

## ORIGINAL RESEARCH ARTICLE

# Suppression of *Helicobacter pylori*-induced gastric carcinogenesis by emodin in GES-1 cells through the PI3K/AKT and NRF2 signaling pathways

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## Abstract

*Helicobacter pylori* is recognized as a microbial carcinogen among Gram-negative bacteria and is considered the most significant risk factor for the development of human gastric cancer (GS). Consequently, inhibiting the growth of *H. pylori* has become a critical strategy for preventing GS. This study focuses on the inhibitory effects of emodin (EMN) against *H. pylori*-induced gastric carcinogenic signaling in human gastric epithelial cells (GES-1). *In vitro* cytotoxicity assessments revealed that a concentration of 40  $\mu$ M of EMN provided remarkable protection to gastric cells, resulting in 85% cell viability without inducing toxicity. Furthermore, EMN prevented the *H. pylori*-induced depletion of antioxidants, which was mediated by reactive oxygen species generation, DNA damage, and nuclear fragmentation. Our findings indicate that EMN significantly suppresses the expression of phosphorylated forms of phosphatidylinositol 3-kinase (PI3K)/AKT, phosphorylated p38 kinases (p-p38), phosphorylated extracellular signal-regulated kinase-1 (p-ERK1), phosphorylated c-Jun N-terminal kinase (p-JNK) in GES-1 cells infected with *H. pylori*. In addition, EMN notably enhances the expression of antioxidant proteins nuclear factor erythroid factor-2 (NRF2) in *H. pylori*-infected cells. In summary, EMN demonstrates promising potential in preventing *H. pylori*-associated infections and the subsequent development of resistance, making it a viable candidate for the prevention of *H. pylori*-induced GS.

**Keywords:** Emodin; Gastric cancer; *Helicobacter pylori*; GES-1 cell line; Inflammation

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## 1. Introduction

Gastric cancer (GC) is one of the most prevalent cancer types globally, primarily triggered by microbial infections. In 2015, it was ranked as the third leading cause of cancer-related deaths. Several risk factors contribute to the development of GC.<sup>1</sup> Notably,

*Helicobacter pylori* stands out as a significant factor in the development of GC. Other causative agents contributing to heightened GC risks include Epstein–Barr virus infection, high-salt diets, low vegetable consumption, smoking, and severe gastritis with intestinal metaplasia.<sup>2</sup> *H. pylori*, a Gram-negative bacterium, has received extensive attention for its role in carcinogenesis when it infects gastric cells over an extended period.<sup>3</sup> Out of the one million registered GC patients, approximately 700,000 cases have been conclusively linked to *H. pylori* infection, with nearly 89% of these cases exhibiting clinical signs of inflammation resulting from *H. pylori* infection.<sup>4</sup> Therefore, mitigating *H. pylori* infection is deemed a vital and indispensable approach to preventing bacteria-induced GC in humans.

*H. pylori* infection triggers the production of reactive oxygen species (ROS), leading to detrimental consequences such as inflammatory responses and apoptosis, which contribute to severe stomach conditions such as gastric ulcers and gastric carcinoma.<sup>5</sup> Scientific evidence indicates that microbial pathogens induce oxidative stress by activating ROS-mediated mitogen-activated protein kinase (MAPK) family proteins such as extracellular signal-regulated kinase-1 (ERK), c-Jun N-terminal kinase (JNK), and p38 kinases.<sup>6</sup> *H. pylori* infection, in particular, activates these MAPK family proteins, playing a crucial role in epithelial damage and oxidative stress.<sup>7</sup> Nuclear factor erythroid-related factor 2 (NRF2) is a sensitive transcription factor that plays a pivotal role in safeguarding cells against oxidative stress by enhancing and regulating antioxidant enzymes.<sup>8</sup> NRF2 also regulates the suppression of MAPK family members, thereby impeding oxidative stress.<sup>9</sup>

While the eradication of pathogenic bacteria has reduced the risk of gastric-related PI3K/AKT pathway disorders, the development of antibiotic resistance among these pathogens poses a growing concern.<sup>10</sup> Consequently, the clinical efficacy of antibiotic-based treatments has been increasingly compromised.<sup>11</sup> In addition, the broad-spectrum antibacterial activity of antibiotics used for *H. pylori* eradication can harm the normal gut flora, leading to severe gastrointestinal side effects.<sup>12</sup> Therefore, identifying drug capable of targeting specific pathogens such as *H. pylori* while reducing the risk of infection is a paramount concern within the research community.<sup>13</sup> Anthraquinones are abundantly found in natural products and common in numerous organisms, such as bacteria, plants, fungi, and several other higher organisms. They are considered quinones, and their byproducts constitute a major group of natural benzoquinones.<sup>14</sup> Among the anthraquinones, aloe holds particularly effective properties. Aloe preparations, which consist of the solid filtrate from the transversely cut

plants of various Aloe species, especially *Aloe ferox* and *Aloe barbadensis*, are notable for their medicinal applications. Aloins A and B, the anthraquinone glycosides found in aloe, are responsible for their laxative effects.<sup>15</sup>

