Antibacterial, Antibiofilm, and Anti-inflammatory Activities of Zingiber Officinale Extract Against Helicobacter Pylori

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Article

Antibacterial, Antibiofilm, and Anti-Inflammatory Activities of *Zingiber officinale* Extract against *Helicobacter pylori*

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Abstract: The increased emergence of multidrug-resistant *Helicobacter pylori* influences the prevention of stomach cancer. *Zingiber officinale* is a plant usually used in folk medicine to treat a variety of diseases involving infections, nausea, vomiting, peptic ulcer, dyspepsia, and inflammation. This study was conducted to evaluate the ability of *Z. officinale* extract to combat resistant *H. pylori*. The disc diffusion, microdilution, and microplate assays were performed to evaluate the susceptibility to antibiotics, and the antibacterial and antibiofilm activities of the *Z. officinale* extracts. Using the checkerboard method, the combined effects of the gentamicin and *Z. officinale* extract were investigated. In addition, anti-inflammatory activity and GC-MS analysis were determined by the modified protocol. According to the findings, *H. pylori* isolates exhibited resistance rates of 56.33, 50.0, and 45.85 for metronidazole, gentamicin, and tetracycline, respectively. The methanolic extract of *Z. officinale* had the strongest effectiveness against resistant *H. pylori* isolates with MIC of 20.0 to 50.0 µg/ml against both *H. pylori* isolates and the stranded strain NCTC 11637. *Z. officinale* extract suppress the biofilm formed by *H. pylori* isolates with a percentage of 92.96% at 50.0µg/ml, compared with 97.19% for gentamicin at the same concentration. According to FICI values, combination of methanolic *Z. officinale* extract to gentamicin increases bacterial sensitivity to such drugs. Moreover, the *Z. officinale* extract exhibits strong anti-inflammatory activity. The GC-MS analysis of *Z. officinale* extract exhibits 17 different chemical compounds. Conclusions: The *Z. officinale* extract contain the anti-inflammatory compound gingerol as the main constituent which inhibits the growth of *H. pylori* and its biofilm, are a promising natural therapeutic alternative or enhance antibiotics activity.

Keywords: *H. pylori;* antibiotics resistant; *Z. officinale* antibacterial; biofilm suppression; anti-inflammatory; GC-MS

1. Introduction

The gastrointestinal tract, particularly the stomach, is colonized by *H. pylori* [1]. *H. pylori* is one of the most common causes of human infection, especially in developing countries, where it affects more than 80% of the population. [2]. An infection with *H. pylori* typically persists for the rest of one's life. This bacterium has also been related to mucosa-associated lymphoid tissue (MALT) lymphoma, peptic ulcer, chronic gastritis, and gastric cancer. [3-6]. Despite the fact that the majority of *H. pylori* infections are asymptomatic, those who are infected are at a significant risk of developing the aforementioned illnesses. *H. pylori* was categorized as a human carcinogen category I by the World Health Organization International Agency for Research on Cancer in 1994. [7]. Flagella, biofilm, outer membrane proteins (OMPs) catalase, mucinase, lipase, urease, proteases, and phospholipase), vacuolating cytotoxin A (VacA), cytotoxin-associated gene antigen (CagA), and induced contact of the epithelium A (IceA), among others, are virulence factors that control the pathogenicity of *H. pylori* [1, 7]. Several studies have shown that *H. pylori* strains have the potential to form biofilms in human stomach mucosa both in vitro and in vivo. [8-12]. Amoxicillin, clarithromycin, or amoxicillin and metronidazole are the two antibiotics used in the *H. pylori* treatment regimen, together with a proton pump inhibitor. Due to the emergence of drug-resistant strains, standard treatment was unable to completely eradicate *H. pylori* [1, 12]. The formation of biofilms modifies the outer membrane proteins

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associated with antibiotic resistance, and elevating proteinase K levels alters clarithromycin resistance, according to findings by Hathroubi et al. [13]. Moreover, eDNA in biofilms stimulates microbial adhesion, prevents the diffusion of antibiotics, and chelates cations. [14]. Some extracellular enzymes in the biofilm deactivate antibiotics [15]. The rapid emergence of multi-drug *H. pylori* is a strong reason to search for a new approach to eradicating it [16]. In comparison to treatments derived from chemical sources, using plant extracts for the therapeutic treatment of multi-drug resistant *H. pylori* has benefits. This approach was preferred because, as compared to those developed from chemical sources, these therapeutics had less toxicological and pharmacological side effects. In an attempt to decrease the toxicity of synthetic drugs, researchers in a wide range of disciplines, including gastroenterology and bacteriology, have focused a great deal of emphasis on the pharmacological actions of natural products against *H. pylori* [17, 18]. So, several studies have examined plant compounds that have gastroprotective and anti-*H. pylori* activity. [1, 19, 20]. *Zingiber officinale*, which belongs to the *Zingiberaceae* family, has grown in popularity as a complementary medicine in many parts of the world. The methanol extracts of *Zingiber officinale* prevent the growth of 19 *H. pylori* strains in vitro with a MIC range of 6.25 to 50 μg/ml. Most *H. pylori* strains are inhibited by gingerols, an active fraction with a MIC range of 0.78 -12.5 μg/ml and significant activity against CagA positive bacteria [21]. Moreover, Ohno et al. (2003) shown that plant essential oils can inhibit *H. pylori* isolates from clinical samples and the ATCC strain [22]. An infection with *H. pylori* activates many pro-inflammatory signals. This reaction contributes to the pathophysiology of a number of gastrointestinal illnesses. To treat stomach disease caused by *H. pylori* infection, the inflammatory response must be under control. Strong anti-inflammatory properties and an ability to stop the release of cytokines that induce inflammation are included in the *Zingiber officinale* extract. [23]. The use of combination therapy has several benefits, including the ability to treat mixed infections and infections caused by a specific causal organism, as well as to enhance antimicrobial activity, reduce the need for continuous antibiotic administration, and stop the formation of bacteria that are resistant to several drugs [18]. The current work aimed to identify the main bioactive components of *Zingiber officinale* methanolic extracts and explore its antibacterial, antibiofilm, the ability to enhance antibiotics activity and anti-inflammatory actions against resistant *H. pylori*.

