Article

Gossypol from *Gossypium* spp. Inhibits *Helicobacter pylori* Clinical Strains and Urease Enzyme Activity: Bioactivity and Safety Assessments

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Abstract: This study investigates the inhibitory activities of gossypol, a natural polyphenolic compound from *Gossypium* spp., against *Helicobacter pylori* (HP) clinical strains and a urease enzyme that plays a key role in the pathogenesis of HP. Gossypol was detected to exhibit a bacteriostatic action against all the HP strains tested with minimum inhibitory concentration (MIC) values ranging from 3.51 to 4.14 µg/mL. The activity of HP urease (HPU) was efficiently impeded by gossypol with a 50% inhibitory concentration (IC₅₀) value of 3.3 µM using an Electrospray Ionization–Mass Spectrometry (ESI-MS)-based method. The in vitro cytotoxicity assay showed no significant cytotoxic properties of gossypol against human gastric epithelial cells. Additionally, molecular docking studies were performed to assess the binding mode and the molecular interactions of gossypol with HPU with a binding affinity value of −8.1 kcal/mol compared with an HPU–acetohydroxamic acid (a standard urease inhibitor) docking complex (−6.1 kcal/mol). The overall results reveal that gossypol might help fight against HP infection by two mechanisms of action: inhibition of the growth of HP and inhibition of urease.

Keywords: antibacterial; enzyme inhibition; *Gossypium* spp.; gossypol; *Helicobacter pylori*; *Helicobacter pylori* urease; mass spectrometry; molecular docking; natural products; cytotoxicity; polyphenols

1. Introduction

Gossypol (Figure 1), an orally active polyphenolic compound, is widely distributed in cottonseed (*Gossypium* spp.) and has also been detected in *Hampea integerrima* Schltdl., a tree belonging to the family Malvaceae [1,2]. This compound is formed in the plant by the dimerization of two molecules of hemigossypol and is best classified as a dimeric-sesquiterpenoid [3]. Gossypol has previously been studied in various preclinical and clinical experiments and showed various therapeutic properties, including antioxidant, anticancer, antiviral, antiparasitic, and antimicrobial properties along with lower plasma cholesterol properties. This compound was also observed to act as a male contraceptive [4–8]. Gossypol remains a little-examined molecule and, therefore, further studies need to be performed [9]. Consequently, our study aims to draw attention to the novel biological properties of this compound.

*Helicobacter pylori* (HP), the predominant species of the human gastric microbiome, is the most frequent bacterial infection in the world and poses a significant threat to public health, as an estimated 50% of the world’s population is infected by this pathogen [10]. HP is a Gram-negative bacterium known to cause peptic ulcer diseases (gastric and duodenal ulcers) [11]. Several critical complications were also described to be associated with HP infection, including chronic gastritis, gastric cancer, and gastric-mucosa-associated lymphoid tissue (MALT) lymphoma [12]. Currently, the most effective eradication regimen for HP is mainly based on the use of a proton pump inhibitor (PPI) and a gastric mucosal...
protective agent coupled with one or two antibiotics (such as clarithromycin, metronidazole, levofloxacin, and amoxicillin), resulting in triple or quadruple therapy [13,14]. As with all antibiotics, extended or recurrent use has increased the risk of developing antibiotic resistance, leading to an increase in the failure rate of standard therapy for HP infection [15]. Therefore, to overcome this global problem, new sources such as natural products [16–18] that deliver successful anti-HP drugs with less resistance, reduced undesirable effects, and diverse mechanisms of action are urgently required [19].

