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ORIGINAL ARTICLE

The effect of snakehead fish extract supplementation to first-line eradication regimen on macrophage migration inhibitory factor (MIF) expression in rats induced by *Helicobacter pylori* infection

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ABSTRACT

Objective: This work was organized to assess macrophage migration inhibitory factor (MIF) expression in snakehead fish extract supplementation to first-line eradication regimen in rats induced by *Helicobacter pylori* infection.

Materials and methods: A total of 28 manly rats were haphazardly isolated equally into four groups. Group-1 was the control negative, and groups-2–4 were *H. pylori*-infected groups. Group-2 was the control positive. Groups-3 and 4 were treated with first-line eradication regimen and first-line eradication regimen supplemented with snakehead fish extract, respectively. Immunoreactive scores (IRS) of MIF expression and eradication testing procedure were carried out. The comparison and difference between groups were analyzed by Kruskal–Wallis and *post hoc* Mann–Whitney U-test. A value of $p < 0.05$ was considered to be a limit of significance.

Results: The average IRS of MIF expression in group-2 was the highest among other groups ($p < 0.05$). Group-4 (supplemented by snakehead fish extract) had a lower median value IRS of MIF expression compared to group-3 [1.0 (0.0–2.0) *vs.* 3.5 (2.0–6.0), $p = 0.004$].

Conclusion: MIF expression was higher in rats induced by *H. pylori* infection. Snakehead fish extract supplementation to first-line eradication regimen significantly reduces more MIF expression compared to a single administration of first-line eradication regimen in rats induced by *H. pylori* infection.

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First-line eradication regimen; *Helicobacter pylori* infection; macrophage migration inhibitory factor expression; snakehead fish extract

Introduction

It is estimated currently that most of the world's populace stomach colonized and contaminated by *Helicobacter pylori* [1]. *H. pylori* is a microaerophilic microorganism that is pathologically found within the gastric mucosa or attached to the gastric epithelium. As one of the most prevalent human pathogens, *H. pylori* is clinically linked with gastritis, gastritis ulcer, and gastric malignancy, especially to developing countries, at an average prevalence of around 80% [2].

Virulence factors, inflammatory response, releasing of chemokines, cytokines, and reactive oxygen species (ROS) due to *H. pylori* infection play a role in the formation of gastric pathological lesions above [3]. Virulence factors, as destructive components, including cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), lipopolysaccharides, ureases, and flagella induce the encourage of proinflammatory cytokines within the gastric mucosal to complicated long-term immunoinflammatory responses in producing cytotoxic molecules that lead to gastric mucosal damage and heading to gastric malignancy [4]. *H. pylori* generates an innate immune response that implicates many types of innate immune system constituents. This innate immune response proceeds inflammation to gastric

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mucosa and causes an influent of neutrophils and distinct immune cells through the liberation of various cytokines and chemokines [5].

Macrophage migration inhibitory factor (MIF) constitutes notable cytokine to regulate immune reaction, inflammatory progression, and immune system-mediated diseases such as autoimmune diabetes mellitus, asthma, rheumatoid arthritis, atherosclerosis, and several diseases in the gastrointestinal system [6]. MIF enhances the inflammatory reaction by provoking the expression of other proinflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), IL-8, IL-6, and interferon-gamma (IFN-γ) through attachment to its natural receptor, cluster-differentiated 74 (CD74) [7].

It was stated in a study that the increase of MIF expression in the gastrointestinal system is closely related to the occurrence of gastritis, gastritis ulcer, gastric malignancy, ulcerative colitis, and colon cancer [8]. MIF is generated by stress, endotoxins, exotoxins, and several bacterial infections, including *H. pylori* infection [9]. A study in Turkey found that MIF levels increased during *H. pylori* infection and decreased significantly after eradication with a first-line eradication regimen [10].

Nowadays, proton-pump inhibitor (PPI)-based triple therapy is a first-line regimen in the eradication of *H. pylori* worldwide [11]. The effectiveness of first-line eradication regimen is felt to be less than optimal regarding the problem of patient's immune, patient compliance, nutritional status, and antibacterial resistance issue in certain regions [12]. Besides, the long-term inflammation caused by *H. pylori* infection also becomes the focus of attention [13]. Therefore, the efforts in finding agents or drugs that come from nature as a supplementation to first-line eradication regimen continue to be pursued to overcome those problems.

