(-)-Patchouli alcohol protects against *Helicobacter pylori* urease-induced apoptosis, oxidative stress and inflammatory response in human gastric epithelial cells

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27 *Abbreviations*

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
CAT	catalase
Cys	cysteine
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GES-1	human gastric epithelial cells
Gly	glycine
His	histidine
HPU	<i>Helicobacter pylori</i> urease
IL-2	interleukin-2
IL-4	interleukin-4
IL-13	interleukin-13
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
Lys	lysine
MDA	malonyldialdehyde
Met	methionine
MMP	mitochondrial membrane potential
MTT	methyl thiazolyl tetrazolium
NO	nitrite oxide
PA	(-)-Patchouli alcohol
PI	propidium iodide
ROS	reactive oxygen species
RMSD	root-mean-square deviation
SOD	superoxide dismutase
TNF- α	tumor necrosis factor- α

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Abstract

(-)-Patchouli alcohol (PA), the major active principle of *Pogostemonis Herba*, has been reported to have anti-*Helicobacter pylori* and gastroprotective effects. In the present work, we aimed to investigate the possible protective effect of PA on *H. pylori* urease (HPU)-injured human gastric epithelial cells (GES-1) and to elucidate the underlying mechanisms of action. Results showed that pre-treatment with PA (5.0, 10.0, 20.0 μ M) was able to remarkably ameliorate the cytotoxicity induced by 17.0 U/mg HPU in GES-1 cells. Flow cytometric analysis on cellular apoptosis showed that pre-treatment with PA effectively attenuated GES-1 cells from the HPU-induced apoptosis. Moreover, the cytoprotective effect of PA was found to be associated with amelioration of the HPU-induced disruption of MMP, attenuating oxidative stress by decreasing contents of intracellular ROS and MDA, and increasing superoxide dismutase (SOD) and catalase (CAT) enzymatic activities. In addition, pre-treatment with PA markedly attenuated the secretion of nitric oxide (NO) and pro-inflammatory cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4) and tumor necrosis factor- α (TNF- α), whereas elevated the anti-inflammatory cytokine interleukin-13 (IL-13) in the HPU-stimulated GES-1 cells. Molecular docking assay suggested that PA engaged in the active site of urease bearing nickel ions and interacted with important residues via covalent binding, thereby restricting the active urease catalysis conformation. Our experimental findings suggest that PA could inhibit the cellular processes critically involved in the pathogenesis of *H. pylori* infection, and its protective effects against the HPU-induced cytotoxicity in GES-1 cells are believed to be associated with its anti-apoptotic, antioxidative, anti-inflammatory and HPU inhibitory actions.

Key words: Patchouli alcohol; *Helicobacter pylori* urease; Human gastric epithelial cells; Apoptosis; Oxidative; Inflammatory.

1. Introduction

Helicobacter pylori, infecting over half of the world's population, is a vital etiological factor of many gastrointestinal diseases [1]. *H. pylori* infection produces various virulence factors in the stomach and induces mucosal apoptosis, reactive oxygen species (ROS) and inflammatory response with release of various cytokines, and subsequently cause many diseases of the stomach such as gastritis, peptic ulcer and gastric cancer [2,3].

To date, the most effective treatment against *H. pylori* infection is an antibiotics-based combination therapy. Despite the eradication of the pathogenic organism has been shown to reduce gastric inflammation and disorders, emerging antibiotic resistance and undesirable side effects have increasingly compromised the clinical efficacy of antibiotics-based regimen. Furthermore, the antibiotics currently used for *H. pylori* eradication normally possess a broad antibacterial spectrum, and their use would inevitably affect the normal gut flora, leading to a series of gastrointestinal side effects [4]. Hence, there is an urgent need to search for safer and more effective non-antibiotic agents with selective anti-*H. pylori* action for gastric diseases.

H. pylori urease (EC 3.5.1.5; urea amidohydrolase, HPU) plays an essential role in the pathogenesis of *H. pylori* infection by protecting the bacteria from the acid environment of the stomach, and promoting bacterial colonization via inducing the production of ammonia. Previous reports have shown that HPU promoted apoptosis, oxidative stress and the production of inflammatory mediators in gastric epithelial cells (GES-1), and lead to altered epithelial proliferation and oxidative DNA damage [5,6]. Therefore, HPU is deemed as an important target for selective anti-*H. pylori* agents in the management of the gastric disease [7].

