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Original article

Efficacy of Formononetin Alone and in Combination with Metronidazole Against *Giardia lamblia* Infection via Modulating Serum Electrolytes and Intestinal Inflammation in Mice

Javad Ghasemian Yadegari[1], Fatemeh Ezzatkhah[2], Amal Khudair Khalaf[3], Fatemeh Ataie[4], Zeinab Kavandi[4], Hossein Mahmoudvand[5*]

[1]Department of Pharmacognosy, Lorestan University of Medical Sciences, Khorramabad, Iran.
[2]Department of Laboratory Sciences, Sirjan School of Medical Sciences, Sirjan, Iran.
[3]Department of Microbiology, College of Medicine, University of Thi-qar, Thi-qar, Iraq.
[4]Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran.
[5]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.

Abstract

Background and objectives: This study aimed to investigate the in vitro and in vivo anti-giardial effects of formononetin, as well as its potential synergistic effects against *Giardia lamblia* infection in a murine model. **Methods:** Cell viability assay was used to evaluate the in vitro anti-giardial and cytotoxic effects of formononetin. Infected mice were treated with formononetin and metronidazole for seven days after which stool samples were analyzed to assess the presence and viability of *Giardia* cysts. Additionally, serum concentration of the electrolytes were measured using biochemical assay kits. The expression levels of tumor necrosis factor-alpha (TNF-α), nuclear factor kappa B (NF-κB), and interleukin-1 beta (IL-1β) were quantified using real-time PCR techniques. **Results**: formononetin caused a significant ($p<0.001$) reduction in the viability and growth rate of *G. lamblia* cysts. The IC_{50} values for formononetin, metronidazole, and their combination against *G. lamblia* cysts were 37.1, 29.3, and 11.1 µg/mL, respectively. Formononetin, both alone and in combination with metronidazole, produced a significant decrease ($p<0.001$) in the average count and viability of *Giardia* cysts, modulated the serum electrolytes, and significantly decreased the expression of IL-1β, TNF-α, and NF-κB-p65 genes ($p<0.001$). **Conclusion**: formononetin, particularly in combination with metronidazole, demonstrated efficacy in managing giardiasis both in vitro and in vivo by modulating electrolyte levels and inflammatory responses. However, further research is needed to elucidate the mechanisms through which this compound exerts its therapeutic effects against giardiasis.

Keywords: giardiasis; herbal medicines; inflammation; in vitro; in vivo

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Introduction

Giardia lamblia represents a significant pathogenic protozoan responsible for infecting the gastrointestinal tract of humans and other vertebrates [1]. It is estimated that over 280 million new cases of giardiasis are reported around the world, annually [1]. The distribution of *Giardia* spans various geographic regions; however, its incidence is notably higher in tropical and temperate climates compared to colder areas. In Iran, particularly in the northern provinces, a considerable prevalence of this parasite has been documented [2]. Transmission primarily appears

*Corresponding author: mahmoudvand.h@lums.ac.ir

across the eating of resilient cysts present in contaminated water or food, as well as direct fecal-oral contact [3]. While all age groups are susceptible to infection, children exhibit a heightened vulnerability [3]. Clinically, giardiasis may manifest in either acute or chronic forms. Predominant symptoms include diarrhea and malabsorption, which can lead to significant health consequences, especially among children and infants [3]. Standard treatment regimens typically involve chemotherapeutic agents such as metronidazole and furazolidone [4]. These pharmacological interventions are occasionally associated with adverse side effects and do not consistently achieve satisfactory efficacy. Moreover, certain compounds possess mutagenic and carcinogenic properties, rendering their use inadvisable in sensitive populations, including pregnant women and children [5]. Emerging evidence indicates the development of parasite resistance to these drugs [4,5]. Consequently, there is a growing body of research dedicated to identifying substitute medicinal agents derived from natural sources that demonstrate a lower incidence of adverse effects. Recently, medicinal plants and their bioactive constituents have garnered considerable interest economic viability, minimal toxicity, and the presence of bioactive compounds with proven efficacy [6]. Multiple studies have substantiated the antigiardial activity of various phytochemicals derived from species such as *Thymus*, *Eucalyptus*, and *Allium* [7]. However, the broader application of these agents remains constrained by insufficient robust laboratory data and inconsistent research findings. Within the spectrum of bioactive plant compounds, flavonoids—a class of polyphenolic compounds widely distributed in nature—play a pivotal role in exerting pharmacological effects including anti-inflammatory, antioxidant, and antiseptic activities [8]. Isoflavones, a subclass of flavonoids, share structural similarity with estrogen [9,10]. Among these, formononetin ($C_{16}H_{12}O_4$), predominantly found in plants of the Fabaceae family, is notable [11]. Recent studies have revealed that formononetin possesses a wide range of biological activities encompassing anti-inflammatory, antioxidant, anticancer, antibacterial, antiviral, and antiparasitic effects [12,13]. In the light of the established pharmacological properties of formononetin and its prospective utility in addressing parasitic infections, the present study was designed to evaluate the antigiardial efficacy of this compound and to assess its potential synergistic effects within an experimental murine model of *G. lamblia* infection.