Snakehead fish, known by several names such as *Channa striata*, *Ophicephalus striatus*, Chevron snakehead, and Stripped snakehead, is a medicinal food that is widely consumed by people in Asia-Pacific, especially Indonesia. Snakehead fish in Indonesian called as "ikan gabus," has been known to have properties in accelerating the recovery process after suffering an illness, healing wounds in post-surgery condition and to mother after childbirth, reducing pain, antipyretic, treatment of several skin disorders, and anti-inflammatory [14].

Several studies have documented the benefits of snakehead fish extract in medicine. The usability of snakehead fish extract as an anti-inflammatory has been investigated by Dwijayanti et al. [15]. Other researchers have found the potential effect of snakehead fish extract as an antibacterial and antifungal in a few *in vitro* studies, such as those studied by Kumar et al. [16], Dhanaraj et al. [17], Zulaikha et al. [18], and Andini et al. [19]. Furthermore, a work noted that the supplementation of snakehead fish extract capsules to antituberculosis drugs in pulmonary tuberculosis patients decreases the proinflammatory cytokine levels significantly [20].

Concerning to the medicinal properties of snakehead fish extract, we were interested to examine its beneficial effect, supplemented to first-line eradication regimen, in lowering gastric inflammatory by investigating MIF expression in rats induced by *H. pylori* infection. Based on the literature research, there are still very few studies published regarding the involvement of animal extract in the management of *H. pylori* infection. This can be the primary study to examine the medicinal effect of snakehead fish extract supplementation to the first-line *H. pylori* eradication regimen addressing in the lowering of MIF expression. The existence of this study is expected to be able to contribute to science, knowledge, and complement previous research associated with the management of *H. pylori* infection.

The purpose of this work was to assess MIF expression in snakehead fish extract supplementation to *H. pylori* first-line eradication regimen in rats induced by *H. pylori* infection.

Materials and Methods

Ethical approval

Before this work, we obtain ethical feasibility from the Animal Research Ethics Committee, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia, with number of letter: 0448/KEPH-FMIPA/2019 to perform all trials and procedures of this work.

Experimental animals and study design

We bought and used 28 male albino rats, *Rattus norvegicus*, as experimental animals in Biomedical Research Unit, West Nusa Tenggara Province General Hospital from October 2019 to December 2019. The animal in this work had average body weight and age of 294 gm and 8–12 weeks, respectively. The selected criteria included health, active movement, and good appetite. We excluded subjects in sickness condition or die during this work. They were located in cages in a laboratory environment at room temperature of around 26°C under 12-h cycles in a day, free access to water and standard food. The work began by adapting rats for 7 days in a laboratory. This work applied a post-test only with a control group design, and the determination of samples was done by simple random sampling.

Provision of bacteria

Gastric biopsy specimens of duodenal ulcer patients, which were stored and cultured in Microbiology Laboratory, Biomedical Research Unit, West Nusa Tenggara Province General Hospital, were used to acquire *H. pylori* isolates. Furthermore, *H. pylori* from this human isolate was cultured using Tryptic Soy Agar (TSA) (Cat. No. CM0131B, Oxoid™, Thermo Scientific™, Hampshire, UK) added with 10% fresh sheep blood, Dent Supplement (Oxoid™, Thermo Scientific™, Hampshire, UK), and Vitox Supplement (Oxoid™, Thermo Scientific™, Hampshire, UK). Under microaerophilic atmospheric condition with a concentration of 5% O_2, 10% CO_2, and 85% N_2, the incubation process was performed in a CO_2 incubator for 72 h at 37°C. Later on, we provide *H. pylori* from formed colonies based on colony appearance, equipped with microscopic examination by Gram stain and biochemical analysis. The rats in groups-2, 3, and 4 were inoculated with *H. pylori* suspension containing 5×10^8–5×10^{10} colony-forming unit/ml (CFU/ml) in 0.9% NaCl at 1 ml/rat.