Identification of effective and safe HPU inhibitors derived from medicinal plants is becoming an important area of research for the development of new therapeutic agents against *H. pylori* infections [8]. *Pogostemonis Herba*, the dried aerial parts of *Pogostemon cablin* (Blanco) Benth (Labiatae), is traditionally used in Chinese medicine for the treatment of a wide range of ailments, including gastrointestinal disorders, such as dyspepsia, gastritis and peptic ulcer [9]. Previous researches have revealed that the aqueous extract of *Pogostemonis Herba* could effectively protect the intestinal barrier function [10] and improve the digestive function [11]. (-)-patchouli alcohol (CAS number 5986-55-0, PA), a sesquiterpene officially used as a chemical marker for the quality assessment of *Pogostemonis Herba* in the Chinese Pharmacopoeia [12], has proved to exhibit various biological effects, including free radical-scavenging, anti-inflammatory, and immunomodulatory activities [13-15]. Recent work from our laboratory has also demonstrated the effectiveness of PA as a gastroprotective, anti-*H. pylori* and anti-urease agent. PA pre-treatment cleared *H. pylori* from the stomach of infected mice and relieved gastric inflammation caused by the bacterium. In addition, PA was demonstrated to selectively inhibit *H. pylori* functioning by inhibiting its HPU, while exerted no effect on other major normal gastrointestinal bacteria [16-18]. However, the possible modulatory effect of PA on the HPU infected GES-1 cells has not been investigated.

Based on the critical roles of gastroprotective and anti-*H. pylori* activities in the management of gastric diseases, the present study was, therefore, aimed to evaluate the possible cytoprotective effect of PA on the HPU-induced injury in GES-1 cells and to elucidate the underlying mechanisms of action.

2. Materials and Methods

2.1. Plant materials.

PA (Fig. 1) was isolated in our laboratory from patchouli oil with purity above 99.0% by gas chromatographic analysis and the optical rotation ($[\alpha]_D^{25}$) was measured to be -120° (c 2.0, CHCl_3) as reported previously [15]. Its structure was confirmed by melting point, infrared spectroscopy (IR), as well as by ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) as described in our previous work [19]. PA was dissolved in dimethyl sulfoxide (DMSO) and the solvent concentration was ensured to be less than 0.1% in all experiments [20]. The GES-1 cells grown in the medium containing an equivalent amount of DMSO without PA were used as control.

2.2. Preparation and activity determination of HPU.

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA, USA) were grown in *Brucella* broth supplemented with 10% heat-inactivated horse serum for 24 h at 37°C under microaerobic conditions (5% O_2 , 10% CO_2 , and 85% N_2). HPU was prepared and its activity was determined following the reported regime [16]. The resultant urease solution was added to an equal volume of glycerol and stored at 4°C until use in the experiment.

2.3. Cell culture and treatments.

Human GES-1 cells were kindly provided by the First Affiliated Hospital of Guangzhou University of Chinese Medicine. They were maintained and grown in high glucose DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37°C in humidified atmosphere of 95% air and 5% CO_2 .

To study the possible protective effect of PA, GES-1 cells were divided into control group (without PA and HPU treatment), HPU group (only 17.0 U/mg HPU for 24 h); and HPU plus PA (5.0, 10.0, 20.0 μM) groups. For HPU plus PA groups, GES-1 cells were pre-treated with PA for 4 h prior to co-cultivation with HPU for 24 h. The

dose of PA was selected based on the results of our previous investigation and preliminary experiment.

2.4. Cell viability assay.

Cell viability was assessed by the MTT assay, which is a test of normal metabolic status of cells based on the reduction of MTT to a purple formazon product by mitochondrial dehydrogenases of viable cells.