![Chemical structure of gossypol](image)

**Figure 1.** Chemical structure of gossypol.

Studies on enzyme inhibition continue to play a vital role in drug discovery since these studies have led to the discovery of novel drugs useful in the therapy of numerous diseases [20,21]. Urease is a nickel-dependent metalloenzyme that is naturally synthesized by plants, bacteria, fungi, and algae. It catalyzes the hydrolysis of urea, producing ammonia and carbon dioxide [22,23]. Urease is an essential enzyme produced by HP, where the amount of ammonia released by the urease-catalyzed reaction neutralizes the gastric acid, leading to optimal conditions for the survival and colonization of HP. Therefore, inhibition of HP urease (HPU) represents a valuable method for designing novel anti-HP drugs [24,25]. By using in vitro systems, this study was designed to investigate the antibacterial effect of gossypol against HP and the inhibitory properties against the urease that plays a critical role in its pathogenesis. The cytotoxicity of gossypol was also tested to evaluate its safety. In addition to in vitro tests, molecular docking studies were performed to predict the binding mode and molecular interaction of gossypol with HPU.

2. Materials and Methods

Gossypol (from cottonseed with a purity of $\geq 95\%$ and a molecular weight of 518.6 g/mol) used in all experiments was obtained from Sigma-Aldrich, Berlin, Germany.

2.1. Antimicrobial Assay

2.1.1. Bacterial Strains and Culture Requirements

The HP standard strain 43504 (American Type Culture Collection (ATCC); Manassas, VA, USA) and ten HP clinical isolates (HP1–HP10; hospital-acquired from Motol University Hospital (MUH), Prague, Czech Republic) were used for the antimicrobial assay. All bacterial strains were cultured and grown in Mueller–Hinton agar (MHA) supplemented with 7% horse blood (Sigma-Aldrich, Prague, Czech Republic). Further, the strains were incubated at 37 °C for three days under microaerobic conditions with 5% O$_2$, 10% CO$_2$, and 85% N$_2$ [14,26]. All used clinical strains had previously been identified based on the microaerophilic growth condition, Gram’s stain, morphology, and the presence of virulence factors along with verified enzyme activities (urease, catalase, and oxidase). Moreover, the impacts of temperature, aging, aerobiosis, starvation, and antibiotics on the morphologic conversion rate to coccoid forms along with the culturability of bacterial strains were ascertained as previously recommended by the Clinical and Laboratory Standards Institute (CLSI) [26]. All used strains were susceptible to gossypol and standard antibiotics (clarithromycin, metronidazole, and levofloxacin; purchased from Sigma-Aldrich, Berlin, Germany).
2.1.2. Anti-Helicobacter pylori (HP) Activity

For antimicrobial susceptibility testing, the agar dilution method was used to determine the minimum inhibitory concentration (MIC) values as previously advised by the CLSI [26]. Briefly, the stock solutions of test compounds (gossypol, clarithromycin, metronidazole, and levofloxacin were dissolved in dimethyl sulfoxide (DMSO; 1%) and prepared in serial dilutions with starting concentrations ranging from 0.5 to 10 µg/mL) were added to MHA–7% horse blood and maintained in a microaerobic condition (5% O2, 10% CO2, and 85% N2). All HP strains were subjected to sub-culturing on MHA supplemented with horse blood (7%) under the same microaerobic condition for three days at 37 °C. The bacterial suspensions prepared in MH broth were modified to a final concentration of a McFarland Standard 0.5 (10^8 CFU/mL). Further, 2 µL of the adjusted inocula was transferred to the series of agar plates, including a control plate without test compounds. After 72 h of incubation under the microaerobic condition, the MIC values were assessed as the lowest concentration of test compounds inhibiting visible growth. MIC values were acquired from three individual measurements conducted in triplicate.

