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Research article

Anti-*Helicobacter pylori*, anti-biofilm activity, and molecular docking study of citropten, bergapten, and its positional isomer isolated from *Citrus sinensis* L. leaves

Hala Sh Mohammed [a], Mona H. Ibrahim [b], Marwa M. Abdel-Aziz [c], Mosad A. Ghareeb [d,*]

[a] *Department of Pharmacognosy and Medicinal Plants, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, 11311, Egypt*
[b] *Pharmaceutical Medicinal Chemistry and Drug Design Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, 11311, Egypt*
[c] *The Regional Centre for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt*
[d] *Medicinal Chemistry Department, Theodor Bilharz Research Institute, Kornaish El-Nile, Warrak El-Hadar, Imbaba, P.O. Box 30, Giza, 12411, Egypt*

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ABSTRACT

Introduction: Citrus sinensis L. is a candidate plant with promising antimicrobial potential. In the current study, the phytochemical investigation of *C. sinensis* leaf extract led to the isolation of three coumarins, namely bergapten, xanthotoxin, and citropten.
Methods: The chemical structures of the isolated coumarins were elucidated using NMR and ESI-MS techniques. The total aqueous ethanol leaf extract and the isolated coumarins were evaluated for their antimicrobial effects against *Helicobacter pylori* using the MTT-micro-well dilution method and its anti-biofilm activity using MBEC assay, as compared to clarithromycin.
Results: The results showed that citropten scored the lowest MIC value at 3.9 μg/mL and completely inhibited the planktonic growth of *H. pylori*. In addition, it completely suppressed *H. pylori* biofilm at 31.25 μg/mL. These findings have been supported by molecular docking studies on the active sites of the *H. pylori* inosine 5′-monophosphate dehydrogenase (*Hp*IMPDH) model and the urease enzyme, showing a strong binding affinity of citropten to *Hp*IMPDH with seven hydrogen bonds and a binding energy of −6.9 kcal/mol. Xanthotoxin and bergapten showed good docking scores, both at −6.5 kcal/mol for *Hp*IMPDH, with each having four hydrogen bondings. Furthermore, xanthotoxin showed many hydrophobic interactions, while bergapten formed one Pi-anion interaction. Concerning docking in the urease enzyme, the compounds showed mild to moderate binding affinities as compared to the ligand. Thus, based on docking results and good binding scores observed with the *Hp*IMPDH active site, an *in-vitro Hp*IMPDH inhibition assay was done for the compounds. Citropten showed the most promising inhibitory activity with an IC_{50} value of 2.4 μM. *Conclusion*: The present study demonstrates that *C. sinensis* L. leaves are a good source for supplying coumarins that can act as naturally effective anti-*H. pylori* agents.

* Corresponding author.
E-mail address: m.ghareeb@tbri.gov.eg (M.A. Ghareeb).

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1. Introduction

Helicobacter pylori is a gram-negative bacterium that infects the gastric mucosa and is one of the most common causes of chronic gastritis, which can progress to peptic and gastric cancer [1,2]. In most countries, the prevalence of *H. pylori* infection is still high, ranging from 40 % in developed countries up to 80 % in underdeveloped countries. According to estimates, 10–20 % of *H. pylori*-infected patients develop peptic ulcer disease in different degrees, and 1–2 % of patients are at risk of developing stomach cancer [3,4].

Biofilm-forming pathogens produce persistent and recurring infections that are resistant to traditional therapies. Biofilm-associated infections account for up to 80 % of human microbial infections [3,4]. *H. pylori* could develop biofilm in the gastrointestinal tract and is the second most common cause of human infection [5]. As a result, herbal medications are commonly employed in circumstances when drugs need to be used for long periods [6]. Considerable research on herbal medicine has been published in recent years demonstrating its potential effectiveness in preventing or managing peptic ulcers [7] due to the presence of bioactive phytochemicals such as flavonoids, coumarins, alkaloids, terpenoids, tannins, phenolic acids, and antioxidant nutrients that are useful in treating various infectious diseases with no or low toxicity [8–10].

The genus *Citrus* belongs to the Rutaceae family, this genus provides the most important fruit tree crops in the world, with an annual production of approximately 123 million tons in 2010 [11,12]. *Citrus sinensis* (L. Osbeck) or sweet orange, represents the largest group of *Citrus* cultivars planted worldwide, accounting for about 70 % of the total annual production of *Citrus* species [13]. It is traditionally used as a rich source of vitamin C, being a natural antioxidant that strengthens the body's immune system [14], for the management of constipation, colic, bronchitis, cough, cold, obesity, menstrual problems, heart diseases, anxiety, depression, and stress [15]. *C. sinensis* L. contains important phytochemicals such as limonoids, coumarins, flavonoids such as hesperidin, polyphenols, pectin, and a significant quantity of folacin, calcium, potassium, thiamine, niacin, and magnesium [15]. Recently, coumarins have shown gastroprotective effects [16–20], making them potential molecules for anti-*H. pylori* treatment. Molecular docking is a powerful computational tool for characterizing the interactions between small molecules and a target protein or enzyme. It also shows the best conformation or orientation of the docked compounds in the protein's active site. This makes many researchers directed towards and relying on in-silico molecular docking to understand the possible mechanism of action [21,22].

In this study, we performed a phytochemical investigation of *Citrus sinensis* L. leaves and evaluated the anti-*H. pylori* and anti-biofilm activity of the total aqueous ethanol extract as well as the isolated coumarin compounds. Furthermore, molecular docking approaches for the isolated compounds were conducted on the *Hp*IMPDH model and the urease enzyme to explore the probable binding conformation. Moreover, an *in-vitro* assessment of the compound's inhibitory potential on *Hp*IMPDH was performed.

2. Materials and methods

2.1. Plant material

Fresh *C. sinensis* L. leaves were collected in December 2019 from Sharqiyah Governorate, Egypt (31.1166o^{o} N and 30.63333° E). The plant was kindly identified by Dr. Therese Labib, botanical specialist, Department of Flora and Taxonomy, El-Orman Garden, Giza, Egypt. A voucher specimen (Voucher No. 19) of the plant was deposited in the Pharmacognosy Department's Herbarium at Al-Azhar University's Faculty of Pharmacy in Cairo, Egypt. The plant name has been verified using http://www.theplantlist.org/on April 18, 2012.