Emodin (EMN), a natural phytochemical belonging to the anthraquinone family, is derived from Chinese medicinal plants such as *Aloe vera* Berg, *Rheum palmatum*, and *Polygonum cuspidatum*.<sup>16</sup> It possesses beneficial properties for human health and exhibits potential antioxidant effects. In addition, EMN demonstrates various pharmacological activities, including anti-inflammatory, antitumoral, and antidiabetic properties.<sup>17</sup> Studies have reported that EMN inhibits the growth of several cancers, such as ovarian cancer, breast cancer, lung cancer, and leukemia.<sup>18–21</sup> Furthermore, EMN possesses growth inhibition properties against numerous types of tumor cells, acting on several cell lines through diverse mechanisms.<sup>22,23</sup> Cumulative clinical outcomes indicate that inflammation plays a significant role in the formation of cancer, particularly in the early phases. Nearly all tumors are triggered by inflammation, and it is recognized that inflammatory mediators can lead to cancer. Therefore, targeting the anti-inflammatory action of EMN might be crucial in cancer management. Previous studies confirm the anti-inflammatory action of EMN, showing that it regulates cell growth and proliferation through the downregulation of crucial oncogenic growth signaling.<sup>23–25</sup> However, the impact of EMN treatment on *H. pylori* infection-mediated gastric injury, gastric carcinogenesis, and associated signaling events remains unexplored. In this study, we aimed to elucidate the inhibitory effect of EMN on *H. pylori* infection-induced gastric carcinogenic signaling. Our investigation focused on targeting oxidative stress, inflammation, and the PI3K/AKT and NRF2 signaling pathways in gastric epithelial cells (GES-1) cells.

## 2. Materials and methods

### 2.1. Chemicals, reagents, and antibodies

Emodin, Dulbecco's modified eagles medium (DMEM), 2,7-diacetyl dichlorofluorescein (DCFH-DA), Hoechst, 2,5-diphenyltetrazolium bromide (MTT), and ethidium bromide were purchased from Sigma-Aldrich (USA). Primary monoclonal antibodies such as phosphorylated JNK (p-JNK), phosphorylated PI3K (p-PI3K), AKT, phosphorylated extracellular signal-regulated kinase-1 (p-ERK1), NRF2, and phosphorylated p38 kinases (p-p38) were obtained from Cell Signaling and Technology (Haryana, India). All other reagents, solvents, and fine chemicals used in this study were of molecular grade.

## 2.2. Cell lines and *H. pylori* infection

GES-1 cell lines, chosen for the induction of *H. pylori* infection-mediated gastric damage, were purchased from the American Type Culture Collection (USA). These cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 U/mL penicillin and incubated at 37°C in a 5% CO<sub>2</sub> environment. The *H. pylori* bacterial strain was cultured on Campylobacter-selective agar plates containing 10% goat serum and incubated at 37°C under sterile conditions for 24 h. Bacterial density was determined by measuring the optical density using a spectrophotometer. To establish co-cultures, GES-1 gastric cells were exposed to *H. pylori* at varying multiplicities of infection.

## 2.3. Cell viability assay

The cytotoxic potential of EMN at various concentrations in *H. pylori*-induced gastric cells was assessed using a colorimetric-based MTT cytotoxic assay.<sup>26</sup> GES-1 cells infected with *H. pylori* were uniformly seeded into all 96-well plates and allowed to grow for 24 h. Subsequently, the cells were treated with different concentrations of EDN (10 – 100 µM) and incubated for an additional 24 h in a CO<sub>2</sub> incubator. Cytotoxicity levels were determined using the standard protocol.<sup>27</sup> The absorbance of the reaction mixture was measured at 540 nm using a UV spectrophotometer (Tecon, Infinite M200, Switzerland).

## 2.4. DCFH-DA staining

ROS production and fluorescence intensity were determined using the DCFH-DA staining method.<sup>28</sup> GES-1 cells infected with *H. pylori* were uniformly seeded into 6-well plates and allowed to grow for 24 h. Subsequently, the cells were treated with EMN for 24 h, and DCFH-DA was added to all the wells. The images were captured by a fluorescence microscope, and the fluorescence intensity was measured using a multimode reader (Tecon, Infinite M200, Switzerland).

## 2.5. Hoechst staining for nuclear fragmentation analysis

The assessment of apoptotic nuclear fragmentation in GES-1 cells involved Hoechst staining and subsequent microscopic examination following a previously described methodology.<sup>29</sup>

## 2.6. Comet assay

The alkaline-based comet assay, also known as single-cell gel electrophoresis, was employed to detect EDN-induced oxidative DNA damage in *H. pylori*-exposed cells, following the methodology described by Balupillai *et al.*<sup>27</sup> Comet attributes were analyzed using Comet Assay Software Programming (CASP Lab Software 112, Total Lab, UK).

