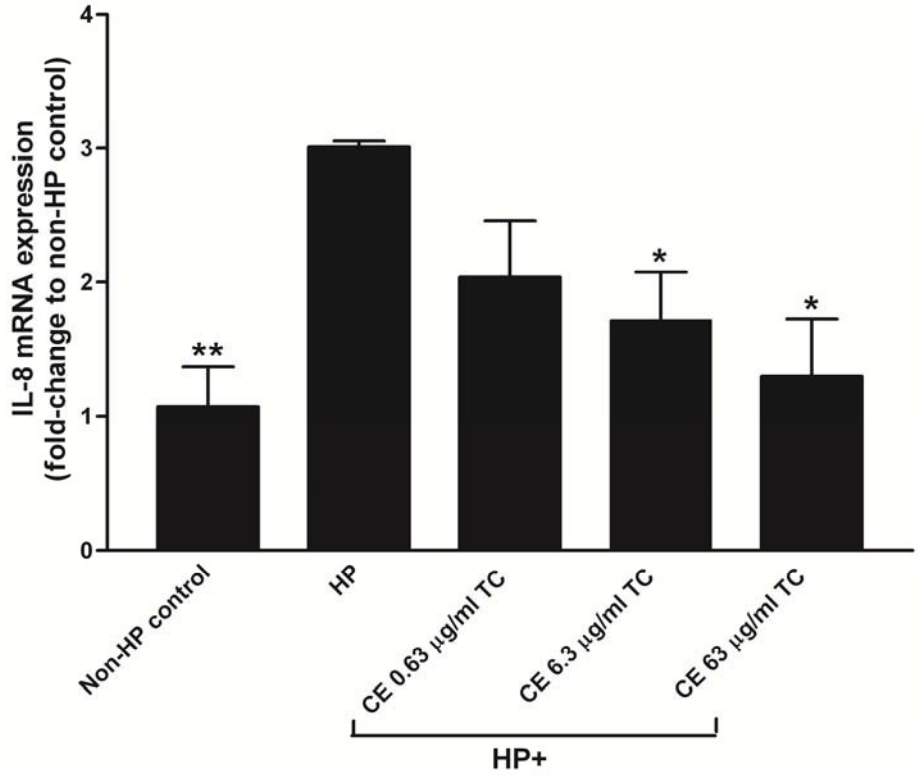

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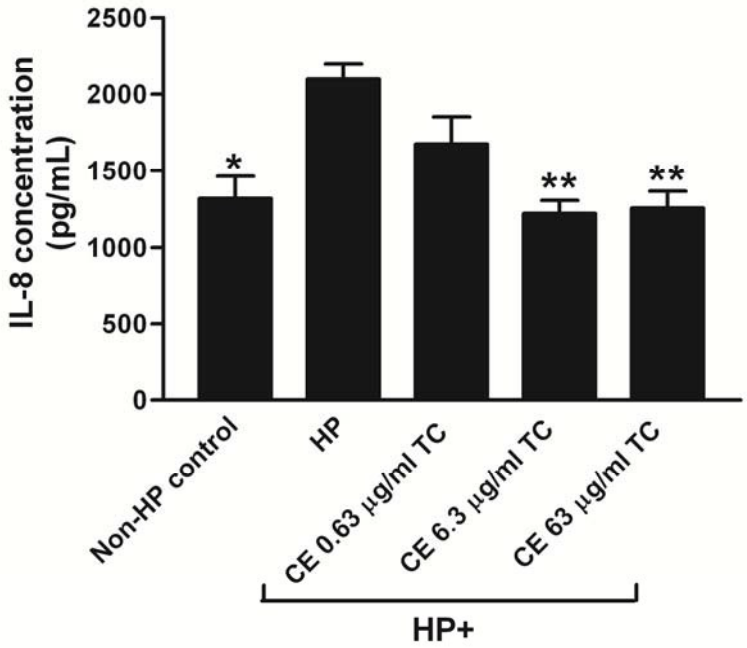