2.2. Extraction, fractionation, and purification

Air-dried leaf powder (700g) was extracted with 70 % aqueous ethanol (3x1L) at 25 ± 2 °C [23], and a rotatory evaporator was used to concentrate the extract (Buchi Co., Switzerland) at 40 °C till dryness to give 230g of total extract. Subsequently, the obtained extract was subjected to extraction using n-hexane (2 × 500ml), chloroform (2 × 500ml), and finally diethyl ether (2 × 500 ml). The diethyl ether fraction (200 mg) was coumarin-rich according to detection by UV-light & spray reagents on TLC, which was subjected to successive Sephadex LH-20 CC using absolute ethanol to obtain three pure coumarins. The isolated compounds were identified by NMR spectrometer (400 & 100 MHz for ^{1}H & ^{13}C). All samples were prepared in suitable deuterated DMSO-d_6 using TMS as an internal reference (Faculty of Pharmacy, Cairo University, Cairo). Sample analysis was performed using liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with an ExionLC AC system for separation and a SCIEXTriple Quad 5500+ MS/MS system equipped with electrospray ionization (ESI) for detection. For separation, an Ascentis® C18 column (4.6150 mm, 3 μm) was used. The mobile phases consisted of two eluents A: 0.1 % formic acid; B: acetonitrile (LC grade). The mobile phase gradient is programmed as follows: 10 % B at 0–2 min, 10–90 % B from 2 to 30 min, 90 % B from 30 to 36 min, 10 % at 36.1, 10 % from 36.1 to 40 min. The flow rate was 0.7 ml/min, and the injection volume was 10 μl. For MS/MS analysis, a negative ionization mode was applied with a scan (EMS-IDA-EPI) from 100 to 1000 Da for MS1 with the following parameters: curtain gas: 25 psi; Ion Spray voltage: 4500; source temperature: 500 °C; ion source gas 1 & 2 were 45 psi and from 50 to 1000 Da for MS^{2} with a declustering potential: 80; collision energy: 35; collision energy spread. Component identification was performed using MS-DIAL software version 4.70 and the Fiehn HILIC library.

2.3. Biological activity

2.3.1. Microorganism

Helicobacter pylori is an American Type Culture Collection strain (ATCC 43526).

2.3.2. Anti-Helicobacter pylori assay

The antibacterial activity of *C. sinensis* L. leaf extract and its isolated compounds against *H. pylori* was determined, using the MTT-micro-well dilution method. The inoculum of *H. pylori* (10^6 CFU/ml) was suspended in 96-well plates. *C. sinensis* extract and its isolated compounds, as well as the reference drug (clarithromycin), were prepared in dimethyl sulfoxide (DMSO) with subsequent two-fold dilutions (125–0.24 μg) in a 96-well plate. Each well of a microplate contained 40 μl of growth medium (Brain Heart Infusion (BHI) plus 10 % fetal bovine serum (FBS), and 10 μl of *H. pylori* (10^6 CFU/ml) were suspended in 96-well plates. Clarithromycin and DMSO are used as positive and negative controls, respectively. Plates were incubated at 37 °C for three days, in 5 % O_2, 10 % CO_2, and 85 % N_2 atmospheres. After that, 40 μl of 3- (4, 5-dimethyl-thiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml was added to each well and incubated for 30 min. The percentage of inhibition was calculated using the given formula:

%inhibition = 1-[Abs Control-Abs samples × 100/Abs Control]. The MIC was taken to the lowest concentration, at which no color change of MTT (100 % inhibitory percentages) was determined using an ELISA microplate reader at 620 nm [24].

2.3.3. Anti-biofilm activity

The biofilm eradication assay was performed by adopting the previously described method by Luca et al. [25]. Briefly, 150 μl of *Helicobacter pylori* culture (1 × 107 CFU/ml) was added to 96 peg-lids and incubated for 24 h to allow biofilm formation on the pegs. After incubation, the pegs were rinsed twice to remove planktonic cells. After that, peg-lids were transferred to another 96-well plate containing different concentrations of citropten as MIC-based concentrations (MIC, 2 MIC, 4 MIC, 8 MIC, 16 MIC, 32 MIC, and 64 MIC μg/ml) and incubated for 2 h at 37 °C. After sample treatment, the pegs were washed and transferred to a new 96-well plate, each containing 200 l of 0.5 mg/ml MTT in PBS and incubated for 4 h at 37 °C. Afterward, 50 μl of 25 % sodium dodecyl sulphate (SDS) was added to each well. MTT reduction was determined using a microplate reader (TECAN, Inc.) at 620 nm. Biofilm-treated samples were assessed for their biofilm eradication % by the following formula: % of inhibition = [1-(ODt/ODc)] x 100 % where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The Minimum biofilm eradication concentration (MBEC) was detected as the minimum concentration needed to eradicate biofilms.

2.4. HpIMPDH enzyme inhibition assay

Bergapten, xanthotoxin, citropten, and the standard drug (clarithromycin) were tested in triplicate at 20 μM. The assay was performed in a final volume of 200 μl on a Black 96-Well Plate (Tarsons Products Pvt. Ltd., Kolkata, India) with 50 mM Tris-HCl (pH 8.6), 100 mM KCl, and 1 mM dithiothreitol (DTT). 100 nM *Hp*IMPDH was tested with and without test substances. 250 μM IMP and 300 M NAD+ (substrate buffer) were added to the assay mixture and incubated for 10 min at 37 °C. The experiment took 45 min at 37 °C. Fluorescence (340 nm excitation, 440 nm emission) was read at 1min intervals for NADH measurement (Waltham, MA). The positive