2.5. Annexin V-FITC-propidium iodide (PI) assay.

The number of apoptotic cell death induced by the HPU was measured by flow cytometry using Annexin V-FITC-PI kit (Genzyme). Samples were analyzed within 1 h using a flow cytometer (BD Biosciences, CA, USA). The Annexin V and PI emissions were detected in the FL1-H and FL2-H channels of a FACS Vantage low cytometer, using emission filters of 525 and 575 nm, respectively. Fluorescence minus one controls were used to set the positive/negative cell gates and validate the flow cytometric results.

2.6. Measurement of the mitochondrial membrane potential (MMP, $\Delta\psi M$).

The changes in the mitochondrial transmembrane potential (MMP, $\Delta\psi M$) were determined using JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, a MMP probe). GES-1 cells were seeded in 6-well culture plates at a density of 5×10^5 cells/well. At the end of the above treatment scheme, cells were washed twice with PBS solution and incubated with JC-1 (final concentration 10 $\mu\text{g/mL}$) for 30 min at 37 °C in the dark. The changes in MMP were assessed using fluorescence microscope (Olympus, Japan).

2.7. Measurement of intracellular reactive oxygen species (ROS).

The formation of ROS was evaluated by means of the probe 2,7-dichlorodihydro-fluorescein diacetate (H₂DCF-DA) **as per** a previously described method [21]. ROS levels were measured as the fluorescence intensity of 2,7-dichlorodihydro-fluorescein (DCF) by flow cytometry (BD Biosciences, CA, USA) **with** excitation and emission wavelengths **being** 488 and 525 nm respectively.

2.8. SOD, CAT and MDA assay.

After the treatment, the GES-1 cells were washed twice with ice-cold PBS, harvested by centrifugation at 1000× g for 4 min, pooled in 0.5 mL PBS, and homogenized. The homogenate was centrifuged at 4000× g for 15 min, and the supernatant was collected for SOD, CAT and MDA assay using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) **according to** the manufacturer's instructions.

2.9. Nitrite oxide assay.

The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction method described elsewhere [22].

2.10. Cytokine determination.

After HPU treatment for 24 h, the supernatants were collected and used for cytokine assays. The levels of IL-2, IL-4, IL-13 and TNF-α in the supernatant were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Co., Carlsbad, CA, USA) **as per** the manufacturer's instructions. The levels of IL-2, IL-4, IL-13 and TNF-α were expressed as the percentage of the non-treated control.

2.11. Protocol of molecular docking study.

Docking studies were carried out **as per** our earlier investigation [23]. Molecular

docking simulations were performed using the software Auto-Dock version 4.0 along with AutoDockTools (ADT 1.5.2) using the hybrid Lamarckian Genetic Algorithm (LGA). The three-dimensional (3D) crystal structure of HPU (PDB code: 1E9Y) was obtained from the RCSB Protein Data Bank, and its resolution was 3.00 Å. The standard 3D structure (PDB format) of PA was obtained with chem3D Ultra 8.0 software. The cubic grid box of 60 Å size (x, y, z) with a spacing of 0.375 Å and grid maps were built. The center of the grid was set to the average coordinates of the two Ni²⁺ ions. All other parameters were used according to default settings of AutoDock 4.0.

2.12. Statistical analysis.

The data were expressed as the Mean ± Standard Error (S.E.M). Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test to detect differences between treatment groups and the control. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effect of PA on GES-1 cells by MTT.

The effect of PA on the viability of GES-1 cells cultured for 24 h was detected by MTT assay. As shown in Fig. 2, no significant changes were observed on the viability of GES-1 cells treated with individual PA in the range of 10-75 µM, while 100 µM caused obvious cell damage. The concentrations from 5-20 µM of PA were chosen for the subsequent experiments.

3.2. PA inhibited the HPU-induced GES-1 cell death.

The protective effect of PA against the HPU-induced cell injury was measured with the MTT assay. As shown in Fig. 3, stimulation with HPU alone resulted in a significant decrease (74%, $p < 0.05$) in cell viability as compared with control group.

In contrast, the cell viability in PA (5, 10 and 20 μ M) pre-treatment group was notably higher (10%, 20% and 13%, all $p < 0.05$) than those of cells exposed to HPU alone after 24 h culture. Among the test concentrations, 10 μ M of PA showed the best protective effect, which increased the cell survival rate from $58.7 \pm 8.2\%$ (vehicle group) to $79.2 \pm 3.3\%$. These data indicated that PA could improve the cellular viability of GES-1 cells and ameliorate the damage caused by HPU.