## 2.7. Protein extraction and western blotting

Total cell lysates were prepared using RIPA lysis buffer, and the protein concentration was quantified using the standard bicinchoninic acid method. Twenty micrograms of protein extracts were loaded into 8 – 12% SDS-PAGE gels (HiMedia, Mumbai, India), separated, and subsequently transferred to PVDF protein transfer membranes (Sigma, Mumbai, India).<sup>27</sup> The membranes were blocked for 1 h at a cold temperature, followed by rinsing with TBST (HiMedia, Mumbai, India). Primary antibodies, including tumor necrosis factor - $\alpha$  (1:200), COX-2 (1:1000), p-PI3K (1:1500), IL-6 (1:2000), AKT (1:1000), PTEN (1:2000), p-ERK1 (1:1000), Nrf2 (1:2000), p-JNK (1:1000), and p-p38 (1:1000), were applied at recommended dilutions and incubated overnight. Following another round of TBST washes, the membranes were exposed to horseradish peroxidase-conjugated secondary antibodies for 45 min. PVDF membranes were washed again with TBST, and protein band expression was visualized by adding a chemiluminescence (ECL) substrate (Protech Consultants, Chennai, India).

## 2.8. Real-time polymerase chain reaction

Total RNA extraction from GES-1 cells was carried out using the TRIZOL reagent (Invitrogen, USA) following the manufacturer's guidelines. The obtained RNA showed an A260/280 ratio ranging from 1.8 to 2.0, as assessed using the NanoDrop2000 spectrophotometer (Thermo Fisher, USA). For quantitative real-time polymerase chain reaction (qRT-PCR), the Stratagene Mx 3000PTM qPCR system (Stratagene, USA) was utilized. The qRT-PCR assay employed the SYBR RT-PCR kit from Takara Bio (Takara Biomedical Corporation, Beijing, China). The thermal cycling protocol consisted of an initial step at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 6 s. Subsequently, a melting curve analysis was performed with temperature settings of 95°C for 1 min, 55°C for 30 s, and a final step at 95°C for 30 s.

## 2.9. Statistical analysis

Statistical analysis was performed using SPSS 17 software (SPSS Incorporation, Chicago, USA). Data were presented as mean  $\pm$  SD. The analysis included analysis of variance and Duncan's multiple range test to compare variations and differences among treatment groups. Statistical significance was established when the  $P < 0.05$ .

## 3. Results

### 3.1. Cytotoxic effects of emodin on GES-1 cells

We assessed the cytotoxic potential of EMN against GES-1 cells using the MTT assay after 24 h of incubation. [Figure 1A](#) demonstrates that concentrations of EMN ranging

from 10 to 50  $\mu\text{M}$  did not significantly affect the viability of GES-1 cells. That is, [Figure 1B](#) control group cells depict normal structured morphology of GES-1 cells. And *H. pylori* alone group cells exhibit decreased cell number, cytotoxic round appearance, shrunk and expanded gap among the cellular space, this assures the pylori infection harm the GES-1 cells. While cells cultivated with the *H. pylori* along with EMN 40 $\mu\text{M}$  group showed some positive effect where the cell number increased with regular morphology and the cell burst is controlled. At the same time alone group showed near normal structural morphology in comparison with normal control GES-1 cells. [Figure 1C](#) shows the graphical bar diagram representation of EMN on *H. pylori* infection-mediated cytotoxicity in GES-1 cells. Data were presented as means  $\pm$  SD from three experiments in each group. This outcome signifies that EMN 40 $\mu\text{M}$  do not damage the GES-1 cells. Microscopic outcomes remarkably demonstrates that GES-1 cells exposed to *H. pylori* became irregular in dimensions, attained an uneven shape and exhibited shrinkage of GES-1 cells. Conversely, treatment with EMN

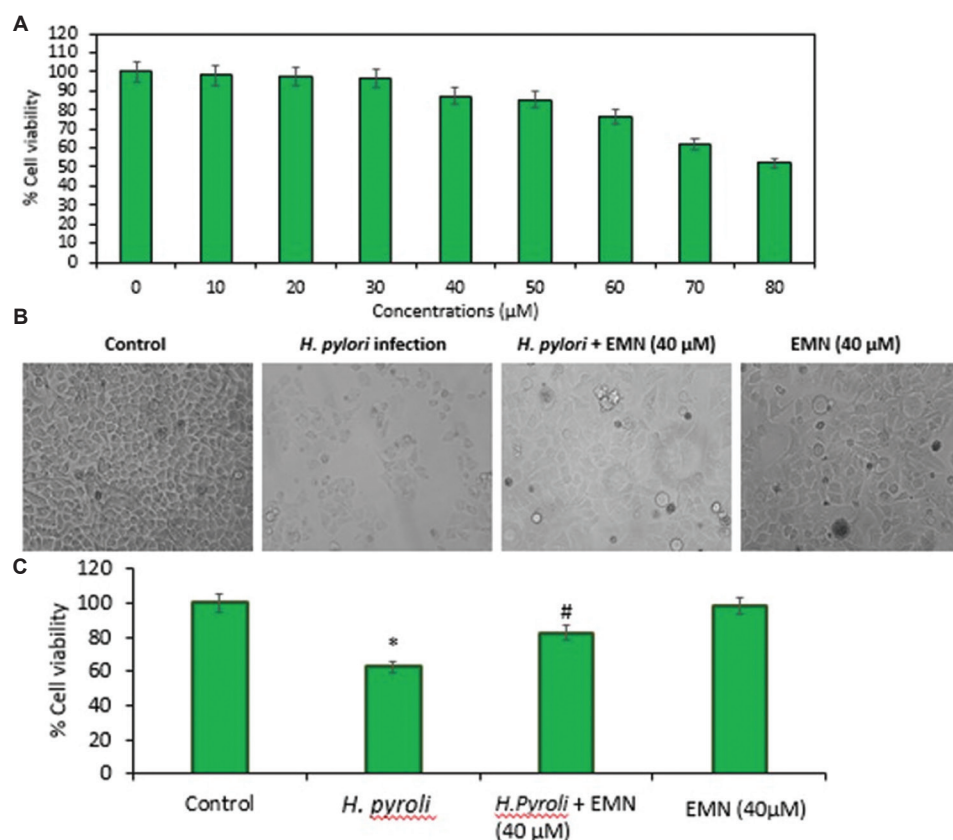
at 40  $\mu\text{M}$  significantly protected GES-1 cells from *H. pylori*-induced damage. Consequently, we selected 40  $\mu\text{M}$  EMN for subsequent experiments, as it effectively inhibited *H. pylori* growth and maintained cell viability of 85% in GES-1 cells.