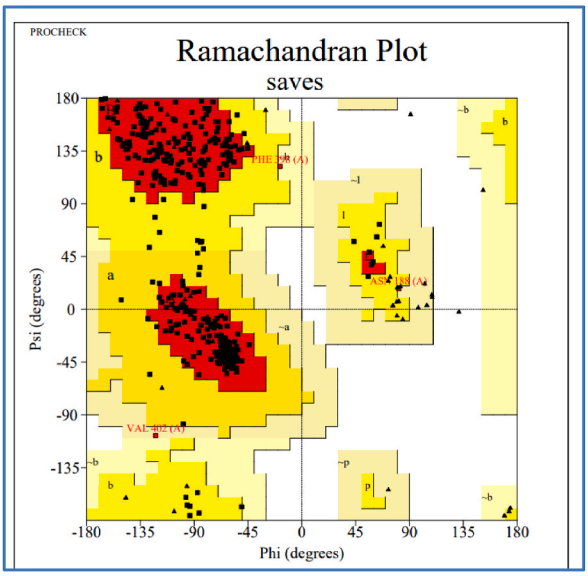

Fig. 1. Ramachandran plot of the homology model of *Hp*IMPDH enzyme.

control was clarithromycin, and the vehicle control was DMSO. The IC_{50} value is the sample concentration that inhibits *Hp*IMPDH by 50 % [26].

2.5. Computational studies

2.5.1. Homology modeling

The sequencing of *Hp*IMPDH (481 amino acids) was obtained from the UniProtKB data entries E1S8Z1 and BLAST, resulting in 54.6 % identity. The *Hp*IMPDH tetramer homology model was generated using the template *Streptococcus pyogenes* IMPDH (PDB ID 1ZFJ, Resolution 1.9) [27] discovered by SWISSMODEL. The compounds citropten, bergapten, and xanthotoxin were then docked into their biological targets to explain the potential binding mode of these compounds, which have diverse activity patterns, and to interpret the experimental data.

2.5.2. Molecular model evaluation

The final model was validated using the PROCHECK tool Fig. 1S and Ramachandran's plots (Fig. 1) [28] and ProSA (Fig. 2S) [29]. Fig. 1 shows that 91.80 % of the amino acids are found in the most favored sites, 8.20 % in the permitted regions, and 0 percent in the disallowed regions.

2.5.2.1. Binding site prediction. To predict the active site of the 3D model of *Hp*IMPDH, we used the *Hp*IMPDH inhibitor compound C91 [26]. C91 has been shown to bind to the protein with the highest docking score of – 6.9 kcal mol^{-1} [30].

2.5.2.2. Molecular docking. Crystal structures of urease (PDB ID: 6zja) were obtained from the Protein Data Bank. Docking attempts were conducted using Auto Dock Vina 4.2, which requires that both the receptor and the ligands be in PDBT format. Ligand/protein files, grid, and docking parameters were prepared according to previous reports [31]. A 3D grid box of 60 × 60 × 60 Å size (x, y, z) with the spacing of 0.375 Å centered at 96.86, 62.55, and 19.13 Å for docking into the *HP*IMPD model and at 195.31, 166.19 and 248.78 Å for docking into the Urease enzyme. Before docking, M.G.L tools were necessary to synthesize enzymes, co-crystalized ligand, and three lead compounds into the correct format [31]. The calculated RMSD between the docked and co-crystalized compounds was 0.60 (urea) after re-docking the co-crystalized ligand into the enzyme (Fig. 3S), indicating that the docking procedure was valid. The Discovery Studio 4.5 visualizer was used to visualize the docking results. For each docked compound, the docking process generated ten poses; the one with the highest affinity and the best docking score was selected. Rescoring was done using the MOE software and the AGL-SCORE website to further validate the chosen poses (see Supplementary Table 1S).

3. Results

3.1. Phytochemical investigation

The isolation of three coumarin components was achieved through column chromatographic analysis of a *C. sinensis* aqueous ethanol leaf extract. Based on their spectral data (Figs. 6S–13S), the isolated compounds were identified as 5-Methoxy-2H-furo [3, 2-g] chromen-2-one, Bergapten (5-methoxy psoralen) [32], 8-OMe-psoralen (xanthotoxin) [33,34], and 5, 7-di-methoxy -2H-chromen-2-one which is known as citropten [35,36]. Additionally, Table 1 contains the spectroscopic data of the isolated compounds (^{1}H and ^{13}C NMR). While Fig. 2 depicts the structures of the isolated compounds, and Fig. 3 represents the fragmentation pattern.

Table 1
^{1}H- &^{13}C NMR spectroscopic data of the isolated compounds.

	Compound 1 (Bergapten)		Compound 2 (xanthotoxin)		Compound 3 (citropten)
No.	δ_H	δ_C	δ_H	δ_C	δ_H
2		160.56		160.1	
3	6.30 (1H, d, J = 9.8Hz)	112.66	6.34 (1H, d, J = 9.8Hz)	114.7	6.33 (1H, d, J = 9.8Hz)
4	8.16 (1H, d, J = 9.8Hz)	139.86	8.19 (1H, d, J = 9.8Hz)	144.7	8.17 (1H, d, J = 9.8Hz)
5		149.87	7.69 (1H, s)	112.9	7.40 (1H, d, J = 2.3Hz)
6		112.74		127.6	
7		158.22		149.8	8.10 (1H, d, J = 2.3Hz)
8	7.30 (1H, s)	93.49		132.3	
9		152.56		140.2	
10		106.10		116.8	
2′	8.02 (1H, d, J = 2.4Hz)	146.7	8.09 (1H, d, J = 2.3Hz)	146.7	
3′	7.38 (1H, d, J = 2.2Hz)	106.00	7.39 (1H, d, J = 2.3Hz)	106.1	
5-OCH_3	4.25 (3H, s)	60.65			4.18 (3H, s)
7-OCH_3					4.04 (3H, s)
8-OCH_3			4.18 (3H, s)	60.2	

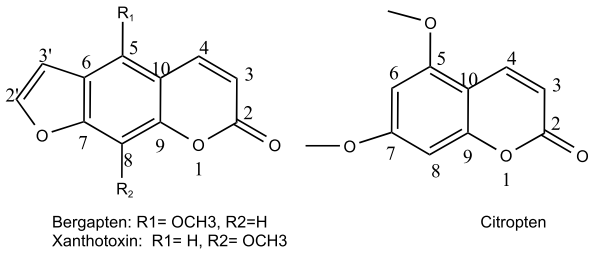

Fig. 2. Isolated coumarins from *C. sinensis* L. leaves.