3.3. PA inhibited the HPU-induced GES-1 cell apoptosis.

Flow cytometry was used to investigate the apoptosis of GES-1 cells induced by HPU and quantify the extent of total apoptotic cells. As depicted in Fig. 4A, there were primarily two populations of cells after treatments: cells that were viable and not undergoing apoptosis (Annexin V-FITC⁻PI⁻) and cells undergoing apoptosis (Annexin V-FITC⁺PI⁻). A minor population of cells were observed to be Annexin V-FITC⁺PI⁺, indicating that they were in the late stage of apoptosis or necrosis.

As shown in Fig. 4B, the rate of apoptotic cells was greatly increased in the HPU-treated group (28.4%) compared to that in the control group (2.3%, $p < 0.05$). **Pre-treatment** with PA (5, 10 and 20 μ M) significantly attenuated the HPU-induced cellular apoptosis and the percentage of apoptotic population decreased to 17.8%, 13.4%, and 12.1%, respectively.

3.4. PA inhibited the HPU-induced dissipation of $\Delta\psi$ M of GES-1 cells.

The protective effect of PA on mitochondrial function in the HPU-treated GES-1 cells was measured by JC-1 staining. Control cells stained with JC-1 emitted mitochondrial bright red fluorescence (Fig. 5A). However, when cells were exposed to the HPU (17.0 U/mg) for 24 h, the $\Delta\psi$ m depolarized, as shown by the increase in green fluorescence and the concomitant relative disappearance of red fluorescence.

Pre-treatment with PA (5, 10 and 20 μ M) reduced the changes in $\Delta\psi_m$ as indicated by repression of green fluorescence and restoration of red fluorescence, suggesting that PA was able to protect GES-1 cells by preventing the HPU-induced mitochondrial damage. Quantitative results from fluorescence microplate assay also suggest that PA pre-treatment (5, 10 and 20 μ M) could significantly (0.77 ± 0.11 , 0.79 ± 0.12 and 0.69 ± 0.13 , respectively, all $p < 0.05$) mitigate the loss of $\Delta\psi_m$ induced by HPU (1.50 ± 0.29), thereby preserving the dissipation of the membrane polarity of mitochondria.

3.5. PA inhibited the HPU-induced ROS production in GES-1 cells.

Previous studies have indicated the involvement of ROS in the HPU-induced apoptosis [24]. To determine the effect of PA on the HPU-induced ROS production, the intracellular ROS was visualized by detecting DCF derived from the oxidation of H_2DCF . A representative fluorescence pattern from flow cytometry (Fig. 6A) illustrated that a marked elevation of oxidative stress was evidenced upon treating GES-1 cells with HPU for 24 h (by 58% of control, $p < 0.05$). Whereas PA pre-treatment left shifted the fluorescence peak closer to the peak generated by control cells, and significantly attenuated the HPU-induced ROS production (21%, 47% and 40% respectively, all $p < 0.05$). As shown in Fig. 6C, the DCF fluorescence intensity in the HPU group was significantly increased (2771.13 ± 512.64 , $p < 0.05$) when compared with the control group (1815.92 ± 237.45) in fluorescence microplate test, indicating that the HPU induced noticeable escalation in ROS level. However, the overproduction of intracellular ROS was markedly attenuated by pre-treatment with 10 and 20 μ M of PA (2023.52 ± 185.77 and 2143.58 ± 335.90 , both $p < 0.05$), a result congruent with that from the fluorescence microscope (Fig. 6B).