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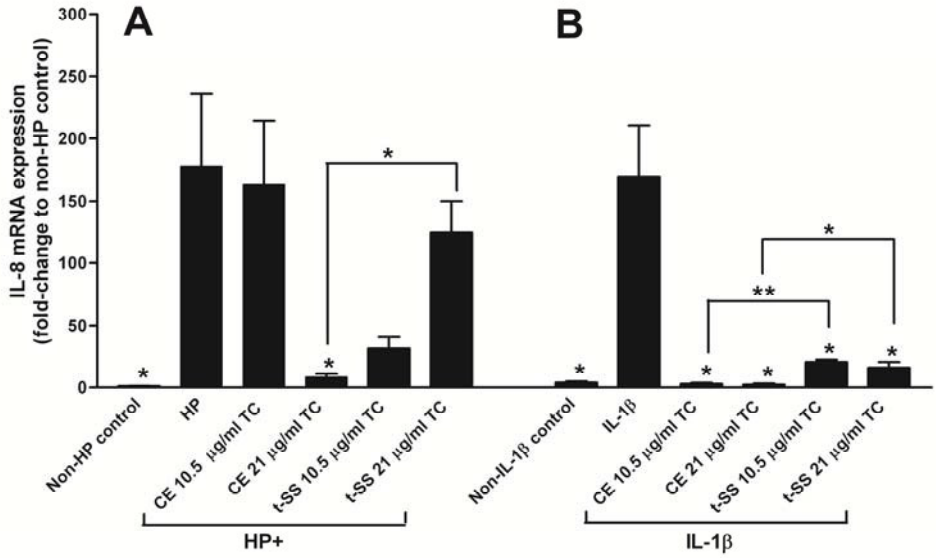

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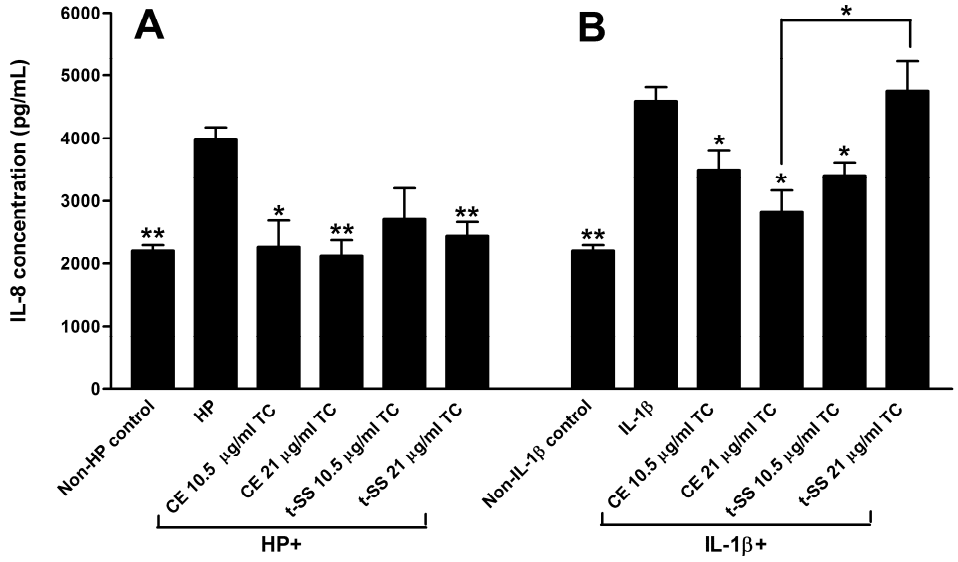

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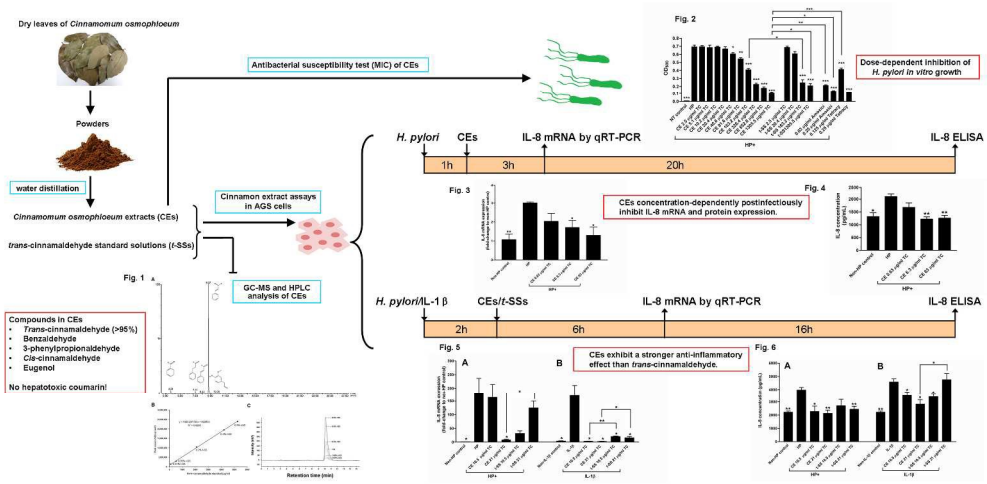

Extracts from dry leaves of *Cinnamomum osmophloeum* concentration-dependently inhibit *Helicobacter pylori* growth and postinfectious interleukin-8 expression in human gastric epithelium
800x400mm (167 x 167 DPI)