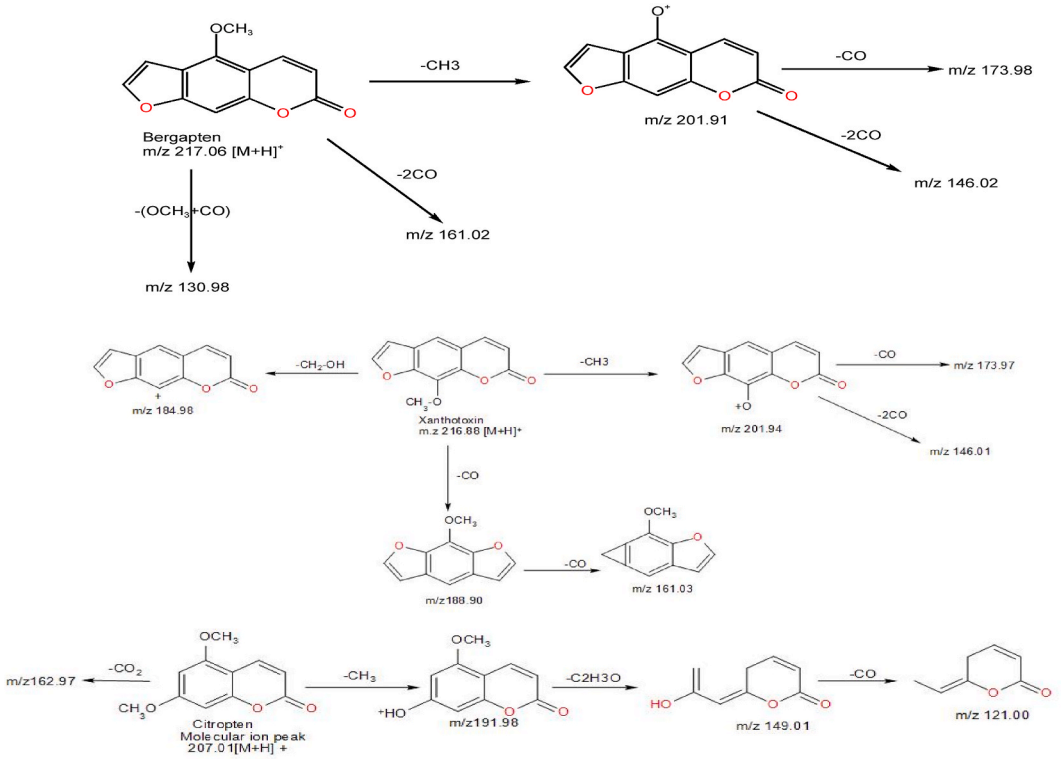

Fig. 3. MS/MS fragmentation patterns of bergapten, xanthotoxin, and citropten.

3.2. Anti-Helicobacter pylori & anti-biofilm activity

Both total aqueous ethanol leaf extract and isolated compounds were tested for their anti-*H. pylori* effects. Fig. 4 showed that xanthotoxin and citropten were more active against *H. pylori* compared with bergapten and the total extract. Xanthotoxin and citropten, both at a concentration of 7.81 μg/mL inhibited the growth of *H. pylori*, by 100 %, as compared to 32.14 and 59.85 % inhibition by the extract and bergapten, respectively, at the same concentration (**Table 2S**). However, the reference compound, clarithromycin, was more active than both the extract and the tested compounds. Regarding MIC values for the extract and the compounds, citropten showed the best results against *H. pylori* with a MIC value of 3.9 μg/mL. The results also confirmed the moderate antimicrobial activity of bergapten (MIC = 31.25 μg/mL), while the extract showed the weakest anti-*H-pylori* activity (MIC = 62.5 μg/mL). Citropten was the most effective in inhibiting the planktonic growth of *H. pylori,* so it was selected for evaluation against *H. pylori* biofilm. Fig. 5 showed that citropten exhibited potential anti-biofilm activity, eliminating microbial biofilm at a concentration of 8 MIC, i.e., 31.2 μg/mL (**Table 3S**).

3.3. Molecular Docking Study

Molecular docking is a computational technique that shows the binding affinities and possible interactions of docked compounds with the active sites of target proteins [37]. Coumarin-type compounds were docked at the binding sites of the *Hp*IMPDH 3D model and the urease enzyme to predict their mechanism of action as anti-*H. pylori*. Since an X-ray structure of *Hp*IMPDH was not available, a 3D

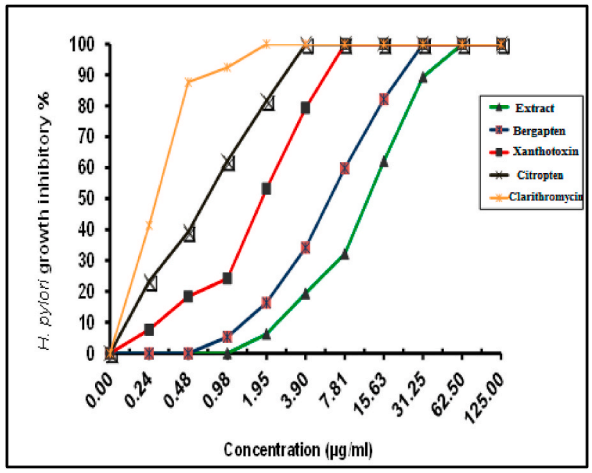

Fig. 4. Percentage of *H. pylori* growth inhibition at different concentrations of the extract, the isolated compounds (bergapten, xanthotoxin, and citropten) as compared to clarithromycin.