3.6. Effect of PA on SOD, CAT and MDA levels of GES-1 cells.

As shown in Fig. 7, the enzymatic activities of SOD (11.90 ± 1.11 U/mg prot) and CAT (13.57 ± 1.14 U/mg prot) were dramatically decreased after GES-1 cells were cultured in media containing HPU (17 U/mg) for 24 h (SOD, 8.60 ± 0.79 U/mg prot; CAT, 8.07 ± 1.31 U/mg prot). The cellular MDA level in HPU group was significantly higher (3.80 ± 0.18 nmol/mg prot) when compared with the control group (2.53 ± 0.19 nmol/mg prot, $p < 0.05$). It was observed that these variations produced by HPU were markedly improved in PA pre-treatment group. Pre-treatment with PA observably increased the levels of SOD and CAT, and decreased the MDA contents. Of which, PA at high dose (20 μ M) exhibited stronger antioxidative capacity (SOD, 10.96 ± 1.20 U/mg prot; CAT, 11.92 ± 1.03 U/mg prot; MDA, 2.99 ± 0.33 nmol/mg prot, all $p < 0.05$).

3.7. Effect of PA on NO release and cytokine productions in the HPU-injured GES-1 cells.

Results indicates the incubation of GES-1 cells with 17.0 U/mg HPU for 24 h caused a significant accentuation in NO release (by 52.7%, $p < 0.05$), pro-inflammatory cytokines IL-2 (48%, $p < 0.05$), IL-4 (97%, $p < 0.05$) and TNF- α production (40%, $p < 0.05$) in the medium, and a noticeable decrease in the anti-inflammatory mediator IL-13 (34%, $p < 0.05$). While the incubation of the cells with PA (5, 10, and 20 μ M) not only significantly attenuated the NO production (12%, $p < 0.05$; 10%, $p > 0.05$; 23%, $p < 0.05$) and IL-2 (20%, $p > 0.05$; 22%, $p < 0.05$; and 30%, $p < 0.05$), IL-4 (33%, $p > 0.05$; 47%, $p < 0.05$; and 44%, $p < 0.05$) as well as TNF- α levels (13%, $p > 0.05$; 20%, $p < 0.05$; and 20%, $p < 0.05$) (Fig. 8), but also reversed the suppressed level of IL-13 secretion (6%, 14% and 13%, all $p < 0.05$) elicited by HPU.

3.8. Molecular docking study.

To gain further insight into the molecular working mechanism of the HPU inhibitory effect of PA, molecular docking of possible interaction between PA and HPU was carried out. The best possible binding modes of PA with HPU are shown as cartoon modes, enzyme surface (Fig. 9A), and interaction diagram (Fig. 9B), respectively. In the docked conformation of PA, the logarithm of free binding energy (ΔG) of PA to the HPU active site was -5.67 kcal/mol. PA was shown to interact with the bi-nickel center of HPU, with the oxygen atom of hydroxyl group coordinating with Ni atoms (2.9 and 3.1 Å, respectively). Similarly, interacting hydrogen bonding could be observed by Ala 169 (O...H distance = 2.8 Å) and Asn 168 (O...H distance = 2.5 Å) residues with oxygen of hydroxy group, jointly contributing to the restriction of the active conformation of HPU active site. In addition, PA was found to interact with HPU active sites His 248, His 221, His 138, Cys 321, Kcx 219, Ala 365, Asp 362, Met 366, Gly 279, Arg 338 via van der Waals (vdW) interaction (Fig. 9B).

4. Discussion

In recent years, a series of natural urease inhibitors were isolated from plants, such as betulinic, oleanolic acid, sulforaphane and resveratrol, among others [25-27]. However, few of them have ever been investigated for the possible cytoprotective effect against the HPU-induced injury in human gastric epithelial cells.

In our previous investigation, PA was demonstrated to possess *in vivo* and *in vitro* selective anti-*H. pylori* and anti-HPU activities [16]. Besides, PA exhibited significant gastroprotective effect against gastric ulceration in rats [17]. In the present follow-up work, the possible cytoprotective effect and mechanism of PA on the HPU-induced GES-1 cell injury were probed. GES-1 was employed to establish a HPU-damaged gastric epithelial cell model, which mimicked the *in vivo* environment

of the HPU-induced pathogenesis and infection of *H. pylori* towards gastric mucosa; and the possible modulatory role of PA was evaluated in this model. Gastric cells were exposed to 17.0 U/mg HPU with or without the addition of PA. Results of the current study revealed that PA (5, 10 and 20 μ M) visibly increased the viability of GES-1 cells injured by HPU ($p < 0.05$), suggesting that PA might act as an effective cytoprotectant against the HPU-induced GES-1 cell injury.