### 3.2. Reduction of *H. pylori*-induced ROS levels by emodin in GES-1 cells

We utilized the fluorescent probe DCFH-DA to detect EMN and/or *H. pylori*-mediated ROS production in GES-1 gastric cells ([Figure 2](#)). Our findings revealed that *H. pylori* co-cultured with GES-1 cells exhibited significantly higher ROS production compared to GES-1 cells in the control group. In contrast, treatment with EMN at 40  $\mu\text{M}$  significantly reduced ROS production induced by *H. pylori* in GES-1 cells.

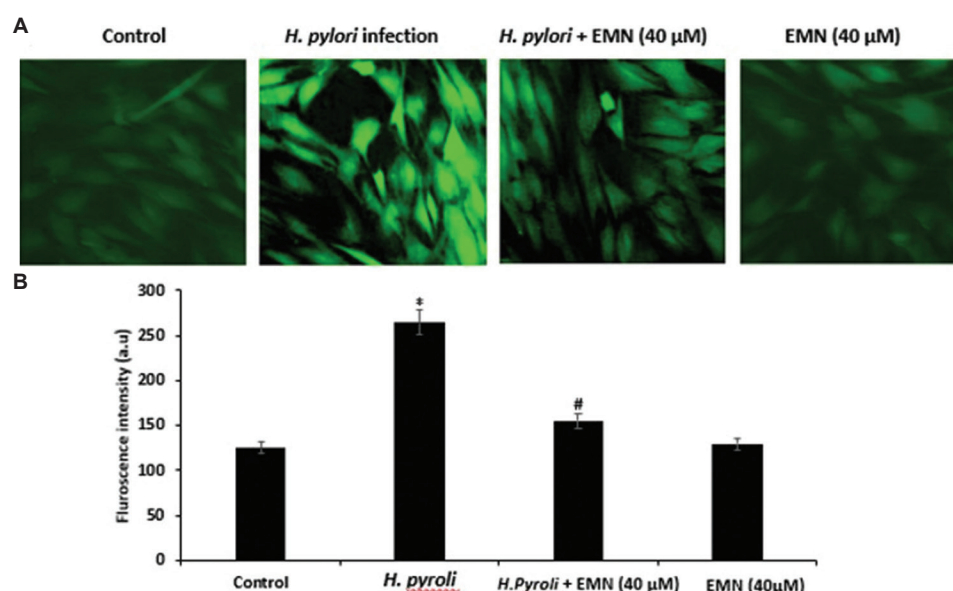
### 3.3. Mitigation of *H. pylori*-induced DNA damage and nuclear fragmentation by emodin

The impact of EMN on DNA damage induced by *H. pylori* was assessed using the comet assay. We observed increased



**Figure 1.** MTT assay was employed to determine the non-toxic concentration of emodin (EMN). (A) The effects of EMN and its non-toxic concentration on GES-1 cells without *Helicobacter pylori* infection. (B) Microscopic images depicting the impact of EMN on *H. pylori*-induced cytotoxicity in GES-1 cells. Magnification:  $\times 40$ . (C) The effects of EMN on *H. pylori* infection-mediated cytotoxicity in GES-1 cells. Data were presented as means  $\pm$  SD from three experiments in each group. Values marked with asterisks (\*) and hashtags (#) indicate significant differences against control at  $P \leq 0.05$  in the groups of *H. pylori* and *H. pylori* + EMN, respectively.





**Figure 2.** The impact of emodin (EMN) on intracellular reactive oxygen species (ROS) generation was assessed through DCFH-DA staining in *Helicobacter pylori*-infected GES-1 cells. (A) ROS generation was observed using a fluorescence microscope (Nikon, Japan) with a green lamp. Magnification:  $\times 40$ . (B) The bar diagram illustrates fluorescence intensity, measured with excitation and emission wavelengths set at  $485 \pm 10$  and  $530 \pm 12.5$  nm, respectively, using a spectrofluorimeter (Shimadzu, Kelvin Labs, Kerala). Values marked with asterisks (\*) and hashtags (#) indicate significant differences against control at  $P \leq 0.05$  in the groups of *H. pylori* and *H. pylori* + EMN, respectively.