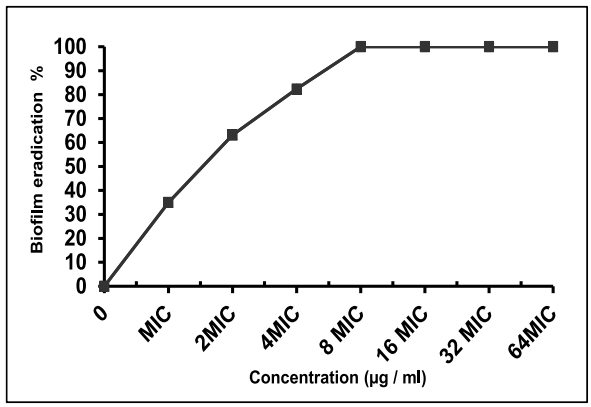

Fig. 5. Percentage of *H. pylori* biofilm eradication at different MIC-based concentrations of citropten.

model of *Hp*IMPDH was constructed to ensure a good description of the ligand-receptor interaction of *Hp*IMPDH. The validity of the 3D model is validated by the presence of 91.8 percent of amino acids, most of which are in the favorable regions, 8.2 percent in the permitted areas, and zero percent in the disallowed regions Fig. 1. Sequence alignment and RMSD are presented in the supplementary files (Fig. 4S and ***5S***). The *Hp*IMPDH model and Hpurease enzyme**s** were docked to reported inhibitors that interacted with the binding site**s** of the *Hp*IMPDH and *Hp*urease enzyme (See Supplementary Fig. 14S, Fig. 15S Tables 4S and 5S). Regarding the binding mode, Fig. 6 revealed significant interactions between the ligands and the related *Hp*IMPDH residues. As shown in Fig. 6, the non-competitive inhibitor C91 [26] interacted with Asp333, Ala46, Cys300, Met48, and Ser245 hydrophobically (energy score −6.9 kcal mol^{-1}). Clarithromycin established hydrogen bonds with Arg337, Ser298, Ser357, and Arg415. The isolated coumarin compounds are anchored to the binding site next to IMP and form hydrogen bonds with Asn272 and Asp333. Citropten has been found to form 7 hydrogen bonds, with the highest score being - 6.9 kcal mol^{-1}. Xanthotoxin and bergapten have a good docking score of 6.5 kcal mol^{-1}. Xanthotoxin formed four hydrogen bonds and was stabilized by hydrophobic interactions with residues Asp333, Ala46, and Met48. Bergapten anchored in the binding site of *Hp*IMPDH formed hydrogen bonds with Ala47, Asp333, Ala246, and Asn272, as well as a single Pi-anion interaction. [1-(3,5-Dimethylphenyl)-1H-imidazole-2-yl] sulfanyl-N-hydroxyacetamide (SHA) is a urease inhibitor [38]. Ni 601 binds to its hydroxamic acid group, and the rings engage with the flab area, particularly with the conserved residues Cys321 and His322 **(**Fig. 7**)**. The tested compounds form metal acceptor interactions with Ni 601 and 602, hydrophobic contacts with Cys321 in the case of Citropten and Bergapten, and bind with His322 in the flab region in the case of Xanthotoxin Fig. 7. In comparison to the ligand (SHA) and Clarithromycin, the compounds bergapten, xanthotoxin, and citropten have a low affinity for the urease enzyme Table 2 and Fig. 7. Citropten, xanthotoxin, bergapten, and the reference inhibitor C91 [26] anchored to the active site of *Hp*IMPDH with docking scores of −6.9, −6.5, −6.5, and −6.9 kcal mol^{-1}, respectively, (Table 2 and Fig. 6). The compounds bergapten, xanthotoxin and citropten have limited affinity for urease enzyme as compared to ligand (SHA) and clarithromycin (Table 2 and Fig. 7).

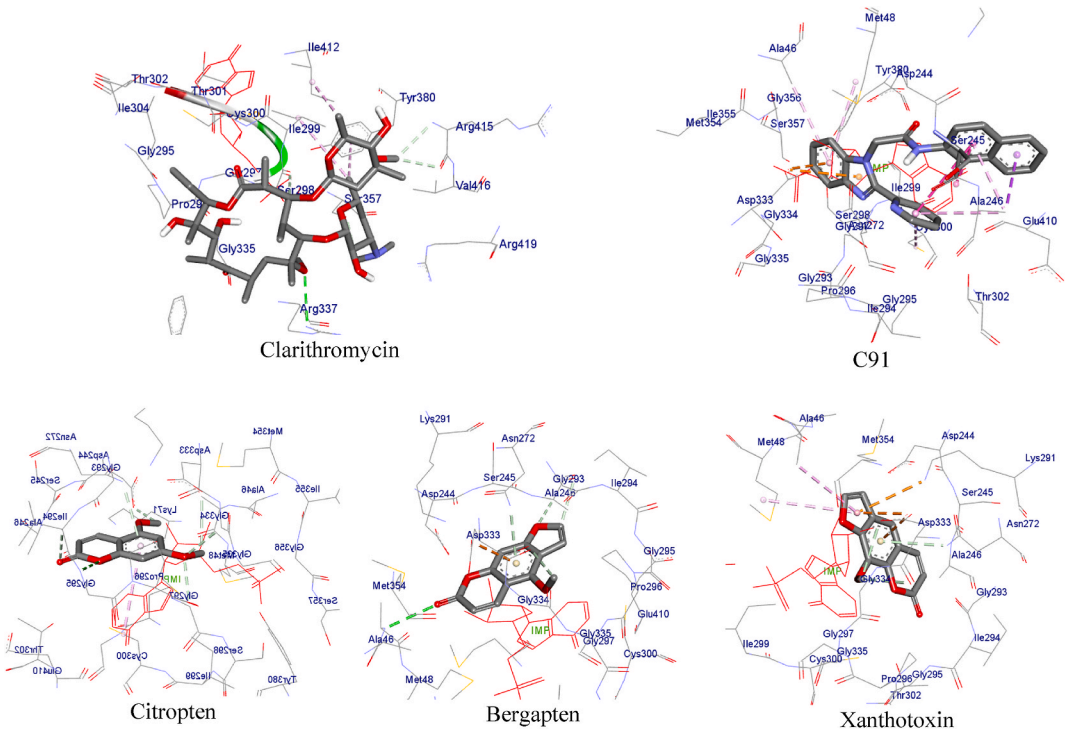

Fig. 6. 3D representation of predicted binding mode for C91, clarithromycin bergapten, xanthotoxin & citropten with 3D-structure of *Hp*IMPDH.