Snakehead fish extract and first-line eradication regimen

Snakehead fish extract was a factory ready-made product by name Channa® capsules (PT Mega Medica Pharmaceuticals, Jakarta, Indonesia). The product packed up in capsules, where each 500 mg of capsule comprises pure snakehead fish extract powder. The composition of chemical active substance of Channa® has been analyzed at the Faculty of Pharmacy, Muhammadiyah University, Surakarta, Central Java, Indonesia. Snakehead fish extract, which was diluted in 0.5% (w/v) carboxymethyl cellulose (0.5% CMC), was given orally by intragastric gavage to rats in groups-3 and 4 at a dose of 300 mg/kgBW (kg body weight), 1 ml/rat daily. We determined a dose of 300 mg/kgBW as a treatment dose based on the previous studies [21,22]. First-line eradication regimen refers to PPI-based triple therapy composed of amoxicillin, clarithromycin, and omeprazole (PT Indofarma, Jakarta, Indonesia) in aqueous solution in a dose of 50, 25, and 20 mg/kgBW, respectively, diluted in 0.5% CMC [23]. All agents and drugs in this study were purchased privately from a commercial drug store.

Experimental procedure

After passing a week of adaptation period, all rats were then distributed randomly into four groups of seven rats each: group-1 as control negative (without *H. pylori* inoculation), group-2 as control positive (with *H. pylori* inoculation), group-3 as *H. pylori* inoculation + first-line eradication regimen group (aqueous solution of amoxicillin 50 mg/kgBW + clarithromycin 25 mg/kgBW + omeprazole 20 mg/kgBW), and group-4 as supplementation group, *H. pylori* inoculation + first-line eradication regimen (aqueous solution of amoxicillin 50 mg/kgBW + clarithromycin 25 mg/kgBW + omeprazole 20 mg/kgBW) + snakehead fish extract with a dosage of 300 mg/kgBW.

Rats in groups-2, 3, and 4 were supplemented with an aqueous solution of streptomycin (PT Indofarma, Jakarta, Indonesia) from tap drinking water (5 mg/ml) for 72-h before the inoculation of *H. pylori*, whereas animals in group-1 were normally given drinking water. All rats on the following day were not given food for a day.

The rats in groups-2, 3, and 4 were then inoculated with the aqueous suspension of *H. pylori* (1 ml to each animal) orally to gaster through gavage for two times per day at an interim of 4 h for 3 successive days, whereas group-1 was treated with a solution of 0.9% (w/v) natrium chloride (0.9% NaCl) at 1 ml to each animal orally to gaster through gavage for two times per day at an interim of 4 h for 3 successive days. All rats in groups-2, 3, and 4 were given oral omeprazole (PT Indofarma, Jakarta, Indonesia) at 1 ml/rat once a day by intragastric gavage with a dose of 400 µmol/kgBW dissolved in 0.5% CMC, 3 h before the first *H. pylori* inoculation and during the following 6 days. Similar to the procedure in three previous groups, group-1 was given 0.5% CMC suspension instead of omeprazole. Two weeks after the last day of *H. pylori* inoculation, all rats were not given any food for 12 h before the next procedure. On the next morning, a rat from each group was randomly selected to evaluate the success of creating *H. pylori* infection. They were sacrified under toxic dose of diethyl ether. Furthermore, a selected rat from all groups was performed surgical procedure. With regard to detect *H. pylori* in gastric tissue, gastric antrum area about 4 mm^2 was cut and take on an urease test (Pronto Dry®, Gastrex, France). We found the positive urease test results in rats that represented inoculation groups from this procedure.

On the following days, the remaining rats from each group were then administered with drugs and/or extracts for 7 consecutive days through intragastric gavage. Groups-1 and 2 were given 0.5% CMC suspension once a day. Meanwhile, group-3 was given first-line eradication regimen at 1 ml/rat once a day, group-4 was given first-line eradication regimen and snakehead fish extract at an interval of 6 h once a day. Four weeks after this procedure, all rats were then sacrified under a toxic dose of diethyl ether. The laparotomy procedure was done to take a small fragment of gastric antrum region to perform the rapid urease test as the previous description. It was noted that all rats in the infected groups were successfully infected with *H. pylori*. Later on, the remaining gastric antrum portion was provided for immunohistochemistry (IHC) examination and eradication testing procedure.

Immunohistochemistry procedure and analysis

Gastric mucosal tissue from the antrum region was taken and fixed with 10% buffered neutral formaline solution with pH 7.4 for 24 h. Afterward, tissue processing and embedding were settled on the paraffin block. The paraffin block was cut into a thickness of 5 µm and located on the slides before staining. A part of the 5-µm paraffin blocks was deparaffinized, rehydrated with 96%, 90%, and 80% alcohol, and warmed with a buffer citrate pH 6.0 for 20 min in a microwave. The samples were dropped with primary antibody such as MIF polyclonal rabbit IgG antibody, 25 µl (N1C3, Cat No. GTX101162, GeneTex, Inc., Irvine, CA) with a dilution of 1:250 at 4°C overnight. The samples were then dropped with secondary antibody, incubated for 20 min, given diaminobenzidine, and counterstained with hematoxylin.