Material and Methods

Ethical considerations

In vivo experiments were conducted in strict compliance with the National Institutes of Health (NIH) guideline for the care and use of laboratory animals. Also, all study protocols were implemented after review and approval by the Research Ethics Committee of Lorestan University of Medical Sciences (Khoramabad, Iran) with the ethical code IR.LUMS.REC.1403.454.

Chemicals

Formononetin (purity>99%, 7-hydroxy-4'-methoxyisoflavone), MTT powder, and metronidazole were purchased from Sigma-Aldrich (Germany). Other materials including dimethyl sulfoxide (DMSO), Dulbecco's modified medium (DMEM), fetal bovine serum (FBS), anesthetics ketamine and xylazine, and eosin dye were purchased from Merck (Germany). Sodium (Na^+) and potassium (K^+) measurement kits were purchased from Pars Azmoun Iran, and RNA extraction, complementary DNA (cDNA) synthesis, and SYBR Green PCR master mix kits were purchased from Iranian companies Favaorgen and Yekta Tajhiz Azma. All chemicals used were of the highest laboratory grade purity.

Giardia lamblia cyst collection and isolation

Stool samples were collected from a patient diagnosed with giardiasis infection and referred to Shahid Rahimi Hospital in Khorramabad. Confirmation of the presence of the parasite was performed by direct microscopic examination and formalin-ether concentration method. Then, for further purification, sucrose gradient method (0.85 M) was used based on the method described elsewhere [14]. The final density of the cysts was adjusted to 1×10^5 cysts per ml.

In vitro antigiardial effects of formononetin

To investigate the antiparasitic activity of formononetin, a volume of 100 µL of the suspension containing 1×10^5 cysts per mL was transferred to the wells of a 96-well plate. Then, different amounts of formononetin and metronidazole were added to the wells at

concentrations ranging from 5 to 200 µg/mL and the samples were incubated for 48 h at 24 °C; the further process was carried out based on the previous study [14]. The 50% inhibitory concentration (IC_{50}) was obtained using probit analysis in SPSS software (version 26.0).

The synergic effect of formononetin and metronidazole

To determine the possible interaction between formononetin and metronidazole, the fractional inhibitory concentration index (FICI) was acquired as:

FIC = (IC_{50} of the combination / IC_{50} of the individual drug)

In interpreting the results, a FICI value≤0.5 indicates the presence of a synergistic effect, values between 0.5 and 1 indicate an additive effect, and values higher than 1 indicate an antagonistic interaction between the two drugs [15].

Cytotoxic effects of formononetin

Normal human intestinal epithelial cells (NCM460) were obtained from the Pasteur Institute of Iran. These cells were cultured in DMEM medium containing 10% FBS and maintained at 37 °C with an atmosphere of 5% CO_2. To measure cytotoxicity, the MTT assay was performed and the absorbance at 590 nm was recorded with an ELISA plate reader [14].

In vivo effects of formononetin on giardiasis in mice

Animals

This study used 64 male Balb/C mice, aged approximately 8 to 10 weeks and weighing between 25 and 30 grams. The animals were maintained under standardized environmental conditions, including regulated temperature, optimal humidity, and a 12-hour light/dark cycle. Throughout the duration of the experiment, the mice were provided with unrestricted access to standard laboratory chow and water.

Study design

In order to improve parasite colonization in the gastrointestinal tract, a four-day pretreatment period with antibiotics was first performed. For this purpose, solutions containing vancomycin (0.5 mg/mL), neomycin (1 mg/mL), and ampicillin (1 mg/mL) were added to the animals' drinking water. Mice were then orally challenged with 0.2 mL of a solution containing 1×10^4 *G. lamblia* cysts per mL. To confirm infection, stool samples were examined by direct smear and formalin-ether fixation to determine the presence of *Giardia* cysts in the animals' feces [15]. After six days of infection, the mice were divided into eight groups and each group received one of the following treatment regimens for seven days:

Group 1: normal saline; group 2: metronidazole 15 mg/kg/day; group 3: formononetin 12.5 mg/kg/day; group 4: formononetin at 25 mg/kg/day; group 5: formononetin at 50 mg/kg/day; group 6: metronidazole at 7.5 mg/kg/day + formononetin 12.5 mg/kg/day; group 7: metronidazole at 7.5 mg/kg/day + formononetin at 25 mg/kg/day; group 8: metronidazole at 7.5 mg/kg/day + formononetin at 50 mg/kg/day.