2. Materials and Methods

2.1. Zingiber officinale

Zingiber officinale rhizomes were obtained from the local market in Cairo, Egypt, in 2022. The rhizomes of *Z. officinale* were washed with distilled water, after drying at room temperature. After drying, the rhizomes of *Z. officinale* are cut into small parts and ground to powder using an electrical blender.

2.1.1. Extract of Z. officinale (ZO) preparation.

Ten grams of powdered *Z. officinale* were mixed with 100 ml of methanol (Sigma-Aldrich, St. Louis, USA) to generate the methanol extract, which was then allowed to soak for 24 hours at room temperature. Throughout the time spent soaking, the extracts were filtered using Whatman No. 1 filter paper, and the filtrate was then evaporated at 50 °C under decreased pressure in a vacuum evaporator. The extracts were kept in opaque vials and frozen at -10 °C until further research.

2.2. Antibiotic susceptibility of H. pylori isolates.

Seventy-six *H. pylori* isolates previously identified and the reference strain NCTC 11637 were grown at 37 °C in Mueller-Hinton broth (MH) medium (Oxoid, UK) supplemented with 5% horse blood until they reached the exponential phase. The turbidity of bacterial growth was measured using a spectrophotometer set at 620 nm and examined every 30 minutes to identify the exponential phase. The inoculum density in each bacterial solution was then adjusted to 0.5 McFarland Standard (1.5×10^6 CFU/ml) in sterile saline (0.84% NaCl). Antibiotic susceptibility testing of *H. pylori* isolates was performed using the disc diffusion technique, as described in the Clinical and Laboratory

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Standards Institute (CLSI 2020) guidelines (M7-A5) [24]. In brief, 50µl of the bacterial suspension at the above-mentioned turbidity concentration were inoculated onto (MH) agar medium enriched with 5% horse blood. Antibiotic discs representing different classes of antibiotics (antibiotics panel were ciprofloxacin 5µg/ml, levofloxacin 5µg/ml gentamicin 10µg/ml, neomycin 30µg/ml, clarithromycin 15µg/ml, erythromycin 15µg/ml, tetracycline 30µg/ml, amoxicillin 25µg/ml, metronidazole 5µg/ml and amoxicillin/clavulanic acid 20/10µg/ml), were gently loaded on the prepared plates using sterile forceps. Then, they were incubated for 24 h under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) using a gas pack system (Mitsubishi, Japan) at 37°C. The diameter of the inhibition zone was measured in millimeters (mm), compared with the standard zone diameter given in the protocol chart. It can be determined whether the bacterial isolate is resistant, intermediate, or susceptible to the tested antibiotics.

2.3.1. Anti H. pylori activity of (ZO) extract

Zingiber officinale extract were investigated for antibacterial activity against resistant *H. pylori* isolates and *H. pylori* NCTC 11637. One hundred microliters of bacterial growth (1.5 x10^6 CFU/ml) were inoculated into Muller Hinton Blood Agar (MHBA) medium. Paper discs (8mm) were saturated with 50µl of crude extract containing 10 mg of (ZO) in DMSO per ml. The saturated paper discs were plated on the surface of the inoculated MHBA plate, and a control antibiotic, gentamicin paper discs containing 25 g/ml, was used on the same plates. The plates were incubated at 37 °C for 48 hours under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) using a gas pack system (Mitsubishi, Japan), and the diameter of the inhibitory zone was estimated with (mm). This experiment was performed in three replicates [25].

2.3.2. Determination of minimum inhibitory concentrations (MICs) of (ZO) extract.

The microbroth dilution technique was used to evaluate the MIC of the methanolic extract (ZO) against *H. pylori* isolates and standard strain NCTC 11637 using gentamicin (HiMedia Laboratories Pvt. Ltd., India) as an antibacterial control. In each well of a 96-well microplate, gentamicin and a methanolic extract of (ZO) were coated in twofold serial dilutions. A saline suspension of the test strain was added to each well, and the cultures were incubated at 35 °C for 3 days in a microaerophilic atmosphere (10% CO_2, 5% O_2, and 85% N_2). *Z. officinale* extract was investigated at concentrations ranging from 0.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, and 60.0 µg/ml, and gentamicin was started at 0.30, 0.613, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 g/ml. The experiment was performed according to the criteria of the CLSI 2020 guidelines (M7-A5) [24, 26]. Wells containing a negative control (medium + ZO) extract or gentamicin at the tested concentrations) were performed to determine the differences in optical density (OD) at 630 nm. MIC was defined as the lowest concentration of the *Z. officinale* extract or gentamycin that can inhibit the visible growth of bacteria.

2.3. Checkerboard assay

Checkerboard is a technique used to determine the synergistic effect between gentamicin and ZO extract. Using the broth microdilution method, two-dimensional checkerboard serial dilutions were carried out, with the concentration of gentamicin decreasing vertically and the concentration of the of (ZO) decreasing horizontally. The previously made stock solution was diluted with Mueller-Hinton broth. Gentamicin concentrations started at 1/ 128 MIC in the rows, and (ZO) concentrations started at 1/ 128 MIC in the columns. At a density of 10^6 (CFU)/ml/well, bacterial isolates were inoculated. and the plate was incubated for 24 hours at 37°C. A positive control well included only media with bacteria inoculated and no gentamicin /(ZO) combinations. The negative control wells contained only medium containing the used combinations with no bacteria inoculated. By comparing the growth of tested bacteria in the wells with the positive and negative controls, the MIC for the antibacterial agents was identified. Spectrophotometer was analyzed the optical density (OD) of the microplate at 620 nm. To determine the correlation between gentamicin and (ZO), fractional inhibitory concentration index (FICI) was determined with the formula [18].