The examination of MIF expression used immunoreactive score (IRS), which was the outcome of multiplication between the percentage score of immunoreactive cells and color intensity scores on immunoreactive cells at IHC staining. The percentages of immunoreactive cells are grouped as follows: score 0 determined as no staining cells found, score 1 determined as staining cells below 10%, score 2 determined as staining cells between 10% and 50%, score 3 determined as staining cells between 51% and 80%, and score 4 determined as staining cells above 80%. Color intensity is characterized by the appearance of brown cells, whereas negative staining results are marked with a paler color. Color intensity is grouped as follows: score 0 determined colorless, score 1 determined as weak intensity, score 2 determined as moderate intensity, and score 3 determined as strong intensity. The MIF expression is classified into four groups, namely, negative (IRS 0–1), weak (IRS 2–3), moderate (IRS 4–8), and strong (IRS 9–12) [24]. The observation of MIF expression was carried out using Olympus® CX22 LED binocular light microscope with a magnification of 100× and 400× equipped with Vivo® 9 camera. Microscopic examination was conducted blindly by two pathologists.

Eradication testing procedure

A piece section of gastric mucosal from the antrum area about 4 mm^2 was put into normal saline solution, then crushed, and homogenized. Furthermore, the dilutions of 1/10, 1/100, and 1/1000 were made, and each 1 ml of those dilutions was taken to be spread on the surface of plates containing TSA culture media (Cat. No. CM0131B, Oxoid™, Thermo Scientific™, Hampshire, UK) added with 10% fresh sheep blood. The plates were then incubated in a CO_2 incubator at 37°C in microaerophilic conditions for 72 h. We identified *H. pylori* colonies morphologically and biochemically. The number of colonies per plate was calculated and expressed as CFU logs per hull wall. The calculation of formed colonies was done using a colony counter.

Statistical analysis

The normality of quantitative variable was decided by the Shapiro–Wilk test. A descriptive analysis was reported as mean ± standard deviation (SD) or median (minimum value–maximum value). The one-way analysis of variance (ANOVA) test was used to specify the differences between quantitative variables in normal distribution and Kruskal–Wallis test to specify the differences between quantitative variables in non-normal distribution with *post hoc* Mann–Whitney U-test. The SPSS software v.22 was applied to perform the calculation. A value of $p < 0.05$ was determined as a limit of significance.

Results

This work resulted in a positive urease test in all rats those inoculated by *H. pylori*. A microscopic assessment of MIF expression in gastric epithelial cells observed according to the changes in color intensity on IHC staining. Color intensity was represented by the appearance of brown cells, whereas negative staining results were marked with a paler color. The expression of MIF was stronger in *H. pylori* inoculation groups (group-2 to group-4) than the negative control group (group-1). This is shown in Figure 1.

The IRS of MIF expression was decided in accordance with the results of multiplication between the percentage score of immunoreactive cells with color intensity scores. The calculation results regarding this are shown in Table 1, which was statistically computed by Kruskal–Wallis test.

To find out the difference in average IRS of MIF expression between the groups, Mann–Whitney U-test was performed. The results of analysis are shown in Table 2.

Eradication testing procedure is an effort to assess the success of *H. pylori* eradication. The output of this assessment is shown in Figure 2 and Table 3.

In Figure 2 and Table 3, the growth of *H. pylori* was only seen in group-2, and no *H. pylori* growth was found in the other groups.

Discussion

Pretreatment with streptomycin and omeprazole to inhibit the growth of other bacteria and reduce gastric acidity respectively turned out to produce a positive rapid urease test in *H. pylori*-inoculated groups. This effort is in accordance with several previous studies [25,26].