Evaluation of parasite excretion in feces

One day after the end of treatment, fecal samples of animals were collected and evaluated for the presence or absence of *Giardia* cysts and their reduction. Eosin staining was used to determine the viability of the cysts; pink cysts were considered dead and colorless cysts were considered alive [15].

Collecting intestinal tissues and blood sample

After the anesthetizing and euthanizing of mice via intraperitoneal administration of a ketamine and xylazine mixture at dosages of 100 mg/kg and 10 mg/kg, respectively, the duodenum was excised, longitudinally incised, and harvested following the protocol described by Masoori et al. [16]. The tissue specimens were promptly preserved at −80 °C for subsequent RNA extraction. Additionally, blood samples were obtained through cardiac puncture following an abdominal incision; after centrifugation, the serum was collected and stored for biochemical analyses.

Evaluating the serum electrolytes

Serum sodium (Na^+) and potassium (K^+) levels were quantitatively measured using commercial biochemical kits according to the manufacturer's instructions.

Assessment of the expression level of inflammatory-related gene by real-time PCR

Total RNA was extracted from mouse duodenal tissue using an RNA extraction kit. The purified

RNA was then converted to cDNA. Real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green master mix and primers specific [16] for TNF-α, NF-κB, and IL-1β genes (Table 1). The PCR protocol comprised an initial denaturation phase of 5 minutes at 96 °C, succeeded by 35 amplification cycles, each including a denaturation step of 30 seconds at 96 °C and a primer annealing step of 60 seconds at 62 °C. The procedure concluded with a final elongation phase lasting 5 minutes at 73 °C. Quantitative data analysis was performed utilizing the $2^{-\Delta\Delta Ct}$ method in conjunction with the Bio-Rad iQ5 Optical System software (USA).

Statistical analysis

All datasets were analyzed utilizing SPSS software, version 26.0. To compare group means, one-way analysis of variance (ANOVA) and Tukey's post hoc test were applied. Statistical significance was established at a threshold of $p<0.05$.

Results and Discussion

As shown in Figure 1A, the use of formononetin and metronidazole dose-dependently caused a significant ($p<0.001$) decrease in the survival and growth rate of *G. lamblia* cysts compared to the control group. It is noteworthy that the combination of these two compounds showed the strongest inhibitory effect on the growth and viability of cysts. The IC_{50} values for formononetin, metronidazole and their combination were estimated to be 37.1±4.66, 29.3±3.47 and 11.1±2.19 µg/mL, respectively. Based on the results of calculating the drug interaction index (FICI), which was obtained for formononetin and metronidazole as 0.299 and 0.378, respectively, the existence of a significant synergistic effect between these two compounds was confirmed. Figure 1B shows the cytotoxic effects of formononetin on normal NCM460 cells by MTT assay.

Table 1. The sequence of primers used in real-time PCR

Primer	Sequence (5'–3')
TNF-α	F: TGAACTTCGGGGTGATCGGT R: GGTGGTTTGTGAGTGTGAGGG
IL-1β	F: AACCTGCTGGTGTGTGACGTTC R: CAGCACGAGGCTTTTTTGTTGT
NF-κB p65	F: AGGCAAGGAATAATGCTGTCCTG R: ATCATTCTCTAGTGTCTGGTTGG
β-Actin	F: GTGACGTTGACATCCGTAAAGA R: GCCGGACTCATCGTACTCC