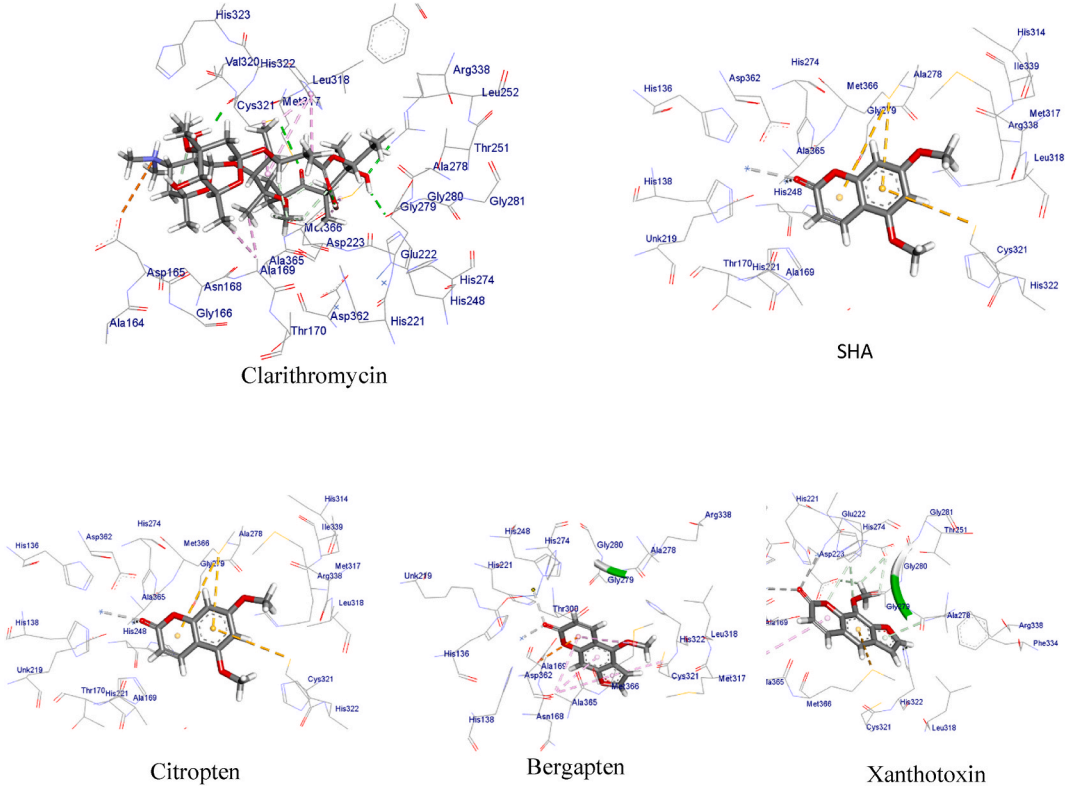

Fig. 7. 3D representation of predicted binding mode for compounds SHA, clarithromycin, bergapten, xanthotoxin & citropten with Urease (PDB ID: 6zja) active site.

Table 2
Types of interactions of bergapten, xanthotoxin & citropten compounds within *Hp*IMPDH 3D model and Urease (PDB ID: 6zja) active site.

	*Hp*IMPDH		Urease	
Cpd.	Amino acids/Bond name/Distance in angstrom (Å)	Energy scores (kcal/mol)	Amino acids/Bond name/Distance in angstrom (Å)	Energy scores (kcal/mol)
C91	Asp333/Pi-Anion/3.41 Asp333/Pi-Anion/4.09 Ala246/Pi-Sigma/3.64 Ser245/Amide-Pi Stacked/4.04 Ala46/Pi-Alkyl/4.78 Met48/Pi-Alkyl/5.14 Ala246/Pi-Alkyl/4.05 Ala246/Pi-Alkyl/5.34 Cys300/Pi-Alkyl/5.26	−6.9	–	–
SHA	–	–	His221/^{1}H-Bond/2.16 His248/^{2}H-Bond/2.75 NI601/Metal-Acceptor/1.97 Cys321/Pi-Sulfur/3.57 Cys321/Pi-Sulfur/4.34 His248/Pi-Sulfur/4.98 His322/Pi-Pi Stacked/4.34 Met317/Alkyl/4.93 Met366/Alkyl/4.93 Met366/Pi-Alkyl/4.94	−5.9
Clarith.	Arg337/^{1}H-Bond/3.23 Ser298/^{2}H-Bond/3.23 Ser357/^{2}H-Bond/3.57 Arg415/^{2}H-Bond/3.64 Ile299/Alkyl/3.64 Ile412/Alkyl/3.78 Try380/Pi-Alkyl/5.18	−6.4	Asp165/Attractive Charge/5.46 Cys321/^{1}H-Bond/3.68 Arg338/^{1}H-Bond/2.50 Gly279/^{1}H-Bond/2.02 Cys321/^{1}H-Bond/1.98 Asp223/^{2}H-Bond/2.61 Asp223/^{2}H-Bond/2.52 Asp223/^{2}H-Bond/2.76 Ala169/Alkyl/3.88 Ala169/Alkyl/4.16 Cys321/Alkyl/4.63/4.63 Met366/Alkyl/4.86 Cys321/Alkyl/4.01 His322/Pi-Alkyl/5.28 His322/Pi-Alkyl/3.47 His322/Pi-Alkyl/5.00	−5.9
Citropten	Gly295/^{1}H-Bond/3.19 Ile294/^{2}H-Bond/3.60 Gly297/^{2}H-Bond/3.46 Gly334/^{2}H-Bond/3.71 Asp333/^{2}H-Bond/3.50 Asp244/^{2}H-Bond/3.68 Asn272/^{3}H-Bond/3.90 Cys300/Pi-Alkyl/5.42	−6.9	NI601/Metal-Acceptor/2.66 NI602/Metal-Acceptor/2.85 Cys321/Pi-Sulfur/5.43 Met366/Pi-Sulfur/4.73 Met366/Pi-Sulfur/5.91	−3.5
Xanthotoxin	Asp333/^{2}H-Bond/3.72 Asp333/^{2}H-Bond/3.54 Lys291/^{4}H-Bond/4.12 Asp333/Pi-Anion/3.66 Asp333/Pi-Anion/3.59 Asn272/^{3}H-Bond/4.05 Ala46/Pi-Alkyl/4.84 Met48/Pi-Alkyl/5.19	−6.5	His248/^{2}H-Bond/3.23 His248/^{2}H-Bond/3.22 Glu222/^{2}H-Bond/2.67 Glu222/^{2}H-Bond/2.81 Asp223/^{2}H-Bond/2.67 His322/^{2}H-Bond/2.43 NI601/Metal-Acceptor/2.79 NI602/Metal-Acceptor/3.12 Arg338/^{3}H-Bond/3.20 Met366/Pi-Sulfur/5.60 Ala365/Pi-Alkyl/5.35	−4.1
Bergapten	Ala47/^{1}H-Bond/3.14 Ala246/^{2}H-Bond/3.52 Asp333/^{2}H-Bond/3.50 Ala246/^{2}H-Bond/3.52 Asp333/Pi-Anion/3.86 Asn272^{3}H-Bond/3.74	−6.5	NI602/Metal-Acceptor/2.16 NI601/Metal-Acceptor/2.69 Asp362/Pi-Anion/4.31 Ala365/Pi-Alkyl/4.44 Ala365/Pi-Alkyl/4.53 Met366/Pi-Alkyl/5.45 Cys321/Pi-Alkyl/4.55 Ala365/Pi-Alkyl/5.28	−3.9
	^{1}Conventional Hydrogen Bond ^{2}Carbon Hydrogen Bond ^{3}Pi-Donor Hydrogen Bond ^{4}Pi-Cation; Pi-Donor H-Bond SHA (Urease inhibitor) [38]			