H. pylori infection leads to long-term inflammation and produces gastric pathological lesions that clinically manifest as gastritis, peptic ulcer disease to gastric cancer. A

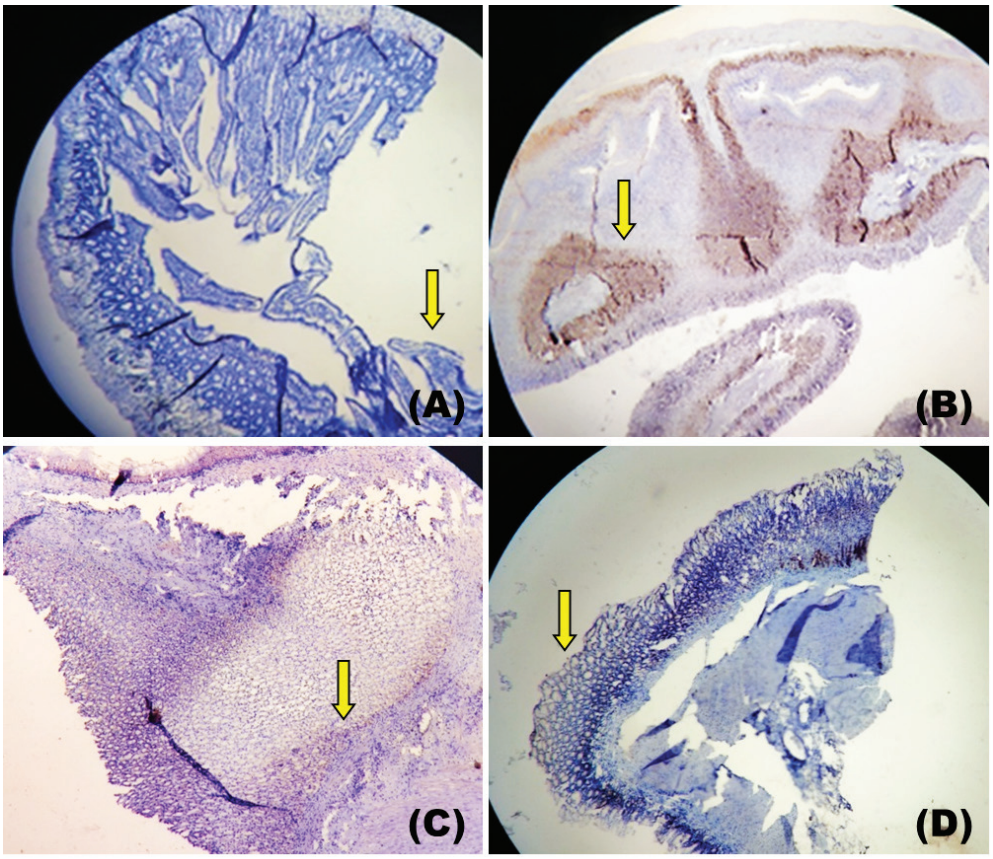

Figure 1. Representation of macrophage MIF expression in IHC staining (Magnification 100×). MIF expression is valued by IRS, which is the result of multiplication between the percentage score of immunoreactive cells with color intensity. Color intensity is characterized by the appearance of brown cells, whereas negative staining results are marked with a paler color. MIF expression is classified into four groups: negative, weak , moderate, and strong. The yellow arrows showed the difference of immunoreactive cells with color intensity. (A) Group-1 (control negative, representation of negative MIF expression); (B) Group-2 (control positive, representation of strong MIF expression); (C) Group-3 (first-line eradication regimen, representation of moderate MIF expression); (D) Group-4 (first-line eradication regimen + snakehead fish extract, representation of weak MIF expression).

Table 1. Average IRS of MIF expression in each group.

Groups	Immunoreactive score	*p* value
Group-1	0.17 ± 0.41	0.000*
Group-2	7.50 ± 1.64	
Group-3	4.00 ± 1.67	
Group-4	1.00 ± 0.63	

Data were expressed as mean ± SD. Statistical test using Kruskal–Wallis. Number of subject per group 6. *$p < 0.05$.

long-term inflammation is inseparable from the role of lymphokines such as MIF. MIF was derived from several immune cells such as granulocytes, T-cells, B-cells, dendritic cells, macrophages, and monocytes [27]. MIF regulates innate immunity system and adaptive immunity system. Releasing and increasing of MIF activity due to *H. pylori* infection will induce the activation of other proinflammatory cytokines comprising TNF-α, IL-1β, IFN-γ, IL-6, and IL-8 [28]. These cytotoxic mediators tend to recruit neutrophils, macrophages, and T-cells into gastric mucosa, which will cause long-term inflammation and various pathological lesions in the gastric mucosa [9]. The work noticed that the inoculation of *H. pylori* produced an increase of MIF expression, and this was associated with the enhancement density and growth of *H. pylori* in the eradication testing procedure as shown in group-2. We observed that, by performing an eradication testing procedure, there was no *H. pylori* growth in groups treated by first-line eradication regimen (groups 3 and 4) as well as the decrease of MIF expression in both groups.