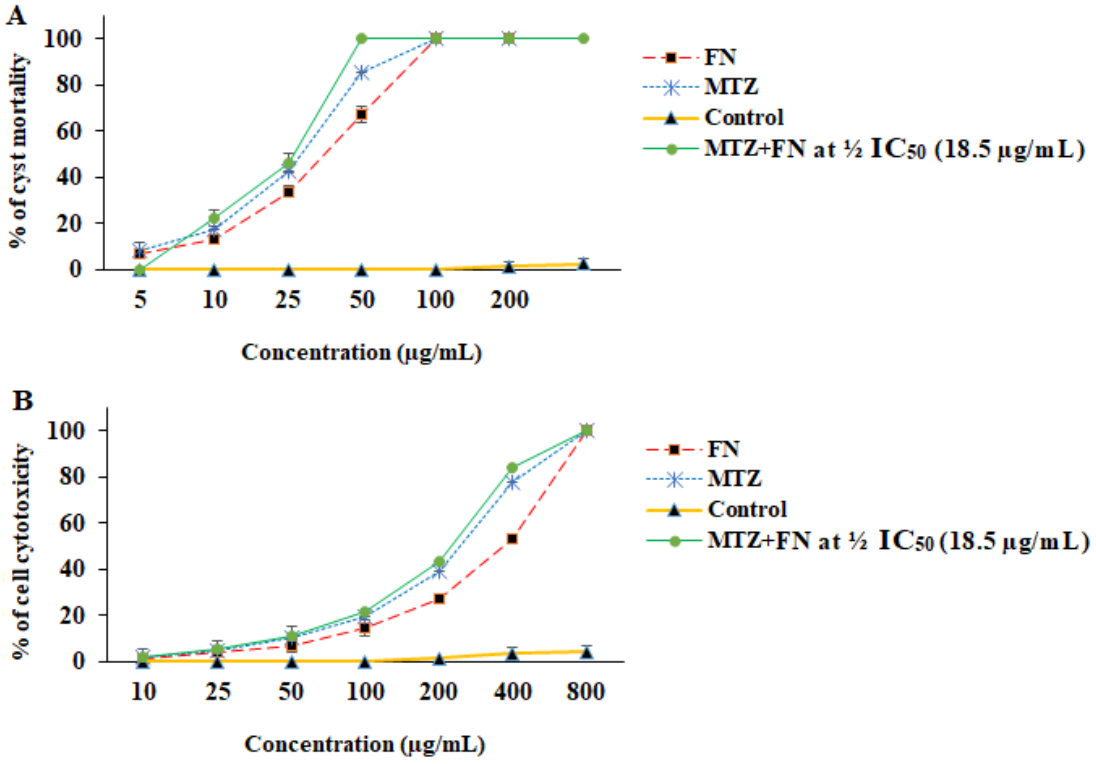

Figure 1. (A) Antigiardial and (B) cytotoxic effects of different concentrations of formononetin (FN) and metronidazole (MTZ), alone and in combination on *Giardia lamblia* and the normal human intestinal epithelial cells, respectively; the results are presented as mean ± standard deviation (SD) with a sample size of n=3.

Table 2. In vivo effects of formononetin on the number and the viability of *Giardia lamblia* cysts in mice with giardiasis

Treatment	No. of *Giardia* cysts (mean±SD)	% of Cysts viability (mean±SD)
Normal saline	439.6±13.6	91.6±4.6
MTZ 15 mg/kg/day	3.6±2.1***	2.3±1.5***
FN 12.5 mg/kg/day	114.3±6.3***	49.8±6.3***
FN 25 mg/kg/day	46.6±3.5***	31.6±5.4***
FN 50 mg/kg/day	28.4±2.8***	23.3±3.4***
MTZ 7.5 mg/kg + FN 12.5 mg/kg	1.7±1.5***###	1.9±0.6***###
MTZ 7.5 mg/kg + FN 25 mg/kg	0.0±0.0***###	0.0±0.0***###
MTZ 7.5 mg/kg + FN 50 mg/kg	0.0±0.0***###	0.0±0.0***###

FN: formononetin; n=8 mice/group; *** p<0.001 compared to normal saline; ### p<0.001 compared to metronidazole (MTZ) by post hoc analysis

Table 3. In vivo effects of formononetin on the serum electrolytes level in mice with giardiasis

Treatment	Sodium level (mmol/L) mean±SD	Potassium level (mmol/L) mean±SD
Normal saline	57.8±4.3	4.1±1.50
MTZ 15 mg/kg/day	119.5±5.6***	8.2±0.6***
FN 12.5 mg/kg/day	69.3±3.7*	5.9±0.4**
FN 25 mg/kg/day	82.2±4.1**	6.3±0.3**
FN 50 mg/kg/day	89.5±3.7***	6.6±0.4***
MTZ 7.5 mg/kg + FN 12.5 mg/kg	129.6±8.6***###	6.9±0.5***###
MTZ 7.5 mg/kg + FN 25 mg/kg	137.8±9.8***###	7.1±0.8***###
MTZ 7.5 mg/kg + FN 50 mg/kg	153.4±11.2***###	8.3±0.7***###

FN: formononetin; n= 8 mice/group; *p<0.05; ** p<0.01; *** p<0.001 compared to normal saline; ### p<0.001 compared to metronidazole (MTZ) by post hoc analysis