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$$\mathbf{FICI = FIC^{gentamycin} + FIC^{ZO}}$$

where, $\mathbf{FIC^{gentamicin}}$ = (MIC of gentamicin in the presence of ZO)/ (MIC of gentamicin alone) and $\mathbf{FIC^{ZO}}$ = (MIC of ZO in the presence of gentamicin)/ (MIC of ZO alone).

Then, the interpretation ranges were applied to the FICI value. (Synergy ≤ 0.5, additive > 0.5 and ≤ 1.0, Indifference > 1 and ≤ 4.0, and Antagonism > 4.0)

2.4. Antibiofilm activity of methanolic extract Z. officinale.

The ability of *Z. officinale* methanolic extracts to inhibit biofilm formation by *H. pylori* isolates at concentrations of 25 and 50µg/ml and using gentamicin as a control at the same concentrations were assessed using microtiter plate assays. In brief, 0.5 McFarland turbidity was adjusted from previous 24-hour cultures of each isolate to a 100-fold dilution using TBS (Liofilchem, Italy). Then, 100µl of this dilution was inoculated in triplicate on a 96-well flat-bottomed polystyrene plate (China) and incubated for 24 h at 37°C. Each well had its contents removed, and phosphate-buffered saline was used to repeatedly wash the wells (PBS). The plate was then air-dried after a 15-minute methanol fixing procedure. Each well was stained with 100µl of 1% crystal violet solution in water and incubated at room temperature for 30 min. Afterward, the stain was solubilized by 100µl of glacial acetic acid (GAA) (33%), plates were washed with distilled water three times, and then they were dried. The optical density (OD) of each well was read at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Based on the OD average values, the results of the biofilm formation were interpreted as follows; (OD≤ODC) = negative (non-biofilm formation), ODC optical density of control; (ODC≤ OD≤2ODC) = weak biofilm formation, (2ODC≤OD≤4ODC) = moderate biofilm formation; and (4ODC≤OD) = strong biofilm formation [27].

2.5. Anti-inflammatory assay by human RBCs

Using a human red blood cell (HRBC) technique, the anti-inflammatory activity of *Z. officinale* extract were examined. An equal volume of Alsever solution (consisting of 0.8% sodium citrate, 2% dextrose, 0.42% sodium chloride, and 0.5% citric acid) was added to the blood of a healthy human volunteer who had abstained from using non-steroidal anti-inflammatory drugs (NSAIDS) for the two weeks before the study began. The mixture was then centrifuged at 3,000 rpm. After being iso-saline washed from the precipitated cells, a 10% suspension was made. Deionized water was used to prepare different extract concentrations, including 4, 8, 16, and 32 µg/ml. To each concentration, 1 ml of phosphate buffer, 2 ml of hypo-saline, and 0.5 ml of HRBC solution were added. After a 30-minute incubation period at 37 °C, they conducted a 20-minute centrifugation at 3,000 rpm. Spectrophotometrically determine the supernatant's hemoglobin concentration at 560 nm [28]. The following equation was used to calculate the inhibition percentage.

$$\text{Inhibition percentage } \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \text{ x } 100$$

2.6. Identification of chemicals that constitute Z. officinale extract by GC-MS.

Gas chromatography-mass spectrometry (GC-MS) was used to evaluate, count, and identify the main active components in *Z. officinale* extract, with a few minor adjustments according to El-Sherbiny et al [29].

2.7. Statistical analysis

Using the statistical package-extended Minitab 18 software and Microsoft Excel 365, the data were computed as the mean ±SD value.

3. Results

3.1. Antibiotics profile of H. pylori isolates.

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Of the 76 previously identified *H. pylori* isolates and the reference strain, NCTC 11637 was investigated for antibiotic susceptibility using disc diffusion techniques. The findings demonstrate that *H. pylori* isolates are resistant to metronidazole, gentamicin, and tetracycline with 56.33%, 50.0%, and 45.85%, respectively. The antibiotics with the highest activity against those isolates were amoxicillin-clavulanate and ciprofloxacin, with ratio sensitivity of 73.36% and 58.95%, respectively. While the standard strain was shown to be susceptible to all antibiotics tested as shown in Table 1. *H. pylori* rapidly develops resistance to one treatment and requires combination therapy with different antibiotics. The combination therapy used in the treatment program should be chosen based on the country's predicted national drug resistance rates [1]. Globally, resistance to metronidazole and clarithromycin has grown over the past several years, lowering the efficacy of conventional first-line treatment regimens and raising the number of treatment failures brought on by drug-resistant *H. pylori* [30]. A significant number of isolates (73.9%) were previously demonstrated to be resistant to metronidazole, which was followed by amoxicillin (54.3%), clarithromycin (47.8%), ciprofloxacin (13.3%), and tetracycline (4.3%), according to Rasheed et al. [31]. Additionally, a study of antibiotic susceptibility by Tan and colleagues [32] in 2018 on 34 *H. pylori* strains revealed metronidazole resistance as the most frequent antibiotic resistance (79.4%), followed by clarithromycin (70.6%) and ciprofloxacin (42.9%). Among macrolides, including gentamicin, clarithromycin has been employed in front-line regimens for *H. pylori* eradication given its two pharmacokinetic features in the stomach, acid stability and amelioration absorption in the gastric mucus layer [33]. By reversibly binding to the peptidyl transferase loop of the 23S ribosomal RNA's domain V in the bacterial cell, macrolides inhibit protein synthesis and have antibacterial effects. Overall, point mutations in domain V of the 23S rRNA gene are the main cause of clarithromycin resistance in *H. pylori*, [34]. Tetracyclines have been used in a variety of regimens to eradicate *H. pylori*. The several Tet protein homologs mediate the molecular mechanisms causing *H. pylori* tetracycline resistance. Despite the fact that *H. pylori* encodes numerous assumed PBPs and -lactamase-like proteins, it causes amoxicillin resistance mainly by decreasing the binding affinity to a specific PBP without generating significant -lactamase activity [33]. Fluoroquinolones (moxifloxacin, levofloxacin, and ciprofloxacin) have been used as an alternative first- and second-line *H. pylori* eradication treatment. These antibiotics have a bactericidal effect by inhibiting two key bacterial type II topoisomerases, topoisomerase IV, and DNA gyrase, which modify the chromosomal supercoiling necessary for DNA synthesis, transcription, and cell division [35]. Bacterial resistance to fluoroquinolones is commonly caused by three distinct but non-exclusive mechanisms: target-mediated resistance caused by mutated topoisomerase IV or DNA gyrase, plasmid-mediated resistance caused by plasmids encoding DNA mimics that compete with natural drug targets; enzymes that lower antibiotic activity through acetylation and efflux systems; and chromosome-mediated resistance caused by altered antibiotic uptake and intrinsic efflux systems [33]. *H. pylori*, which is naturally absent of these genes, fluoroquinolone resistance is due to target-mediated mechanisms attributed to mutations in single or dual *gyr*A and *gyr*B genes encoding *Gyr* subunits A and B [36].