DNA damage in cells infected with *H. pylori*, as indicated by a higher amount of tail DNA (Figure 3A). EMN treatment effectively reduced tail DNA levels in *H. pylori*-infected GES-1 cells. No changes were observed in the control or EMN-only groups. In addition, we conducted Hoechst staining to evaluate the effect of EMN on *H. pylori*-induced nuclear fragmentation, a characteristic feature of apoptosis. Figure 3B illustrates the percentage of DNA damage was calculated using CASP online software, which was depicted as bar graphical representation. As shown in Figure 3C, GES-1 cells infected with *H. pylori* displayed increased nuclear fragmentation and higher Hoechst staining intensity in the nucleus. However, EMN treatment reduced nuclear fragmentation in GES-1 cells co-cultured with *H. pylori*.

#### 3.4. Modulation of MAPK and NRF2 protein expressions by emodin in *H. pylori*-infected GES-1 cells

*H. pylori* infection has been associated with the overexpression of oxidative stress proteins such as ERK1, JNK, and p38 kinases, contributing to gastric carcinogenesis. In this study, *H. pylori* infection of GES-1 cells led to increased ERK1, JNK, and p38 kinase protein phosphorylation (Figure 4). Notably, treatment with EMN inhibited the *H. pylori*-mediated phosphorylation of these proteins in GES-1 cells. Furthermore, *H. pylori*-infected cells exhibited decreased expression of NRF2,

while EMN treatment restored NRF2 levels in *H. pylori* co-cultured GES-1 cells.

#### 3.5. Modulation of PI3K/AKT signaling proteins by emodin in *H. pylori*-infected GES-1 cells

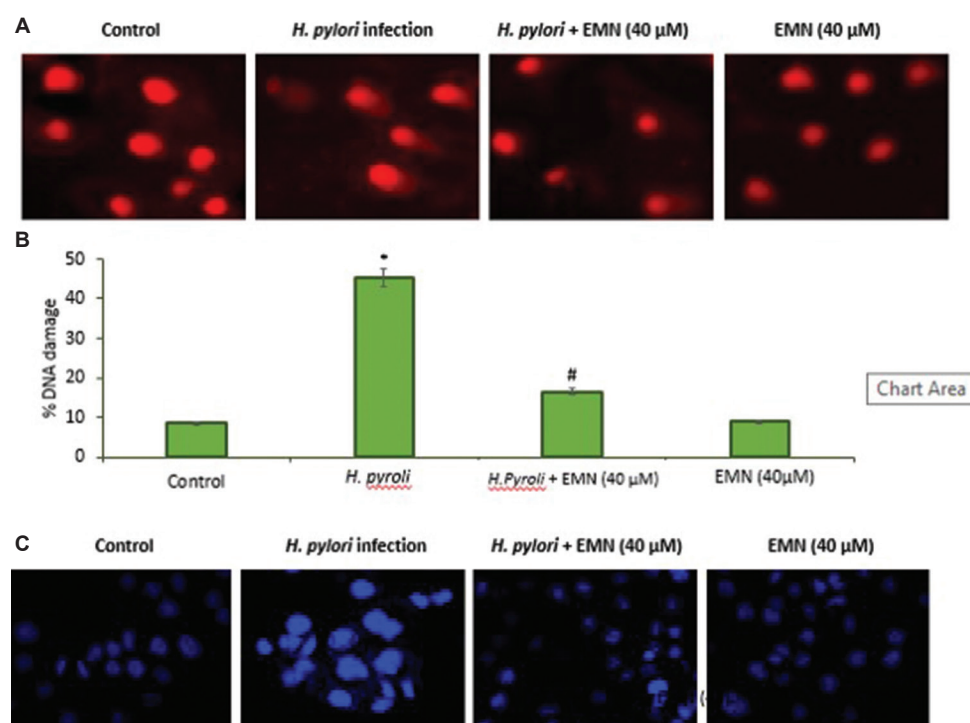
We quantitatively analyzed the protein expression levels of p-PI3K, AKT, and PTEN using Western blot analysis (Figure 5). Co-culture of *H. pylori* with GES-1 cells led to increased protein expression of p-PI3K and AKT. However, treatment with EMN reduced the protein expression of p-PI3K and AKT in *H. pylori*-infected cells.