3.4. In vitro HpIMPDH inhibition assay

Based on the *in-vitro* anti-*H. pylori* activity results, the tested compounds, and clarithromycin were investigated for their *Hp*IMPDH inhibition. Monitoring NADH generation at 20 μM–0.78 μM of IC_{50} determined enzyme inhibition (Table 2S). To create concentration-response curves, the average fluorescence responses at the plateau of the inhibitor fluorescence–time curves were plotted against their respective concentrations. The activity of the compounds (inhibition ratio and IC_{50}) was plotted versus their concentrations. The activity data (% inhibition and IC_{50}) for the compounds and the reference drug (clarithromycin) are given in Table 3 and Fig. 8. Citropten, and xanthotoxin have strong activity against the *Hp*IMPDH enzyme with an IC_{50} of 2.4 and 2.8 μM, respectively. On the other hand, bergapten was less active with an IC_{50} of 3.8 μM, respectively. The activity of clarithromycin (IC_{50} = 1.82 μM) could be attributed to its enzymatic toxic effect and not as an enzyme inhibitor, as previously reported by Galal et al. [39].

4. Discussion

A phytochemical investigation of the aqueous ethanol leaf extract of *C. sinensis* resulted in the isolation and structural identification of three coumarins, namely; bergapten, xanthotoxin, and citropten. Both bergapten and xanthotoxin were previously isolated from the peel and root of *C. sinensis* L. [40], while citropten was isolated for the first time herein from the current plant. *H. pylori*-associated drug resistance, high side effects, low adherence, and the high cost of *H. pylori* treatment increase the therapy failure of this bacterium. In this context, research for alternative treatments must be developed [41]. Several natural extracts and their purified compounds have shown potential antimicrobial effects [42–45]. Coumarins are a group of natural compounds that are found in several plant sources and display exceptional biological activities, such as antioxidant, antimicrobial, anti-inflammatory, anticoagulant, antiviral, and antitumor activities [46]. In the current study, we evaluated the anti-*H. pylori* activity of *C. sinensis* leaf extract and its subsequent compounds, which showed promising activity, with a MIC range of 62.5–7.81 μg/mL, which agrees with the previous study [47], which confirmed the antibacterial activity of coumarins. The anti-*H. pylori* activity of coumarins may be directly associated with the lipophilicity and structure of the molecule [48]. Rapid proliferation is a key feature of many microbial infections and is aided by the expansion of the guanine nucleotide pool by rapid cell divisions [49]. Inosine 5′-monophosphate dehydrogenase (IMPDH) is a promising, but largely untapped, target for antimicrobial drug development. The IMPDH enzyme catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthine 5′-monophosphate (XMP) while simultaneously reducing nicotinamide adenine dinucleotide (NAD^+), which is a critical stage in the biosynthesis of guanine nucleotides [49]. IMPDH inhibition reduces the supply of XMP and the formation of guanosine monophosphate (GMP) and the guanine nucleotide pool [39].

Urease is an important enzyme in the nitrogen cycle, this type of hydrolase increases the rate of urea hydrolysis into ammonia and carbon dioxide by a hundred trillion times [50]. With the help of urease, *H. pylori* can survive in the acidic pH of the stomach by producing a basic environment around the bacteria [51]. The majority of urease is found in the bacterial cytoplasm, with only a small quantity detected on the bacterial cell surface [52,53]. Specifically, inhibiting urease activity has been proposed as a possible method for eradicating this microbe [54,55].

In this research, the *in-silico* approach using molecular docking was applied to citropten, xanthotoxin, and bergapten isolated from *C. sinensis* leaves showing docking scores of −6.9, −6.5, and −6.5 kcal mol^{-1}, respectively. The binding mechanism revealed important interactions between the ligands and the relevant *Hp*IMPDH residues. The non-competitive inhibitor C91 interacted hydrophobically with Asp333, Ala46, Cys300, Met48, and Ser245 (binding energy: 6.9 kcal mol^{-1}). Clarithromycin formed hydrogen bonds with Arg337, Ser298, Ser357, and Arg415.