Table 1. Antibiotics susceptibility among 76 *H. pylori* isolates.

Class of antibiotics and mode of action	**Antibiotic**	**Potency (µg/disk)**	**Antibiotics Susceptibility**			**Ref. normal value zone diameter breakpoints**			**Ref. normal value** *H. pylori*
			S. **N (%)**	I. **N (%)**	R. **N (%)**	S	I	R	NCTC 11637
Inhibition of bacterial nucleic acid synthesis									
Fluoroquinolone	**Ciprofloxacin**	5	45 (58.95)	0(0.0)	31 (40.61)	≥ 21	16-20	≤ 15	22.40
Quinolone	Levofloxacin	5	39(51.09)	9(11.79)	28 (36.68)	≥ 17	13–16	<12	20.0
Inhibition of bacterial protein synthesis									
Aminoglycosides	Gentamicin	10	22 (28.82)	16(20.96)	38 (50.0)	≥ 15	13-14	≤ 12	19.70
	Neomycin	30	16(20.96)	27(35.37)	33 (43.23)				21.0
Macrolides	Erythromycin	15	30(39.30)	12(15.72)	34 (44.54)	≥ 23	14-22	≤ 13	20.50
	Clarithromycin	15	37 (48.47)	9(11.79)	30(39.30)	≥21	15-20	≤14	21-70

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Tetracyclines	Tetracycline	30	19(24.89)	22 (28.82)	35 (45.85)	≥ 19	15-18	≤ 14	23-80
Inhibitors of bacterial cell wall synthesis									
	Amoxicillin	25	43 (56.33)	8(10.48)	25 (32.75)	≥ 13	11-12	≤ 10	24.30
Penicillin combination	Amoxicillin-clavulanate	20/10	56 (73.36)	2(2.62.)	18 (23.58)	>19	-	<20	26.30
Metabolic antagonism									
Nitroimidazoles	Metronidazole	5	15(19.65)	18 (23.58)	43 (56.33)	>21	16-21	<16	18.70

S = Sensitive, I = Intermediate, R = Resistance.

3.2. Antibacterial activity and minimum inhibitory concentration of Z. officinale extract

To assess the antibacterial activity of *Z. officinale* extract against *H. pylori* isolates and standard strain NCTC 11637 using a disc diffusion assay. The results showed that *Z. officinale* extract possesses potential antibacterial activity, with a mean ± SD imbibition zone ranging from 10±03 to 24±04 mm against both *H. pylori* isolates and standard strain NCTC 11637 are comparable with gentamicin 22±0.04 against standard strain NCTC 11637 as shown in Table 2. Several studies reported the antibacterial activity of *Z. officinale* extract against different bacterial strains isolated from clinical samples and standard strains [37-39]. Also, many studies [21, 40, 41] found *Z. officinale* extract to have antibacterial activity against *H. pylori*. In this study, the minimum inhibitory concentration of *Z. officinale* against resistant *H. pylori* isolates and the standard strain ranged from 20 to 48 µg/ml as shown in Table 3. These findings are consistent with those of Mahady et al. [21] found that a crude methanol extract of *Z. officinale* suppressed the growth of 14 clinical isolates of *H. pylori*, four CagA+ strains, and the ATCC-43504 strain, with a MIC of 50.0 µg/ml. Moreover, Azadi et al. [42] found that the ethanolic *Z. officinale* extract had a MIC of 58 µg/ml against the *H. pylori* CagA+ strain and that a combination of *Z. officinale* and cinnamon extract downregulated the CagA gene level by 1.94 times. According to Chakotiya et al. [38], *Z. officinale* extract changes the permeability and efflux activity of bacterial cells.

Table 2. Antibacterial activity of methanolic *Z. officinale* extract against twenty-fiver MDR *H. pylori* isolates and NCTC 11637.

No	Strains code	Mean of inhibition zone diameter (mm ± SD)		No	Strains code	Mean of inhibition zone diameter (mm ± SD)	
		Z. officinale extract	Gentamicin			*Z. officinale* extract	Gentamicin
1	**HPM4**	**14±00**	0.0	14	HPM48	12±05	0.0
2	HPM7	13±09	0.0	15	HPM51	15±20	0.0
3	HPM9	12±05	0.0	16	HPM54	24±04	0.0
4	HPM12	11±05	0.0	17	HPM57	14±06	0.0
5	HPM15	15±09	0.0	18	HPM62	12±04	0.0
6	HPM16	13±03	0.0	19	HPM63	13±06	0.0
7	HPM19	15±00	0.0	20	HPM65	16±00	0.0
8	HPM26	12±04	0.0	21	HPM66	11±04	0.0
9	HPM37	16±00	0.0	22	HPM70	10±03	0.0
10	HPM44	11±06	0.0	23	HPM72	12±07	0.0
11	HPM48	15±08	0.0	24	HPM73	14±08	0.0
12	HPM52	12±00	0.0	25	HPM75	15±08	0.0
13	HPM56	14±06	0.0	26	*H. pylori* NCTC 11637	17±06	20±04

Table 3. MIC of *Zingiber officinale* extracted against resistant *H. pylori* species.