H. pylori interaction with gastric mucosa is followed by several key events linked to the pathogenesis, including apoptosis of gastric epithelial cells, inflammatory response with release of various cytokines and oxidative stress with accumulative ROS [2,3]. Agents that could mitigate the damages of these cascades of events could exert beneficial effects against *H. pylori* infection. In the present study, apoptosis was investigated by Annexin V-FITC/PI staining. Annexin V-FITC/PI staining of HPU-treated GES-1 cells resulted in an increase in Annexin⁺/PI⁻ and Annexin⁺/PI⁺ cells compared to the control (Annexin⁻/PI⁻), indicating apoptosis as a possible mode of cell death. However, it was significantly ameliorated by PA pre-treatment at three tested doses. Hence, it was indicated that PA possessed cytoprotective effect against the HPU-induced apoptosis in GES-1 cells.

Mitochondrial dysfunction as a result of the decrease of MMP or excessive production of ROS has been implicated in apoptosis [28]. Overproduction of ROS would cause mitochondrial dysfunction, which can reciprocally aggravate ROS production. This vicious circle may cause further deterioration of the disease. ROS has been demonstrated to play a cardinal role in the pathogenesis of different types of gastric mucosal injuries inflicted by *H. pylori* [3].

In the present study, when GES-1 cells were exposed to HPU, depolarization of $\Delta\psi_m$, indicative of mitochondrial dysfunction, was remarkably observed and this malfunction was alleviated significantly by pre-treatment with PA. The presence of

PA (5, 10 and 20 μ M) significantly decreased the number of depolarized cells as indicated by the JC-1 fluorescence in the MMP analysis (Fig. 5). Then damaged mitochondria could cause defects in lipid homeostasis and ROS production, leading to lipid accumulation and oxidative stress [29]. Among many biomarkers of oxidative stress, MDA, SOD and CAT are known to be three sensitive indicators.

Increased intracellular ROS and MDA levels with concomitant decreased SOD and CAT activities were found in the HPU-induced GES-1 cells, suggesting that oxidative stress caused by ROS and the disruption of the oxidation-antioxidation homeostasis was involved in the pathogenesis of the HPU-elicited GES-1 cellular injury. However, pre-treatment with 10 and 20 μ M PA significantly alleviated oxidative stress by reducing the levels of ROS and MDA and enhancing the depressed cellular SOD and CAT activities, thereby contributing to reducing oxidative stress and elevating total antioxidant status. PA pre-treatment at dose of 5 μ M only showed a trend, but did not achieve statistic significance.

The most frequent phenotype associated with *H. pylori* infection is inflammation of the gastric mucosal membrane. During inflammation, many cytokines were up-regulated and accumulated in the stomach; among which, TNF- α , IL-2 and IL-4 have been recently implicated as critical mediators of *H. pylori*-induced cytotoxicity [6]. Accumulating evidence suggests that HPU possesses pro-inflammatory effect and contributes greatly to the inflammation underlying the gastrointestinal disease caused by *H. pylori* [6]. Inhibition of inflammatory cytokine productions was proposed to play a significant role in therapeutic potential for the treatment of gastrointestinal inflammatory diseases. NO is the first line of defense against invading pathogens and causes apoptotic cell death in various cell systems by means of oxidative stress. It was reported that *H. pylori* induced apoptosis in gastric epithelial cells through inducible NO [30].

In the current work, pro-inflammatory cytokines IL-2, IL-4 and TNF- α levels as well as NO release were markedly increased in GES-1 cells treated with HPU, but their levels in the HPU injured GES-1 cells were significantly reduced by 4 h co-incubation with PA (5, 10 and 20 μ M) (Fig. 8), indicating that PA might exert potentially beneficial effect against the HPU-induced GES-1 cell injury by modulation of inflammatory mediator and NO level. Of which, the significant effects were mostly observed at the doses of 10 and 20 μ M. In our previous investigation, we have also demonstrated the effectiveness of PA as an anti-inflammatory agent *in vitro* in the LPS-stimulated RAW264.7 macrophages and *in vivo* in the carrageenan-induced paw edema in rats. Herewith, the overall protective effects observed in this study further corroborate the potential of PA as an anti-inflammatory agent.