Tabel 2. *Post hoc* analysis comparing median IRS of macrophage MIF expression between groups.

Group comparison	Immunoreactive score	*p* value
Group-1 *vs.* Group-2	0.0 (0.0–1.0) *vs.* 7.5 (6.0–9.0)	0.002*
Group-1 *vs.* Group-3	0.0 (0.0–1.0) *vs.* 3.5 (2.0–6.0)	0.003*
Group-1 *vs.* Group-4	0.0 (0.0–1.0) *vs.* 1.0 (0.0–2.0)	0.026*
Group-2 *vs.* Group-3	7.5 (6.0–9.0) *vs.* 3.5 (2.0–6.0)	0.012*
Group-2 *vs.* Group-4	7.5 (6.0–9.0) *vs.* 1.0 (0.0–2.0)	0.003*
Group-3 *vs.* Group-4	3.5 (2.0–6.0) *vs.* 1.0 (0.0–2.0)	0.004*

Data were expressed as median (minimum–maximum). Statistical test using Mann–Whitney. Number of subject per group 6. *$p < 0.05$

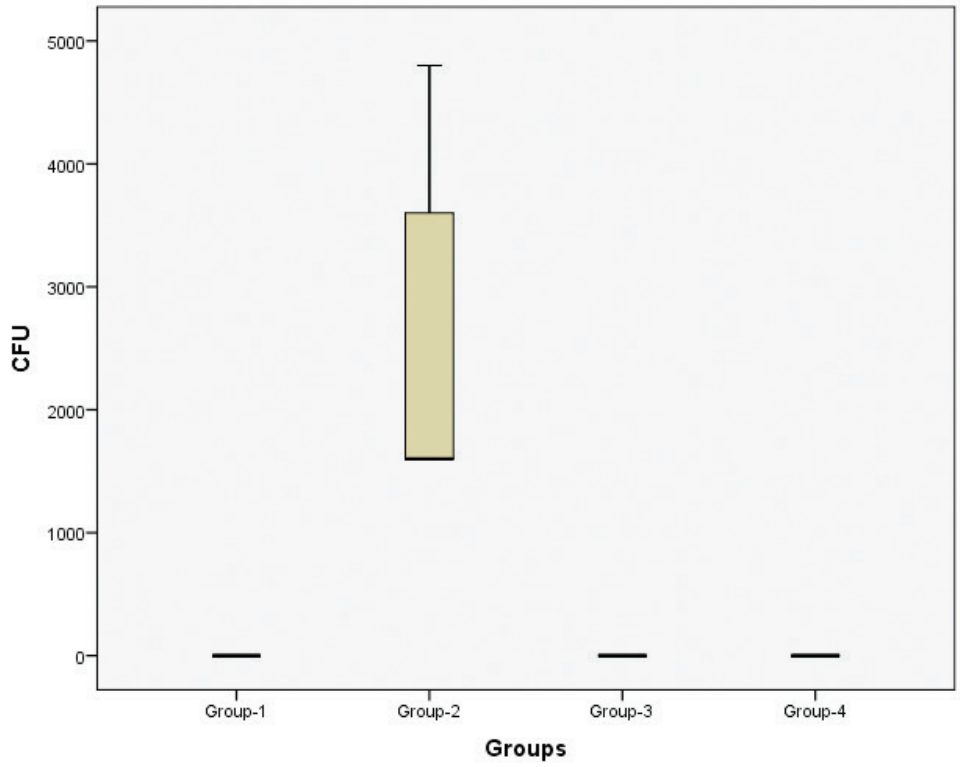

Figure 2. The growth of *H. pylori* in each group after cultured in eradication testing procedure. The growth of *H. pylori* was only seen in group-2, and no *H. pylori* growth was found in the other groups.

In this study, it was encountered that the supplementation of snakehead fish extract to first-line eradication regimen significantly reduced the MIF expression more than the single administration of first-line eradication regimen (group-4 *vs.* group-3).