No	Strains code	Minimum inhibitory concentration (µg/ml)	No	Strains code	Minimum inhibitory concentration (µg/ml)

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		Z. officinale extract			*Z. officinale* extract
1	HPM4	25.0	14	HPM48	39.0
2	HPM7	27.0	15	HPM51	28.0
3	HPM9	22.0	16	HPM54	23.0
4	HPM12	25.0	17	HPM57	29.0
5	HPM15	29.0	18	HPM62	38.0
6	HPM16	32.0	19	HPM63	50.0
7	HPM19	22.0	20	HPM65	27.0
8	HPM26	23.0	21	HPM66	33.0
9	HPM37	33.0	22	HPM70	46.0
10	HPM44	26.0	23	HPM72	42.0
11	HPM48	35.0	24	HPM73	35.0
12	HPM52	37.0	25	HPM75	48.0
13	HPM56	44.0	26	*H. pylori* NCTC 11637	20.0

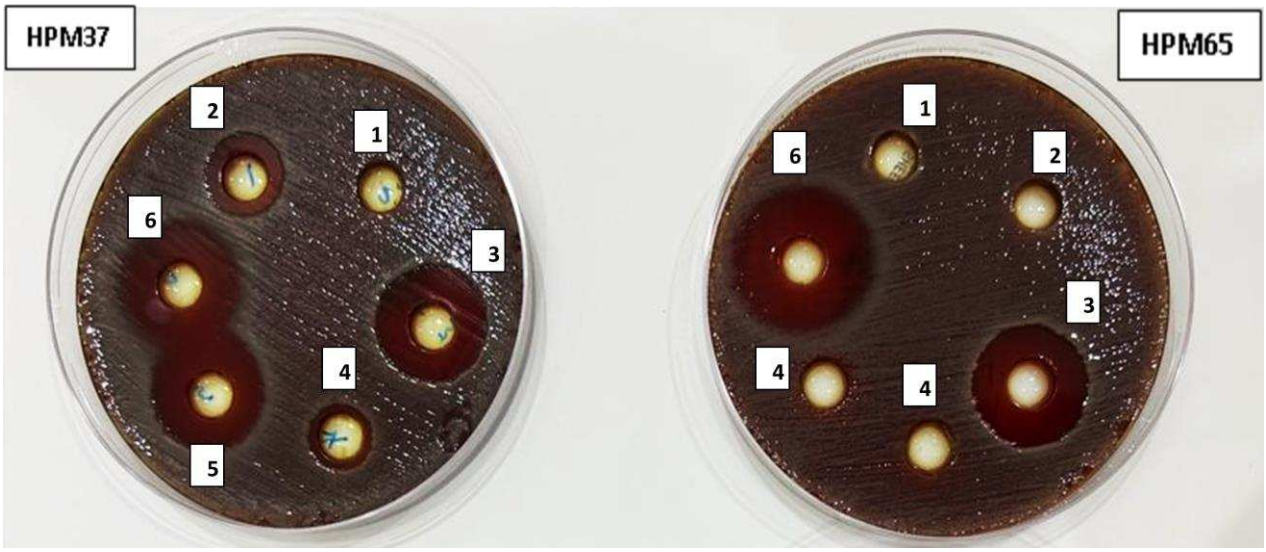

Figure 1. Antibacterial activity of gentamycin (1), amoxicillin (2), ciprofloxacin (3), Dimethyl sulfoxide (DMSO) (4), amoxicillin-clavulanate r (5), *Z. officinale* extract (6) against resistant *H. pylori* isolates (HPM37 and HPM 65) and *H. pylori* NCTC 11637.

3.3. Synergistic effect of gentamicin combination with Z. officinale extract against resistant H. pylori isolate HPM72

In checkerboard technique, the interactions between gentamicin and ZO extract against resistant *H. pylori* isolate HPM72 exhibited thirty-eight treatments causing inhibition of resistant *H. pylori* isolate HPM72. A synergistic effect was considered when gentamicin and ZO extract combination showed FICI value ≤ 0.5, this case was observed with only eleven combinations at different ratios (0.25+0.25, 0.25+0.125, 0.25+0.062, 0.25+0.03, 0.125+0.25, 0.125+0.125, 0.125+0.062, 0.062+0.25, 0.031+0.2, 0.015+0.25 and 0.007+0.25 (µg/µg)/ml of gentamicin and ZO extract, respectively). Also, there were fourteen combinations with FICI ranged from 1.007 to 1.5 meaning additive effects. On the other hand, one combination showed an indifferent effect where the FICI 2 (µg/µg)/ml of gentamicin and ZO extract. Through these interactions, the MIC of gentamicin was reduced from 42 µg /ml to 0.125 µg (336-fold) while the MIC of ZO extract was reduced 33 µg /ml to 0.062µg (532-times). The FIC indexes for the tested combinations and their interpretations are presented in Table 4. Combination therapy has many advantages, including treating mixed infections, infections brought on by a particular causative organism, increasing antimicrobial activity, avoiding the need for prolonged antibiotic use, and preventing the emergence of multidrug-resistant bacteria. Combination therapy is the most frequently recommended empirical treatment for bacterial infections in intensive care

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units [18]. The MIC values against drug-resistant *P. aeruginosa* and clinical isolates of multidrug resistant *Staphylococcus aureus* were dramatically reduced when various antibiotics were combined with crude extracts of several plants [18,43]. Infections brought on by resistant bacterial stains can be treated more effectively when gentamicin and plant extract are combined, according to research done with Khameneh *et al* [44]. The efflux pumps appear to be inhibited by secondary plant compounds through competitive and non-competitive inhibition or by lowering the expression of the efflux genes. Due to the concentration and persistence of antibiotics in the bacteria, herbal extracts are therefore likely to prevent bacterial resistance to antibiotics. Moreover, *Z. officinale* extract possesses significant nephroprotective activity which is induced by gentamicin [45].