Previously, PA was found to display highly selective anti-*H. pylori* activity based on potent inhibitory potential against its urease. From the molecular docking study, it was evident that PA molecule engaged deeply in the active site of urease bearing nickel ions, interacting with Ni atoms via hydrogen bond. Apart from the nickel active site, other residues of the binding pocket, such as Asn168, Ala169, His221, Ala365 and Cys321, also participate in the substrate binding, stabilizing the catalytic transition state and accelerating the reaction. From the docking mode, the inhibitory effect of PA on HPU was believed to be partly attributable to the covalent binding with important residues mentioned by virtue of hydrogen bonding and van der Waals (vdW) interaction, thereby preventing availability of the active urease catalytic conformation.

The current first-line monotherapeutic agent for *H. pylori* treatment either focuses on the direct anti-*H. pylori* effect (antibiotics) or gastroprotective property (e.g. proton-pump inhibitor), and none of them possess simultaneous anti-*H. pylori*,

HPU-inhibitory and gastroprotective activities. Furthermore, their combined use was usually associated with various side effects, such as antibiotic resistance and gastrointestinal disorders. Taken together with the fact that PA has been widely used as food additive permitted for direct addition to food for human consumption for a long time [9], PA is regarded as having a wide safety margin for potential medicinal application. Based on its simultaneous gastroprotective and selective anti-*H. pylori* properties, PA is believed to possess good potential for developing into a novel non-antibiotic therapeutic agent in the management of *H. pylori*-related gastrointestinal disorders, and the discovery of such new non-antibiotic agent would provide a safe and effective complementary approach to the current conventional medications. Future studies for elucidating the detailed molecular mechanism underlying the cytoprotective effect of PA would likely pave an avenue for successful drug development process.

5. Conclusion

In conclusion, PA was amply demonstrated in our present study to exert protective effect against the HPU-induced cellular injury and apoptosis in GES-1 cells, and the cytoprotective mechanism might be associated with its ability to mitigate cellular apoptosis, decrease intracellular ROS, preserve mitochondrial function, and improve the antioxidant and anti-inflammatory homeostasis as well as direct inhibition of HPU action sites.

Conflict of Interest

The authors declared no conflicts of interest.

Acknowledgements

This work was supported by grants from Guangdong International Cooperation

Project (No. 2012B050300002), National Science Foundation of China (Nos. 81374043, 81173534 & 81503202), Guangdong Natural Science Foundation (No. 2015A030310217), Science and Technology Planning Project of Guangdong Province (No. 2014A020221042), Science and Technological Program for Dongguan's Higher Education, Science and Research, and Health Care Institutions (No. 2012105102009), Ph.D. Programs Foundation of Ministry of Education of China (No. 20134425110009), Science and Technology Innovation Project of Guangdong Provincial Department of Education (No. 2013KJCX0045), the Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme 2011 and the Special Funds from Central Finance of China in Support of the Development of Local Colleges and University - Educational finance Grant No. 276(2014).

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535 **Figure legends**

536

537 **Fig. 1.** Chemical structure of (-)-patchouli alcohol.

538 **Fig. 2.** Effect of PA on the GES-1 cell viability. Cell viability was assessed by MTT
539 assay and the results were presented as a percentage of control viability. Data are
540 expressed as mean $\pm$ S.E.M. ($n = 6$). ** $p < 0.01$ vs. the control by Dunnett's test.

541 **Fig. 3.** Protective effect of PA on the cell viability in the HPU-induced GES-1 cells as
542 measured by MTT assay. GES-1 cells in the treatment groups were treated with or
543 without PA for 4 h before incubation with 17.0 U/mg HPU for 24 h. Data are
544 expressed as mean $\pm$ S.E.M. ($n = 6$). # $p < 0.05$ vs. the control; * $p < 0.05$ vs. the
545 model.