#### 3.6. Prevention of apoptosis by emodin in *H. pylori*-infected GES-1 cells

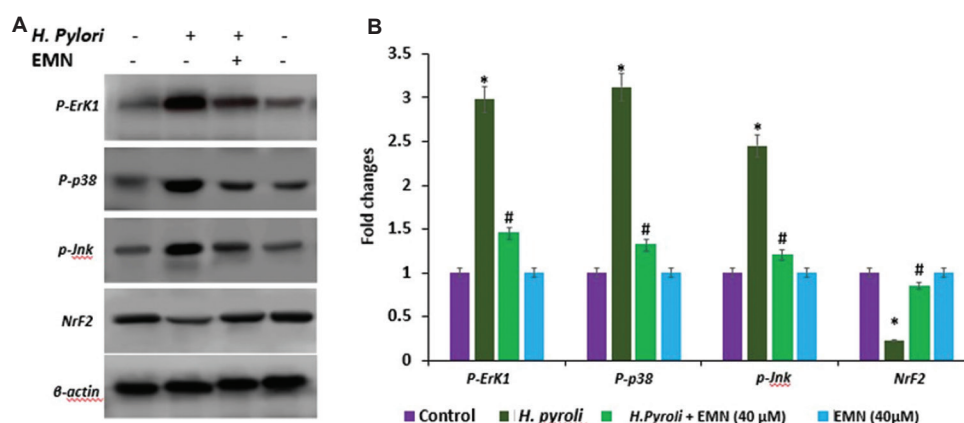
Oxidative stress and PI3K/AKT signaling induced by *H. pylori* have been associated with apoptotic events in cells. We assessed the mRNA expression of apoptotic markers, including BAX, BCL-2, and caspase-3, using qRT-PCR (Figure 6). GES-1 cells infected with *H. pylori* exhibited overexpression of BAX and caspase-3 and downregulation of BCL-2, leading to apoptosis. However, EMN treatment effectively modulated these expressions in *H. pylori*-infected GES-1 cells.

## 4. Discussion

Cancer in the gastrointestinal tract represents one of the most prevalent types of cancer. It is both a hereditary and



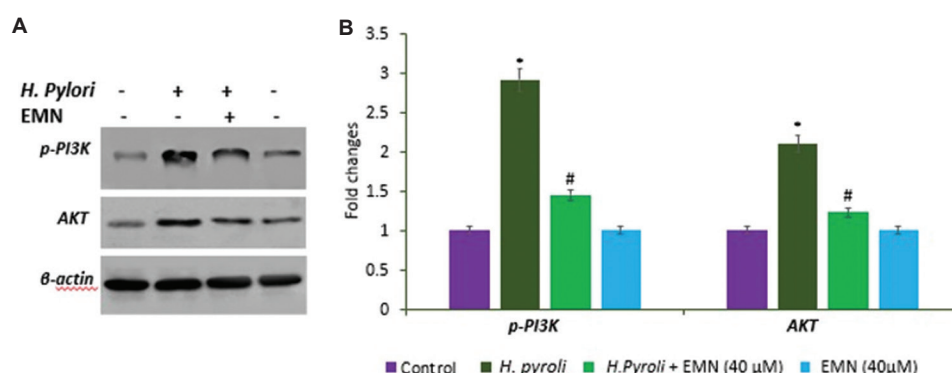
**Figure 3.** Emodin (EMN) protects GES-1 cells from *Helicobacter pylori*-induced oxidative DNA damage and nuclear fragmentation. (A) Microscopic images depict the impact of EMN on *H. pylori*-induced DNA damage, analyzed through the comet assay. (B) The percentage of DNA damage was calculated using CASP online software. Values marked with asterisks (\*) and hashtags (#) indicate significant differences against control at  $P \leq 0.05$  in the groups of *H. pylori* and *H. pylori* + EMN, respectively. (C) The assessment of the influence of EMN on *H. pylori*-mediated nuclear fragmentation (red arrows) was conducted in GES-1 cells stained with Hoechst staining.



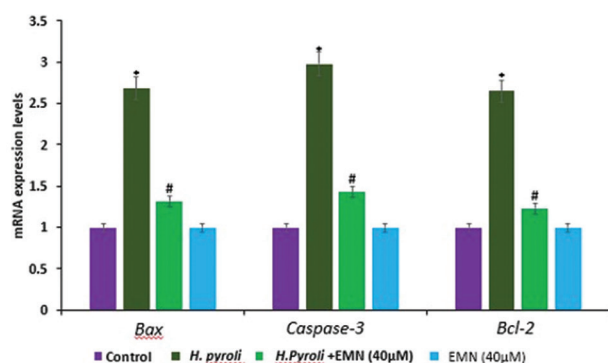
**Figure 4.** Emodin (EMN) regulates MAPK and NRF2 proteins in *Helicobacter pylori*-infected GES-1 cells. (A) Western blot analysis of MAPK protein expression levels (p-ERK, p-JNK, p-p38) and NRF2 in EMN-treated *H. pylori*-infected GES-1 cells;  $\beta$ -actin served as the loading control to ensure equal loading of proteins. (B) Quantification and densitometric analysis of MAPK family proteins and NRF2 were conducted using Image J software, with  $\beta$ -actin as the loading control for normalization. Graphical representations show means  $\pm$  SD from three separate immunoblots. Values marked with asterisks (\*) and hashtags (#) indicate significant differences against control at  $P \leq 0.05$  (Duncan's multiple range test [DMRT]) in the groups of *H. pylori* and *H. pylori* + EMN, respectively.

multifactorial disease influenced by host genetics and environmental factors. It has been estimated that 20% of cancers are triggered by infectious agents, including *H. pylori*, human papillomavirus, and hepatitis B and C

viruses. In addition to these recognized cancer-associated infectious agents, differences in microbiota composition between healthy and cancer patients have been observed. Several studies have proposed that specific microorganisms