2.2. Enzyme Inhibition Assay

2.2.1. Instrumentation and Operational Parameter Settings

The activity of HPU (obtained from MUH, Prague, Czech Republic with a purity of ≥99%) was assessed using a system pump-injector (Agilent 1200, Berlin, Germany) combined with a Sciex-3200QTRAP–hybrid triple quadrupole/linear ion trap mass spectrometer (MS; Toronto, ON, Canada) coupled with Electrospray Ionization (ESI). The instrumental parameter settings were optimized according to the published method [24] (curtain gas (CUR), 25 psi; nebulizer gas (GS1), 50; auxiliary gas (GS2), 40; declustering potential (DP), 15 V; ion spray voltage, −4000 V; turbo temperature, 450 °C). The analysis was initiated by running ESI-MS without a High-Performance Liquid Chromatography (HPLC) column utilizing the flow injection analysis (FIA) mode. To detect and measure the substrate depletion (urea; m/z 61 → 44), MS was used to perform a multiple reaction monitoring (MRM) analysis and was set in positive ion mode using mobile phases (HCOOH (0.1%) and HCOONH4 (1 mM)). The flow rate was set at 0.5 mL/min with 10 µL of injection volume.

2.2.2. Anti-Helicobacter pylori Urease (HPU) Assay

The HPU-catalyzed reaction was determined using an Electrospray Ionization–Mass Spectrometry (ESI-MS) method as previously detailed [24], where the assay is based on the monitoring of the decrease in the substrate concentration (urea concentration) in the presence and absence of inhibitors by observing the changes in the concentration of urea. Briefly, gossypol and acetohydroxamic acid (Sigma-Aldrich, Berlin, Germany; purity ≥98%) at a concentration of 16.1 µM were incubated with a solution consisting of HPU (38.2 µg/mL) prepared in HCOONH4 buffer (1 mM; pH = 7.6) for 20 min to achieve binding equilibrium. Further, the solution mixture was blended with urea (275 µM) and directly injected into the FIA system and the changes in the concentration of urea were detected. The kinetics of urea depletion were analyzed by integrating areas (total counts) under peaks in the FIA system. The repeatability of measurements was confirmed by performing multiple measurements of the same sample. The relative standard deviation (RSD; %) of multiple measured slopes was calculated to determine the precision of time-course analysis. The half-maximal inhibitory concentration (IC_{50}) values for gossypol and acetohydroxamic acid were assessed following the above-mentioned method [24].

2.3. Cytotoxicity Assessment

2.3.1. Cell Lines Preparation

The cytotoxicity of gossypol and cisplatin (a standard cytotoxic drug obtained from Sigma-Aldrich, Prague, Czech Republic; European Pharmacopoeia reference standard) was evaluated using human gastric epithelial cells (GES-1; MUH, Prague, Czech Republic). The GES-1 cells were prepared as previously described [27]. Concisely, GES-1 cells were
cultivated in a culture medium (Roswell Park Memorial Institute RPMI-1640; Sigma Chemicals Co., Saint Louis, MO, USA) enhanced by a mixture consisting of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (25 mM), 10% of heat-inactivated fetal calf serum (FCS; pH = 7.2), L-glutamine (3 mM), streptomycin (200 mg/mL), and penicillin (192 U/mL). The prepared cells were cultivated in a humidified condition with 5% CO$_2$ at 37 °C, and then sub-cultivated two times for 7 days at the same experimental conditions.

2.3.2. Cytotoxicity Test

The cytotoxicity of gossypol was assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide; Sigma-Aldrich, Berlin, Germany) with GES-1 cells following the detailed method [27]. Briefly, dimethyl sulfoxide (DMSO; 1%) was used to prepare the stock solutions of gossypol, while NaCl (0.9%) was employed to prepare the stock solutions of cisplatin. The stock solutions of gossypol and cisplatin were then diluted with nutrient medium to final concentrations of up to 200 µg/mL and 10 µg/mL, respectively, on GES-1 cells. The absorbance of test samples ($\lambda$ 570 nm) was detected by a microplate reader (Infinite M200, Tecan, Salzburg, Austria). The IC$_{50}$ values of test compounds were determined by cell survival diagrams and require the inhibition of 50% of the GES-1 cells’ survival.

2.4. Molecular Docking Studies

For the preparation of investigated ligands and proteins, the SDF file of the three-dimensional (3D) structure of gossypol (CID: 3503) was recovered from the PubChem database, while the 3D crystal structure of HPU in complex with acetohydroxamic acid (PDB ID: 1E9Y) was retrieved from the RCSB Protein Data Bank (www.rcsb.org; accessed on 5 October 2021).