Table 4. Synergistic effect of gentamicin and ZO extract against resistant *H. pylori* HPM72.

No	MIC Gentamicin + MIC ZO extract	Gentamicin + ZO extract (µg/ml)	FICI Gentamicin + FICI ZO extract	FICI	interpretation	MIC Gentamicin + MIC ZO extract
1	**MIC+MIC**	42+33	1+1	2	Indifference	**MIC+MIC**
2	**MIC+1/2MIC**	42+16.5	1+0.5	1.5	additive	**MIC+1/2MIC**
3	**MIC+1/4MIC**	42+8,25	1+0.25	1.25	additive	**MIC+1/4MIC**
4	**MIC+1/8MIC**	42+4.13	1+0.125	1.125	additive	**MIC+1/8MIC**
5	**MIC+1/16MIC**	42+2.06	1+0.062	1.062	additive	**MIC+1/16MIC**
6	**MIC+1/32MIC**	42+1.03	1+0.031	1.031	additive	**MIC+1/32MIC**
7	**MIC+1/64MIC**	42+0,51	1+0.015	1.015	additive	**MIC+1/64MIC**
8	**MIC+1/128MIC**	42+0.25	1+0.007	1.007	additive	**MIC+1/128MIC**
9	**1/2MIC+MIC**	21+33	0.5+1	1.5	additive	**1/2MIC+MIC**
10	**1/2MIC+1/2MIC**	21+16,5	0.5+0.5	1	partial synergy	**1/2MIC+1/2MIC**
11	**1/2MIC+1/4MIC**	21+8,25	0.5+0.25	0.75	partial synergy	**1/2MIC+1/4MIC**
12	**1/2MIC+1/8MIC**	21+4.13	0.5+0.125	0.625	partial synergy	**1/2MIC+1/8MIC**
13	**1/2MIC+1/16MIC**	21+2.06	0.5+0.062	0.562	partial synergy	**1/2MIC+1/16MIC**
14	**1/2MIC+1/32MIC**	21+1.03	0.5+0.031	0.531	partial synergy	**1/2MIC+1/32MIC**
15	**1/2MIC+1/64MIC**	21+0,51	0.5+0.015	0.515	partial synergy	**1/2MIC+1/64MIC**
16	**1/4MIC+MIC**	10.5+33	0.25+1	1.25	additive	**1/4MIC+MIC**
17	**1/4MIC+1/2MIC**	10.5+16.5	0.25+0.5	0.75	partial synergy	**1/4MIC+1/2MIC**
18	**1/4MIC+1/4MIC**	10.5+8.25	0.25+0.25	0.5	synergy	**1/4MIC+1/4MIC**
19	**1/4MIC+1/8MIC**	10.5+4.13	0.25+0.125	0.375	synergy	**1/4MIC+1/8MIC**
20	**1/4MIC+1/16MIC**	10.5+2.06	0.25+0.062	0.312	synergy	**1/4MIC+1/16MIC**
21	**1/4MIC+1/32MIC**	10.5+1.03	0.25+0.031	0.281	synergy	**1/4MIC+1/32MIC**
22	**1/8MIC+MIC**	5.25+33	0.125+1	1.125	additive	**1/8MIC+MIC**
23	**1/8MIC+1/2MIC**	5.25+16.5	0.125+0.5	0.625	partial synergy	**1/8MIC+1/2MIC**
24	**1/8MIC+1/4MIC**	5.25+8.25	0.125+0.25	0.375	synergy	**1/8MIC+1/4MIC**
25	**1/8MIC+1/8MIC**	5.25+4.14	0.125+0.125	0.25	synergy	**1/8MIC+1/8MIC**
26	**1/8MIC+1/16MIC**	5.25+2.06	0.125+0.062	0.187	synergy	**1/8MIC+1/16MIC**
27	**1/16MIC+MIC**	2.62+33	0.062+1	1.062	additive	**1/16MIC+MIC**
28	**1/16MIC+1/2MIC**	2.62+16.5	0.062+0.5	0.562	partial synergy	**1/16MIC+1/2MIC**
29	**1/16MIC+1/4MIC**	2.62+8.25	0.062+0.25	0.312	synergy	**1/16MIC+1/4MIC**
30	**1/32MIC+MIC**	1.31+33	0.031+1	1.031	additive	**1/32MIC+MIC**
31	**1/32MIC+1/2MIC**	1.31+16.5	0.031+0.5	0.531	partial synergy	**1/32MIC+1/2MIC**
32	**1/32MIC+1/4MIC**	1.31+8.25	0.031+0.25	0.281	synergy	**1/32MIC+1/4MIC**
33	**1/64MIC+MIC**	0.65+33	0.015+1	1.015	additive	**1/64MIC+MIC**
34	**1/64MIC+1/2MIC**	0.65+16.5	0.015+0.5	0.515	partial synergy	**1/64MIC+1/2MIC**
35	**1/64MIC+1/4MIC**	0.65+8.25	0.015+0.25	0.265	synergy	**1/64MIC+1/4MIC**
36	**1/128MIC+MIC**	0.22+33	0.007+1	1.007	additive	**1/128MIC+MIC**
37	**1/128MIC+1/2MIC**	0.32+16.5	0.007+0.5	0.507	partial synergy	**1/128MIC+1/2MIC**
38	**1/128MIC+1/4MIC**	0.32+8.25	0.007+0.25	0.257	synergy	**1/128MIC+1/4MIC**

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3.3. Antibiofilm activity of Z. officinale extract.