**Figure 5.** Emodin (EMN)'s impact on p-PI3K and AKT signaling in *Helicobacter pylori*-infected GES-1 cells. (A) Western blot analysis of cell survival protein expression levels (p-PI3K and AKT) in EMN-treated *H. pylori*-infected GES-1 cells; β-actin was used as the loading control to ensure consistent protein amounts. (B) Quantification and densitometric analysis of the proteins of interest were conducted using Image J software. Graphical representations depict means  $\pm$  SD from three separate immunoblots. Values marked with asterisks (\*) and hashtags (#) indicate significant differences against control at  $P \leq 0.05$  (Duncan's multiple range test [DMRT]) in the groups of *H. pylori* and *H. pylori* + EMN, respectively.



**Figure 6.** Emodin (EMN) suppresses the expression of proapoptotic genes in *Helicobacter pylori*-infected GES-1 cells. real-time polymerase chain reaction analysis of apoptotic gene expression (BAX, BCL-2, and caspase-3) in EMN-treated *H. pylori*-infected GES-1 cells; GAPDH served as the loading control to ensure consistent mRNA amounts. Graphical representations show means  $\pm$  SD from three separate experiments. Values marked with asterisks (\*) and hashtags (#) indicate significant differences against at  $P \leq 0.05$  (Duncan's multiple range test [DMRT]) in the groups of *H. pylori* and *H. pylori* + EMN, respectively.

and their associated metabolites can either promote or prevent tumorigenesis through mechanisms that are not yet fully understood. Recent findings have shown that gut microbiota and associated secondary metabolites can function as both cancer inhibitors and promoters. Previous research highlights that dysregulation of non-coding RNA expression by gut microbiota is implicated in gastrointestinal cancer.<sup>30</sup> *H. pylori* infection is a significant risk factor for gastric injuries and carcinogenesis. Conventionally, antibiotic-based treatments have been the primary approach for combating *H. pylori* infections. However, this approach faces challenges due to the emergence of antibiotic-resistant bacteria, leading to the risk of reinfection.<sup>3</sup> Furthermore, antibiotics can

be prohibitively expensive, making effective treatment inaccessible for many individuals. In response to these challenges, non-antibiotic-based treatments, such as plant-based phytochemicals and antioxidant-rich compounds, have gained considerable attention for developing natural remedies against *H. pylori* infections. This study explores the potential of EMN, an active flavonoid, in mitigating *H. pylori*-induced gastric carcinogenesis in GES-1 cells by focusing on oxidative stress, the PI3K/AKT pathway, and inflammatory events.

The involvement of *H. pylori* in GS is a well-known example of a microbial infection linked to cancer in the human gastrointestinal tract. In the 1990s, these flagellated Gram-negative bacteria were categorized as a group I carcinogen by the International Agency for Cancer Research (IARC).<sup>31</sup> *H. pylori* plays a prominent role in GS development through three main mechanisms. First, *H. pylori* introduces two cytotoxins, CagA and VacA, into the host, triggering oncogenic signal transduction pathways.<sup>32,33</sup> Second, it induces the formation of ROS, which further stimulates inflammatory pathways. This process ultimately results in atrophic gastritis, characterized by the destruction of the acid-producing parietal cells. Consequently, there is a compensatory increase in gastrin, which not only creates a more acidic environment but also initiates oncogenic signals.<sup>34</sup> In this study, *H. pylori*-infected GES-1 cells were employed to assess the cytoprotective effects of EMN, evaluated using the MTT toxicity assay. The results clearly demonstrate that EMN, particularly at a concentration of 40 μM, significantly enhanced viability in *H. pylori*-infected GES-1 cells. These findings suggest that EMN may serve as an effective cytoprotectant against *H. pylori*-induced injury in GES-1 cells. The presence of *H. pylori* infection leads to a substantial increase in the production of

ROS and reactive nitrogen species (RNS) within the gastric mucosa. ROS-mediated oxidative imbalance is associated with various gastrointestinal pathologies.<sup>6</sup> In this study, the infection of GES-1 cells with *H. pylori* resulted in elevated ROS levels, which in turn led to increased nuclear fragmentation and DNA damage. *H. pylori*-induced DNA damage and nuclear fragmentation are manifestations of oxidative damage in normal gastric cells. EMN, a flavonoid known for its ROS scavenging properties, effectively protected *H. pylori*-infected GES-1 cells against nuclear fragmentation and DNA damage. Flavonoids, in general, are potent antioxidants with documented roles in anti-inflammatory and anti-carcinogenesis.<sup>35</sup> In addition, astaxanthin, another flavonoid, has been reported to inhibit *H. pylori* growth, thereby mitigating nuclear ROS-dependent nuclear fragmentation and DNA damage in infected cells,<sup>36</sup> supporting our present findings. Previous research on flavonoids from *Rosa roxburghii* Tratt, another ROS scavenger, also demonstrated their ability to inhibit ROS-dependent DNA damage.<sup>37</sup>