The molecular docking analyses were completed using a PyRx virtual screening tool combined with Autodock VINA software (version 0.8, The Scripps Research Institute, La Jolla, CA, USA). The docking analyses and settings were performed according to the previously described protocol with an exhaustiveness of 8 [21,28], where all docking settings, including the preparation of PDBQT files for the receptor and ligands, energy minimization, determination of binding sites, calculations (performed five times and the best-scored result was chosen), the protonation state, and the overall charges, were established and optimized. The cubic grid box with a size of 60 Å (x, y, and z) and a spacing of 0.375 Å along with the grid maps were optimized. The center of the grid was adjusted to the average coordinates of the two Ni$^{2+}$ ions.

The binding affinity values (kcal/mol) of ligand–receptor complexes were utilized to assess the docking results. The binding affinity values are based on hydrogen bonds, hydrophobic interactions, and electrostatic interactions. The validation processes of docking results were confirmed by removing the co-crystallized ligands and re-docking them back into their receptors. The best-scored docking poses were selected, and the docking results were further graphically processed by Discovery Studio Visualizer version v19.1.0.18287 (BIOVIA, San Diego, CA, USA).

3. Results and Discussion

3.1. Evaluation of Anti-Helicobacter pylori (HP) Properties

Agar dilution antimicrobial susceptibility testing was used, where the anti-HP activity of gossypol was evaluated against ten HP clinical isolates along with a laboratory strain. The activity of gossypol was characterized based on the obtained MIC values, where the results demonstrate that this compound was able to inhibit the growth of all HP strains tested (a bacteriostatic effect) with MIC values ranging from 3.51 to 4.14 µg/mL compared with that of standard antibiotics (Table 1). The achieved results also confirm that the collected MICs for test standard antibiotics agreed with the MIC breakpoints reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [29]. On the other hand, we performed additional studies to determine the bactericidal effect of gossypol
and the possible antimicrobial combinatory effects with test standard antibiotics. However, no positive outcomes were observed to disclose any bactericidal, synergetic, additive, or even antagonistic effects, which in turn indicates that gossypol has a bacteriostatic action against HP.

### Table 1. Antibacterial effects of gossypol and standard antibiotics on HP strains.

| HP Strains   | Gossypol (µg/mL) | MIC (µg/mL) | Clarithromycin | Metronidazole | Levofloxacin |
|--------------|------------------|-------------|----------------|----------------|--------------|
| HP43504      | 4.14 ± 0.13      | 0.26 ± 0.02 | 7.91 ± 0.13    | 1.12 ± 0.13    |
| HP1          | 3.91 ± 0.12      | 0.27 ± 0.03 | 8.11 ± 0.13    | 1.11 ± 0.12    |
| HP2          | 3.92 ± 0.14      | 0.24 ± 0.03 | 7.94 ± 0.15    | 0.92 ± 0.03    |
| HP3          | 4.11 ± 0.15      | 0.23 ± 0.03 | 8.10 ± 0.14    | 1.12 ± 0.13    |
| HP4          | 3.82 ± 0.12      | 0.24 ± 0.03 | 8.13 ± 0.10    | 1.13 ± 0.14    |
| HP5          | 3.81 ± 0.14      | 0.28 ± 0.03 | 8.12 ± 0.12    | 0.91 ± 0.04    |
| HP6          | 4.12 ± 0.12      | 0.28 ± 0.03 | 8.20 ± 0.12    | 0.94 ± 0.04    |
| HP7          | 3.91 ± 0.12      | 0.25 ± 0.04 | 7.96 ± 0.14    | 1.10 ± 0.11    |
| HP8          | 3.73 ± 0.13      | 0.26 ± 0.02 | 8.14 ± 0.13    | 1.10 ± 0.11    |
| HP9          | 3.72 ± 0.12      | 0.24 ± 0.03 | 8.00 ± 0.15    | 1.13 ± 0.14    |
| HP10         | 3.51 ± 0.15      | 0.25 ± 0.04 | 8.13 ± 0.13    | 1.11 ± 0.12    |