In this investigation, *H. pylori* isolates formed biofilm with different degrees (93.36%), with the moderate degree being the most common. The antibiofilm efficacy of methanolic extracts of *Z. officinale* in vitro suppressed biofilm formation to various degrees in bacterial isolates at concentrations of 25 and 50 µg/ml compared to gentamicin as the control at the same concentrations Table 5. As shown in the Figure 1, the highest reduction of biofilm formation by *Z. officinale* extract was seen at 50 µg/ml (92.96%). Antibiotic resistance is a result of the structural features of the biofilm and its bacterial constituents. A complex phenomenon, biofilm-related drug resistance may be greatly influenced by biofilms. Biofilms and related infections can be treated with antibiotics, sanitizers, and germicidal chemicals. When compared to their planktonic stage, bacteria that live in biofilms exhibit a 10-1000-fold increase in drug resistance, particularly antibiotic resistance [46]. According to studies by Hoyle *et al*., bacteria in dispersed biofilms are 15 times more sensitive to the antibiotic tobramycin than bacteria in intact biofilms [47]. Numerous studies have revealed the ability of *Z. officinale* extract to inhibit biofilm formation in various bacterial strain isolates from clinical samples. [48-50]. According to Kim et al. [50], *Z. officinale* extract reduces the synthesis of exopolysaccharide in bacterial stains that develop biofilm. *H. pylori*, which forms biofilms, may play an essential role in antibiotic resistance in clinical settings. Additionally, while the fecal-oral pathway is thought to be the primary mode of transmission for *H. pylori*, there is evidence that biofilm-forming strains growing on surface-exposed water may provide another route for infection transmission [51]. Moreover, *H. pylori* can transform into dormant cells known as coccoid forms, for which much higher MICs of different antibiotics are required to achieve bactericidal action [52]. Coccoid development may further exacerbate MDR due to an ultrastructural alteration in the cell membrane and metabolic pathways that reduce antibiotic target exposure and antibiotic penetration. [53].

Table 5. Detection of biofilm 76 *H. pylori* isolates before and after treatment with Z. *officinale* and gentamicin.

Treatment		Number of bacterial isolates biofilm formation (%)	Degree (%)		
			Weak (%)	Moderate (%)	Strong (%)
Untreated	**0.0 µg/ml**	71(93.36)	23.93	60.54	15.48
Z. officinale	25 µg/ml	40(52.6)	30.0	45.0	25.0
	50 µg/ml	5(6.31)	0.0	40.0	60.0
Gentamicin	25 µg/ml	43(56.54)	25.57	44.17	30.22
	50 µg/ml	2(2.63)	0.0	100	0.0

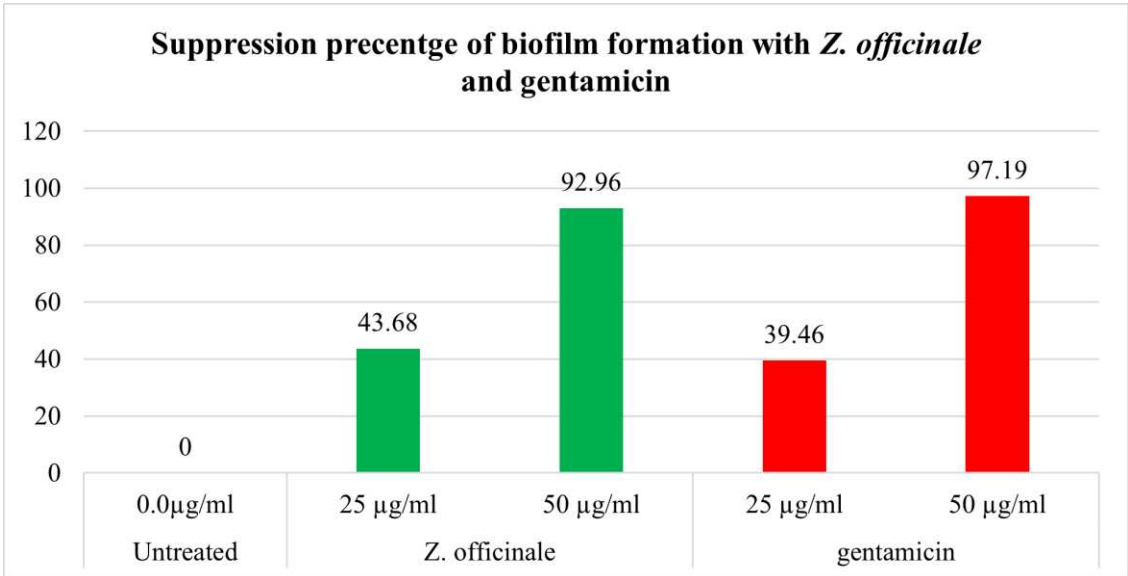

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Figure 2. Percentage of biofilm suppression with Z. *officinale* and gentamicin.

3.4. Anti-inflammatory activity of Z. officinale extract

The anti-inflammatory activity of *Z. officinale* extract was demonstrated in vitro, with the inhibition percentage of red blood cell membrane stabilization increasing from 49.83% to 61.47% at a concentration of 4 to 32µg/ml, comparable to 63.72% to 71.43% as an inhibition percentage of the positive control (sodium diclofenac) at the same concentration, as shown in Table 6. *Z. officinale* has long been used as an anti-inflammatory, and some of its constituents have been shown to have anti-inflammatory properties. [54]. Because of the limiting of cyclooxygenase and cyclooxygenase, *Z. officinale* extract can decrease prostaglandin formation and have the same pharmacological action as non-steroidal anti-inflammatory medications (NSAIDs) [55]. *Z. officinale* also possesses anti-inflammatory properties that help to reduce gingival bleeding [49]. Moreover, *Z. officinale* extract decreased the production of IL-1b, IL-6, IL-8, and TNF-a from LPS-stimulated human PBMCs in the fight against *H. pylori* infection. [56].

Table 6. Assessment of anti-inflammatory activity of *Z. officinale* extract.