546 **Fig. 4.** Effect of PA on the HPU-induced apoptosis in GES-1 cells detected by flow
547 cytometry. After incubation with PA (5, 10 and 20 μ M) for 4 h followed by 17.0 U/mg
548 HPU co-incubation for 24 h, cells were harvested and labeled with combination of
549 Annexin V-FITC and PI, and subjected to FACS analysis. (A) Representative flow
550 cytometry analysis of cells. Dot plot from flow cytometry analysis reveals the four
551 different populations of cells. Top left: necrotic cells (Annexin V-FITC⁻PI⁺); top right:
552 late apoptotic cells (Annexin V-FITC⁺PI⁺); bottom left: live cells (Annexin V-
553 FITC⁻PI⁻); and bottom right: early apoptotic cells (Annexin V-FITC⁺PI⁻). (B)
554 Corresponding cumulative data. Values are shown as means $\pm$ S.E.M. ($n = 4$). # $p <$
555 0.05 vs. the control; * $p < 0.05$ vs. the HPU group.

556 **Fig. 5.** Effect of PA on the HPU-induced loss of MMP in GES-1 cells by JC-1 method.
557 Cells were pre-treated with PA (5, 10 and 20 μ M) for 4 h prior to exposure to HPU for
558 24 h. (A) Representative fluorescence images of GES-1 cells stained with JC-1
559 (magnification $\times 200$). The upper row shows only red fluorescence (monomeric form

560 of JC-1), and the lower row shows green fluorescence (aggregated form of JC-1). (B)
561 Quantitative analysis of disruption of $\Delta\psi_m$ via the ratio of green fluorescence intensity
562 to red fluorescence intensity ratio in GES-1 cells. Data shown are means $\pm$ S.E.M. (n
563 = 6). # $p < 0.05$ vs. the control; * $p < 0.05$ vs. the HPU group.

564 **Fig. 6.** Effect of PA on the HPU-induced ROS in GES-1 cells measured by flow
565 cytometry. After treatment, cells were labeled with H₂DCF-DA for 30 min at 37 °C,
566 and subsequently subjected to FACS and fluorescence microplate analyses for
567 intracellular ROS accumulation. (A) Representative fluorescence images of GES-1
568 cells stained with H₂DCF-DA by FACS. (B) Representative images by fluorescence
569 microplate. (C) Quantitative analysis of ROS accumulation by fluorescence
570 microplate analysis. Data shown are mean $\pm$ S.E.M. ($n = 6$). # $p < 0.05$ vs. the control;
571 * $p < 0.05$ vs. the model.

572 **Fig. 7.** Effect of PA pre-treatment on SOD (A), MDA (B) and CAT (C) levels in
573 GES-1 cells subjected to HPU challenge. Data shown are mean $\pm$ S.E.M. ($n = 6$). # p
574 < 0.05 vs. the control; * $p < 0.05$ vs. the model.

575 **Fig. 8.** Effect of PA on the production of IL-2 (A), IL-4 (B), IL-13 (C), TNF- α (D)
576 and NO (E) in the HPU-stimulated GES-1 cells. Data presented are mean $\pm$ S.E.M. of
577 fold-changes relative to the control ($n = 6$). # $p < 0.05$ vs. the control; * $p < 0.05$ vs.
578 the model.

579 **Fig. 9.** (A) Molecular docking simulations obtained at lowest energy conformation,
580 highlighting potential contacts with PA. (colored by atom: carbon is green; nitrogen is
581 blue; oxygen is red; hydrogen is grey; sulfur is yellow). Hydrogen bonding
582 interactions are shown by dashes. These figures were created by PyMol. The right
583 picture depicts the surface representation of the active-site flap of HPU with PA,

584 shown at the entrance of the binding pocket. (B) Interaction mode displaying reaction
585 of PA with active site of HPU. For clarity, only interacting residues are labeled. (His:
586 Histidine; Arg: Arginine; Lys: Lysine; ALA: Alanine; GLY: Glycine; MET:
587 Methionine; ASP: Aspartate; Cys: Cysteine; Asn: Asparagine) (Autodock4.0).

588

589

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

(A)

Figure 5

(A)

(B)

Figure 6

Figure 7

Figure 8

Figure 9b

(B)

Figure 9a

(A)

Graphical Abstract