Gastric injuries and the development of gastric tumors have been linked to the overexpression of oxidative stress proteins such as ERK1, JNK, and p38 kinases.<sup>38</sup> These MAPKs and their associated proteins are activated in response to excessive intracellular ROS generation and depletion of antioxidant proteins.<sup>39</sup> Under normal physiological conditions, NRF2 plays a crucial role in maintaining redox homeostasis and regulating cell growth by inhibiting the MAPK family and boosting antioxidants.<sup>40</sup> Consequently, NRF2 signaling represents a novel target for preventing carcinogenesis. In this study, *H. pylori* infection led to a significant increase in ROS production, resulting in the phosphorylation of proteins such as ERK1, JNK, and p38 kinases in GES-1 cells. Treatment with EMN effectively inhibited the phosphorylation of these proteins and promoted NRF2 overexpression. Previous research has shown that EMN can block the phosphorylation of ERK1/2, JNK, and p38 kinases in non-small cell lung cancer.<sup>41</sup> *H. pylori*-induced ROS-dependent MAPK overexpression can be neutralized by NRF2, a crucial antioxidant protein. Recent studies have documented the ability of p-coumaric acid to neutralize oxidative MAPK signaling and prevent antioxidant depletion induced by alcohol exposure through the upregulation of apoptosis and NRF2 signaling.<sup>42</sup>

The PI3K/AKT pathway plays a critical role in regulating cell proliferation, metabolism, differentiation, and gastric tumor formation. Activation of PI3K and AKT proteins by extracellular signals is a key step in this pathway. Scientific evidence has implicated the PI3K/AKT pathway in the

development of gastric carcinogenesis during *H. pylori* infection.<sup>43</sup> In addition, ROS have been demonstrated to activate PI3K and its downstream elements. In this study, the co-culture of *H. pylori* with GES-1 cells resulted in increased levels of phosphorylated PI3K and AKT protein expression. However, treatment with EMN effectively reduced phosphorylated PI3K and AKT levels in *H. pylori*-infected GES-1 cells. Scutellarin, a flavonoid, has also been shown to lower PI3K/AKT expression and regulate PTEN expression in GS cells.<sup>44</sup>

Apoptosis, or programmed cell death, is a crucial form of cell death widely observed in nature, characterized by distinct morphological and molecular features. Programmed cell death plays a vital role in regulating the number of epithelial cells in the gastrointestinal tract.<sup>45</sup> Furthermore, dysregulation of the apoptotic pathway is implicated in various intestinal diseases, including carcinogenesis. *H. pylori* infection contributes to the dysregulation of apoptosis, leading to gastric carcinogenesis.<sup>46</sup> GES-1 cells infected with *H. pylori* display an overexpression of BAX and caspase-3, along with a downregulation of BCL-2, resulting in apoptosis. However, EMN treatment effectively modulates these expressions in *H. pylori*-infected GES-1 cells. Overall, *H. pylori* infection leads to a reduction in antioxidant enzymes, resulting in increased MAPK expression and PI3K signaling, ultimately contributing to gastric carcinogenesis. NRF2, a major antioxidant protein, plays a vital role in scavenging radicals and stabilizing genes related to antioxidants when antioxidants are depleted. In this study, EMN demonstrated its potential as a potent antioxidant by inhibiting ROS-mediated MAPK families and PI3K/AKT genes in *H. pylori*-infected GES-1 cells. The inhibitory effect of EMN on oxidative stress and cell survival is associated with the overexpression of the antioxidant protein NRF2. With the global burden of GSs, treatment management targeting multiple characteristics of tumorigenesis or increasing the efficiency of presently permitted drugs is frequently required. Across the management of numerous pathways, plant-based agents have been recommended to provoke their tumoricidal effects without showing the high toxicity displayed by outdated chemotherapeutics.<sup>47</sup> EMN is a potential substitute anticancer drug that has shown effectiveness in pre-clinical representations of liver, pancreatic, and colon cancers. Based on this evidence, we conclude that EMN at a concentration of 40  $\mu$ M exhibits anti-cancer efficiency in GES-1 cells, suggesting potential dosages for human trials. At a higher pre-clinical dose range of 50 – 80 mg/kg,<sup>48-51</sup> the equivalent dose for humans is approximately 4 – 6.5 mg/kg,<sup>52</sup> which is significantly higher than the dosage used in our study. These findings



from our study underscore the potential clinical significance of EMN.

## 5. Conclusion

This study highlights the potential of EMN, a natural flavonoid, in inhibiting ROS, DNA damage, nuclear damage, MAPKs, and PI3K/AKT pathway in *H. pylori*-infected GES-1 cells. Furthermore, EMN enhances the expression of NRF2 protein, effectively inhibiting oxidative stress, induction of apoptosis, and gastric carcinogenic signaling. EMN holds promise as a potential drug for the treatment of *H. pylori*-induced gastric disorders.

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## Conflict of interest

The authors declare that they have no competing interests.

## Author contributions

*Conceptualization:* Palanimuthu Duraisamy

*Formal analysis:* Palanimuthu Duraisamy

*Investigation:* Karthik Mohan

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## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Availability of data

The data used in this work can be made available to readers upon reasonable request to the corresponding author.

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