Coumarin compounds are anchored in a binding site next to IMP and form hydrogen bonds with Asn272 and Asp333. Citropten has been found to form 7 hydrogen bonds, with the highest score of −6.9 kcal mol^{-1}. Xanthotoxin and bergapten have a good docking score of −6.5 kcal mol^{-1}, Xanthotoxin formed four hydrogen bonds and was stabilized by hydrophobic interactions with residues Asp333, Ala46, and Met48. Bergapten anchored in a binding site of *Hp*IMPDH, formed hydrogen bonds with Ala47, Asp333, Ala246, and Asn272, in addition to one Pi-anion interaction. SHA (2-{[1-(3,5-Dimethylphenyl)-1*H*-imidazole-2-yl] sulfanyl}-*N*-hydroxy acetamide) is a urease inhibitor [38], its hydroxamic acid moiety interacts with Ni 601, and the rings engage with the flab area, particularly with the conserved residues Cys321 and His322. The tested compounds form metal acceptor interactions with Ni 601 and 602, hydrophobic contacts with Cys321 in the case of citropten and bergapten, and bind with His322 in the flab region in the case of xanthotoxin. The docking scores of the three compounds were lower than those of SHA and clarithromycin. The IC_{50} values for citropten and xanthotoxin are (2.4 and 2.8 μM, respectively), making them potential proposed inhibitors of the *Hp*IMPDH enzyme. In contrast, bergapten had an IC_{50} of 3.8 μM, making it a less potent compound.

5. Conclusion

One of the most common mechanisms of bacterial resistance is the development of enzymes that break down or alter the antibiotic before it reaches the protein-binding site. Natural products are an interesting investigated candidate as sources for novel drug development due to their efficacy, low cost, convenience, and few side effects. Our findings suggest the potential of citropten as an anti-*H. pylori* agent and its superiority among other tested coumarins such as xanthotoxin, and bergapten. Thus, citropten could be a means to treat and prevent diseases caused by *H. pylori*. Molecular docking on *HP*IMPD and urease enzyme inhibition revealed that the mechanism by which citropten exerted its action may be by blocking the *Hp*IMPDH enzyme. Docking results were verified by an *in vitro* *Hp*IMPDH enzyme assay, which supports the anti-*H. pylori* activity of citropten.

Table 3
*Hp*IMPDH inhibitory activity of the tested coumarins.

Comp. ID.	*Hp*IMPDH enzyme inhibition IC_{50} (μM) ± SD
Bergapten	3.8 ± 1.06
Xanthotoxin	2.8 ± 1.43
Citropten	2.4 ± 1.62
Clarithromycin	1.82 ± 1.50

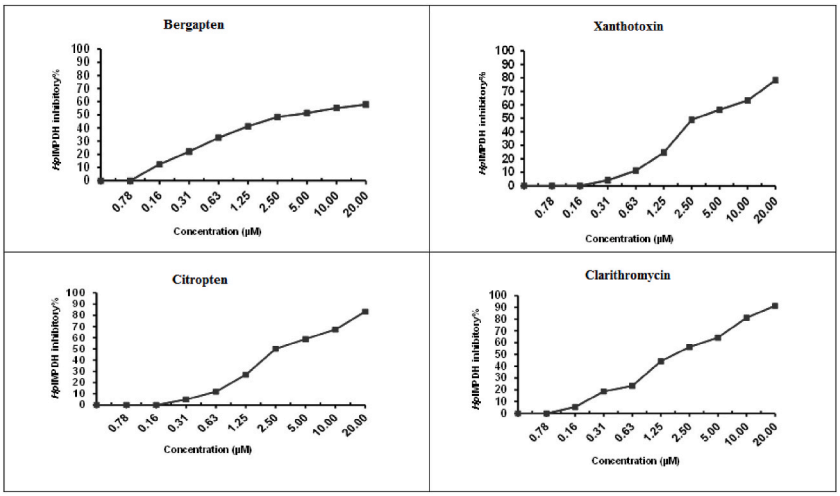

Fig. 8. The *Hp*IMPDH % inhibition of the isolated compounds and clarithromycin at different concentrations.

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Data availability

No data related to this work has been deposited into a publicly available repository. Data involved in article/supp. Material/ referenced in the article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Hala Sh Mohammed: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Mona H. Ibrahim:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Marwa M. Abdel-Aziz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Conceptualization. **Mosad A. Ghareeb:** Writing – review & editing, Writing – original draft, Visualization, Data curation, Conceptualization, Submission..

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

NMR Nuclear magnetic resonance
ESI-MS Electrospray ionization mass spectrometry
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MBEC Minimum biofilm eradication concentration
MIC Minimum inhibitory concentration
HpIMPDH *Helicobacter pylori* inosine 5′-monophosphate dehydrogenase
TLC Thin layer chromatography
CC Column chromatography
MHz Megahertz
DMSO Dimethyl sulfoxide
^{1}H-NMR Proton-nuclear magnetic resonance
^{13}C-NMR Carbon 13-nuclear magnetic resonance
ESI Electrospray ionization
LC Liquid chromatography
ATCC American Type Culture Collection strain
BHI Brain Heart Infusion
FBS Fetal bovine serum
CFU Colony-forming unit
ELISA Enzyme-linked immunosorbent assay
SDS Sodium dodecyl sulphate
OD Optical density
DTT Dithiothreitol
NAD Nicotinamide adenine dinucleotide
IC_{50} Half-maximal inhibitory concentration
BLAST Basic Local Alignment Search Tool
PDB Protein Data Bank
RMSD Root-mean-square deviation
m/z Mass-to-charge
SD Standard deviation
IMPDH Inosine 5′-monophosphate dehydrogenase
IMP Inosine 5′-monophosphate
XMP Xanthine 5′-monophosphate
NAD Nicotinamide adenine dinucleotide
GMP Guanin 5`-monophosphate

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25232.

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