The presented values are means ± standard deviation (SD) of three independent experiments performed in triplicate. * HP, standard strain ATCC 43504; b HP, clinical isolates; MIC, minimum inhibitory concentration. Values in parentheses are the selectivity index (SI, calculated as the ratio IC₅₀/MIC, where an IC₅₀ value represents the cytotoxic effect of gossypol on human gastric epithelial cells (50% inhibition of cell survival), see Table 2). PRISM software (GraphPad Software, Inc., La Jolla, CA, USA; version 8.0) was used to process the collected data.

### Table 2. Cytotoxic effect of gossypol and cisplatin on human gastric epithelial (GES-1) cells.

| Compound     | IC₅₀ (µg/mL) |
|--------------|--------------|
| Gossypol     | 39.32 ± 0.43 |
| Cisplatin    | 6.21 ± 0.22  |

The presented values are means ± standard deviation (SD) of three independent experiments performed in triplicate. IC₅₀, the concentration of compound that exhibits 50% inhibition of cell survival. For statistical analysis, the PRISM software version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) was employed. The differences between treatments with test compounds and the positive control were analyzed by ANOVA followed by post-hoc comparison tests (Dunnett and Student–Newman–Kuels). Statistical significance was set at p < 0.05.

### 3.2. Assessment of Anti-Helicobacter pylori Urease (HPU) Activity

An ESI-MS-based method was employed to evaluate the anti-HPU properties of gossypol in comparison with the standard inhibitor acetohydroxamic acid. In enzymology, it is well-recognized that enzyme activity can be monitored in two ways: through the depletion of substrate or the formation of product. Therefore, in this study, we detected the HPU-catalyzed reaction through the decrease in the substrate concentration (urea) in the presence and absence of inhibitors. Figure 2 shows that the reaction rate constant (RRC) in the presence of the inhibitor (k) is lower than the RRC of the HPU-catalyzed reaction (in the absence of the inhibitor; k₀). The K values in the presence of gossypol and acetohydroxamic acid were determined to be 0.0184/µm and 0.0244/µm, respectively, while the k₀ value was detected to be 0.1081/µm. The IC₅₀ values for gossypol and acetohydroxamic acid were calculated to be 3.3 and 4.7 µM, respectively.

Urease inhibitors are known for their therapeutic values in treating urease-producing bacteria, including HP, by hindering the amount of ammonia released by the inhibition of the urease-catalyzed reaction [30]. Moreover, inhibition of HPU has recently been recognized as an alternative approach for the therapy of HP infection with the ability to overcome the problem of drug resistance [31]. Over the past decade, various urease inhibitors were isolated from natural sources (especially plants) or synthesized with different degrees of inhibition characteristics evaluated in numerous nonclinical experiments and in silico approaches. Unfortunately, most of these inhibitors were prevented from being used...
in vivo because of their cytotoxicity [20,23,32,33]. Therefore, in this work, we present an initial report on the inhibition of HPU by gossypol with a safe degree of cytotoxicity (as shown in the results of the cytotoxicity evaluation).

**Figure 2.** The anti-\textit{Helicobacter pylori} urease (HPU) activities of gossypol and acetohydroxamic acid (standard inhibitor) were assayed by an Electrospray Ionization–Mass Spectrometry (ESI-MS)-based method. The reaction rate constant (RRC) is shown on each slope. As displayed, $k_0$ represents the RRC of the HPU-catalyzed reaction in the absence of inhibitors ($k_0 = 0.1081$ min$^{-1}$) (A) and $k$ represents the RRC of the HPU-catalyzed reaction inhibited by gossypol ($k = 0.0184$ min$^{-1}$) (B) and acetohydroxamic acid ($k = 0.0244$ min$^{-1}$) (C). Changes in urea concentrations are expressed as logarithms of concentration. The precision of the time-course analysis was confirmed by the relative standard deviation (RSD, %) of multiple measured slopes (less than 10%). For figure clarity, multiple measurements are not presented. Gossypol and acetohydroxamic acid effectively inhibited HPU with 50% inhibitory concentration (IC$_{50}$) values of 3.3 and 4.7 $\mu$M, respectively.