The first-line eradication regimen consists of omeprazole, amoxicillin, and clarithromycin. The presence of omeprazole reduces gastric acidity by reducing the pH gradient and producing chemotactic bias in *H. pylori* so that the performance of antibiotics is more optimal [29]. Amoxicillin with its intraluminal topical activity acts by inhibiting bacterial cell wall and membrane synthesis. The administration of a combination of omeprazole and amoxicillin elevates the ability of eradication because the reduction of gastric acidity level leads to the increment of amoxicillin concentration in the stomach beyond the minimum inhibitory concentration to *H. pylori* [3,11]. Clarithromycin, a macrolide antibiotic, works by binding to the 50s bacterial ribosome subunit to inhibit peptide translocation so that the transcription and translational process of *H. pylori* protein is disrupted. Combining clarithromycin with amoxicillin will increase the eradication rate by more than 70%

Table 3. The growth of *H. pylori* in eradication testing procedure.

Groups	*n*	Colony-forming unit	Successful rate (%)
Group-1	6	not detected	–
Group-2	6	1,600 (1,600.00–4,800.00)	0/6 (0%)
Group-3	6	not detected	6/6 (100%)
Group-4	6	not detected	6/6 (100%)

Data were expressed as median (minimum–maximum).

[30]. In this study, it was noted that the single administration of a first-line eradication regimen resulted in complete eradication rate without the issue of antibacterial resistance. Therefore, the first-line eradication of *H. pylori* regimen can reduce MIF expression, and this finding is in accordance with a study from Kebapcilar et al. [10].

Snakehead fish extract has been known as a potential natural anti-inflammatory. Albumin, amino acids, fatty acids, and minerals contained in snakehead fish extract have therapeutic effects due to their anti-inflammatory, antioxidant, and even antibacterial properties [31]. Sulfhydryl cluster (-SH) in albumin and minerals (zincum, cuprum, and ferrum) have antioxidant properties that act as ROS scavenger and cellular protection against oxidative stress, besides that thiol cluster in albumin was known as antibacterial in septicemia condition [32]. An *in vitro* study in Texas, United States, found that albumin can interrupt biofilm formation by inhibiting the bacterial quorum sensing [33]. Some previous *in vitro* study reported the antibacterial activity of some amino acids [34,35]. Amino acids such as lysine, arginine, aspartic, and glutamic acid also perform as antioxidant and anti-inflammatory synergistically with some fatty acids, such as linoleic, arachidonic, stearic, and oleic acid [36]. The generation of proinflammatory cytokines was disturbed by linoleic and arachidonic acids, while stearic and oleic acids serve by reducing the polymorphonuclear leukocyte activity and weakening the expression of endothelial leukocyte adhesion molecules, respectively [37].

This study highlighted that snakehead fish extract supplementation to first-line eradication regimen tends to have a profitable effect in reducing more MIF expression. This finding obviously showed a potential effect of first-line eradication regimen and snakehead fish extract mainly in *H. pylori* eradication and suppression of inflammatory event, respectively. This study is in line with several studies. A research reported significant decreases in proinflammatory cytokine levels after 12 weeks of supplementation of *C. striata* extract capsules into antituberculosis drugs in pulmonary tuberculosis patients [20]. Similarly, another study documented that there was an acceleration in the healing of pulmonary tuberculosis clinically in patients with pulmonary tuberculosis, who administered a combination of antituberculosis drugs and *C. striata* extract capsules [38].

We noticed some limitations that might be found in this study, including the unknown type of *H. pylori* strain and the use of snakehead fish extract without varying doses.

Conclusion

We noted that MIF expression was higher in *H. pylori*-infected groups. The conclusion of this work exhibited that snakehead fish extract supplementation to first-line eradication regimen significantly reduces more MIF expression compared to the single administration of first-line eradication regimen in rats induced by *H. pylori* infection.

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Conflict of interest

The authors announce that they have no conflict of interest regarding creation and publication of this paper.

Authors' contribution

OKY designed and managed the study, did laboratory works, deciphered data, and drafted and wrote the paper. AL was involved in planning and basic checking of the paper. SI analyzed data and critical checking of the manuscript. RLK took part in the collection of data, laboratory works, and planning of the paper. The final paper read, examined, and endorsed by all the authors before submission.

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