Treatment	Concentration (µg/ml)	Absorbance 560 nm	Percentage inhibition
Control	0.0	1.246	0.0
Z. officinale	4	0.625	49.83%
	8	0.536	56.68%
	16	0.501	59.79%
	32	0.480	61.47%
Sodium diclofenac	4	0.452	63.72%
	8	0.405	67.49%
	16	0.384	69.18%
	32	0.357	71.43%

3.5. Chemical Composition of Z. officinale extract

Table 7 and Figure 2 indicated that GC-MS screening of *Z. officinale* methanolic extraction identified approximately 17 distinct chemicals. gingerol (45.05%), zingiberene (16.05%), and thymol (10.50%) were the primary principal chemicals. Elbashir et al. [57] revealed that gingerol is the primary principal detected component (43%) in the ethanolic extraction of *Z. officinale*, followed by zingiberene (14%). *Z. officinale* from the eastern portion of Nigeria had gingerol at peak 12 and ricinoleic acid towards the end. [58]. GC-MS screening of methanol extract of *Z. officinale* from India, detected zingiberene, AR-curcumene, α-bergamotene, gingerol, zingerone, caryophyllene and ç-elemene [59]. Gingerols extracted from *Z. officinale* suppress the development of *H. pylori* Cag A+ strains [21]. Geraniol, another active component in *Z. officinale*, has numerous pharmacological properties, including antibacterial action against *Helicobacter pylori*, anti-inflammatory, and anti-ulcer properties [40]. Gingerol inhibits tumor promotion in mouse skin, inhibits neoplastic transformation and AP-1 stimulation in mouse epidermal JB6 cells treated with epidermal growth factor, inhibits human cancer cell proliferation by inducing apoptosis, and prevents pulmonary metastasis in mice implanted with B16F10 melanoma cells [21].

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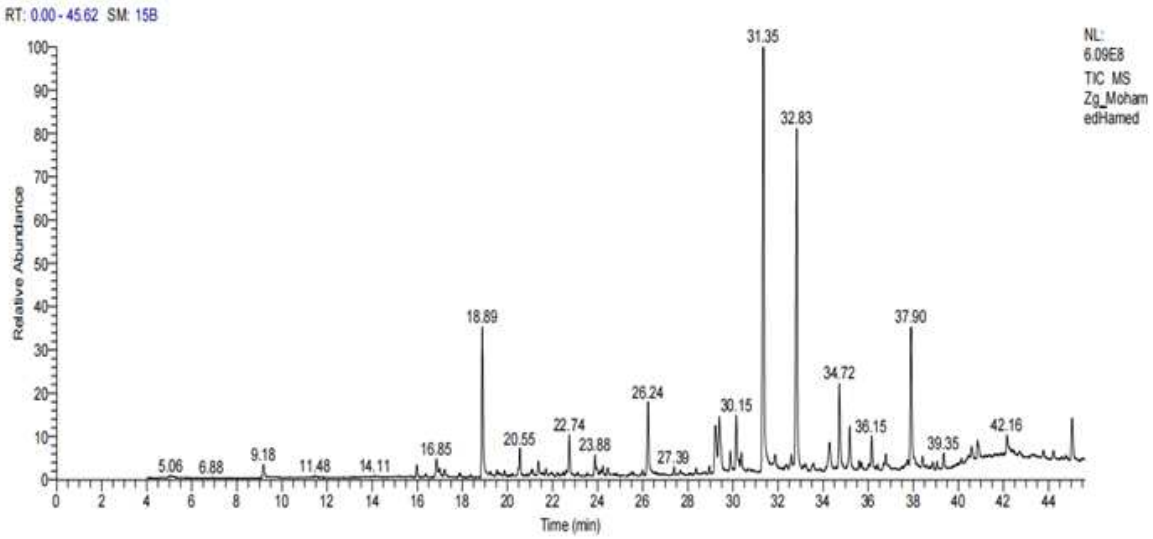

Figure 2. GC-MS analysis of *Z. officinale* methanolic extraction.

Table 7. Chemical profile of *Zingiber officinale* extract by GC-MS.

Peak	Rotation Time	Contents %	Compound name	Molecular Formula	Molecular weight
1	9.18	0.68	Decanal	$C_{10}H_{20}O$	156
2	16.85	0.98	Dodecanamine, N, N-dimethyl-	$C_{14}H_{31}N$	213
3	18.89	10.50	Thymol	$C_{10}H_{14}O$	150
4	20.55	3.26	Phenol, 2-methyl-5-(1-methylethyl)- Carvacrol	$C_{10}H_{14}O$	150
5	22.74	2.32	Eugenol	$C_{10}H_{12}O_2$	166
6	23.88	0.47	Alpha-terpineol	$C_{10}H_{20}O$	156
7	26.24	2.14	Butanedioic acid, 2,3-bis(acetyloxy)	$C_8H_{14}O_8$	182
8	27. 39	0.76	Benzenediamine, 2,5-dimethoxy-alpha	$C_{12}H_{17}NO_2$	207
8	30. 15	6.55	Shogaol	$C_{19}H_{28}O_3$	304
10	31.35	45.05	Gingerol	$C_{17}H_{28}O_4$	246
12	32.83	16.05	Zingiberene	$C_{15}H_{27}$	207
13	34.72	3.44	Beta-bisabolene	$C_{15}H_{22}$	200
14	36.15	2.26	E-11-hexadecenoic acid, ethyl ester	$C_{18}H_{34}O_2$	282
15	37.90	6.69	Cyclohexane, 3-(1,5-dimethyl-4-hexenyl)-	$C_{15}H_{24}$	204
16	39.35.	1.25	Farnesyl acetone	$C_{18}H_{27}O$	269
17	42.16	1.26	Desoxo-9x-hydroxy-7-ketoingol 3,8,9,12-tetraacetate	$C_{28}H_{38}O_{10}$	534

5. Conclusions

Based on our findings, *Z. officinale* exhibits potent antibacterial and antibiofilm properties against *H. pylori* resistance, as well as anti-inflammatory activity. As a result, we suggest utilizing *Z. officinale* extract to combat *H. pylori* infection by developing regimens for *H. pylori* eradication and inflammation.

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"The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of NAME OF INSTITUTE (protocol code XXX and date of approval)." for studies involving humans. OR "The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of NAME OF INSTITUTE (protocol code XXX and date of approval)." for studies involving animals. OR "Ethical review and approval were waived for this study due to REASON (please provide a detailed justification)." OR "Not applicable" for studies not involving humans or animals.

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