### 3.3. Evaluation of Cytotoxicity Properties

The cytotoxic effect of gossypol on GES-1 cells in comparison with the standard drug cisplatin was evaluated using an MTT assay. As shown in Table 2, the acquired results reveal that the treatment of GES-1 cells with gossypol demonstrated a notably low cytotoxicity (IC$_{50} = 39.32$ $\mu$g/mL) compared with cells treated with cisplatin (IC$_{50} = 6.21$ $\mu$g/mL). Since no considerable cytotoxic effect on GES-1 cells was observed, this indicates that gossypol has a moderate safety profile. On the other hand, it is worth mentioning that gossypol is a molecule with reported toxicity against various targets, including the reproductive system, heart, liver, and membranes, with diverse reported concentrations or doses [34]. Therefore, before any possible potential practical application, the non-toxic concentrations or doses that induce therapeutic values should be taken into consideration as key factors and should be evaluated via improved drug delivery techniques to ensure maximum efficacy [35,36].

### 3.4. Evaluation of Molecular Docking Studies

**Interaction of Gossypol with \textit{Helicobacter pylori} Urease**

An HPU–gossypol docking complex (binding mode and molecular interaction) had noticeably been formed with a binding affinity value of $-8.1$ kcal/mol (Figure 3), while the binding affinity value for the HPU–acetohydroxamic acid docking complex was found to be $-6.1$ kcal/mol. The interacting amino acid residues of the HPU active site and the functional groups of gossypol had notably established molecular interactions via crucial contacts. These contacts were observed to be hydrogen bonds (conventional hydrogen
bonds), hydrophobic interactions (Alkyl hydrophobic interactions), electrostatic interactions (Pi–Anion interactions), and Van der Waals interactions. All detected amino acid residues were found to be essential for the activity and stabilization of HPU [37]. It is known that ureases are among the few enzymes that entail nickel ions for activity [38]. The docking results show no interaction of gossypol with nickel ions of HPU, indicating that further in vitro experiments need to be performed to disclose the exact type of inhibition. Since the application of molecular docking studies in drug discovery research has recently received a great deal of attention among scientists, advances in developing new molecular docking methodologies have been achieved. For instance, inverse molecular docking protocols were developed that might help identify potential protein targets for various molecules, including gossypol [39,40]. Consequently, more studies on developing new methodologies would accelerate the progress of drug discovery research.

![Image of molecular docking analysis](image_url)

*Figure 3.* Molecular docking analysis (in a two-dimensional model) indicates the binding mode and the molecular interaction of gossypol with the *Helicobacter pylori* urease (HPU) active site. As shown, numerous crucial interactions between the functional groups of gossypol and amino acid residues of the active site of HPU are formed.

### 4. Conclusions

Although numerous studies have disclosed the pharmacological actions of gossypol against several biological and molecular targets, there are still many activities that have yet to be investigated. The results of this study reveal the pronounced therapeutic utilities of gossypol via its ability to effectively suppress the growth of all HP strains tested along with a remarkable capacity to inhibit urease, a potent virulence factor for HP. Moreover, no remarkable cytotoxicity of gossypol was detected against human gastric epithelial cells evaluated by an MTT assay. The therapeutic efficacy was determined by in vitro microbiological, biochemical, and in silico molecular docking studies. The binding modes and molecular interactions were predicted by molecular docking analyses. Despite the therapeutic value induced by this compound against the investigated targets, additional in-depth in vivo studies are needed to authenticate the findings obtained by in vitro inves-
tigations. Moreover, combined pharmacokinetic and pharmacodynamic experiments need to be performed.

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