The data indicate that formononetin, metronidazole and their combination significantly reduced cell viability. The CC_{50} values for formononetin, metronidazole and metronidazole + formononetin were 382.3±16.26, 251.8±22.45 and 237.8±19.82 μg/mL, respectively, indicating lower cytotoxicity of formononetin compared to metronidazole. Based on the data presented in Table 2, formononetin, administered either as a monotherapy or in conjunction with metronidazole, resulted in a statistically significant (p<0.001) reduction in the mean number of cysts excreted in the feces and a reduction in their viability. The percentage reduction of excretory cysts in the groups treated with metronidazole (15 mg/kg), formononetin (12.5, 25 and 50 mg/kg) and the combination of metronidazole (7.5 mg/kg) with formononetin (12.5, 25 and 50 mg/kg) significantly increased compared to the control group (p<0.001). Also, the percentage of cyst survival in the control group that received only normal saline was recorded as 91.6±4.6%, while this value significantly reduced in all treated groups, especially in the combination treatments (p<0.001).In the biochemical section, the examination of serum electrolyte levels showed that there was a significant decrease in serum sodium and potassium concentrations in infected mice (p<0.001). In contrast, oral administration of formononetin, especially when combined with metronidazole, was able to significantly increase sodium and potassium concentrations compared with the control group (receiving normal saline) (p<0.001; Table 3).

The greatest increase in serum sodium levels was observed in the groups receiving metronidazole 7.5 mg/kg + formononetin 12.5 mg/kg (129.6 ± 8.6 nmol/L), metronidazole 7.5 mg/kg + formononetin 25 mg/kg (137.8 ± 9.8 nmol/L), and metronidazole 7.5 mg/kg + formononetin 50 mg/kg (153.4 ± 11.2 nmol/L). Similarly, the most improvement in serum potassium levels was observed in the metronidazole 7.5 mg/kg + formononetin 12.5 mg/kg (6.9 ± 0.56 nmol/L), metronidazole 7.5 mg/kg + formononetin 25 mg/kg (7.1 ± 0.84 nmol/L), and metronidazole 7.5 mg/kg + formononetin 50 mg/kg (8.3 ± 0.69 nmol/L) groups, clearly indicating the synergistic effects of the combination treatment in improving the electrolyte balance of infected mice.

By real-time PCR, mRNA levels of IL-1β, TNF-α, and NF-κB-p65 genes showed significantly increased in the infected mice (p<0.05) (Figure 2). In contrast, treatment with formononetin, especially in combination with metronidazole, caused a significant decrease in the expression of these inflammatory genes (p<0.001). In mice receiving the combination of formononetin and metronidazole, the expression of IL-1β was about 0.94 to 1.19-fold, TNF-α was between 1.04 and 1.10-fold, and NF-κB-p65 was about 1.11 to 1.31-fold lower than in the infected control group.

Flavonoids, as one of the most important groups of natural compounds, have attracted widespread attention from researchers due to their antibacterial, antiviral, and antiparasitic properties [19]. These compounds have not only shown positive effects in clinical studies, but also have superior safety and patient acceptance compared to synthetic chemical drugs [20]. Drugs such as metronidazole, furazolidone, and quinacrine are commonly used in the treatment of giardiasis; however, the occurrence of numerous side effects and drug resistance has limited their efficacy [4]. Accordingly, the aim of the present study was to investigate the antigiardial efficacy of formononetin, either as monotherapy or in combination with metronidazole, at both in vitro and in vivo levels using a mouse model of *G. lamblia* infection in vitro. The findings demonstrated that the combined treatment of formononetin and metronidazole markedly decreased the viability of *G. lamblia* cysts in a dose-dependent manner relative to the control group. The FICI for formononetin and metronidazole was 0.299 and 0.378, respectively, indicating a synergistic effect between these two compounds. In in vivo studies, administration of different doses of formononetin (12.5, 25, and 50 mg/kg) alone or in combination with metronidazole significantly reduced the number and survival of *Giardia* cysts in infected mice for seven days ($p<0.001$). Regarding the antimicrobial properties of formononetin, the findings of Yang et al. [21] demonstrated that formononetin exhibits substantial inhibitory effects against both Gram-positive and Gram-negative bacteria, encompassing species such as *Escherichia*, *Enterobacter*, and *Pseudomonas*. Wang et al. [22] also reported the antiviral effects of formononetin against Enterovirus-51 and stated and that this compound has the ability to inhibit viral RNA and protein synthesis. In the context of antiparasitic activity, Lavat et al. [23] demonstrated that formononetin effectively inhibits the adhesion, motility, and survival of *G. lamblia* trophozoites. Furthermore, Haghighi et al. [24] reported that concentrations of 15 and 300 µg/mL of formononetin caused complete destruction of hydatid cyst protoscolices after 30 and 60 min of contact, both in vitro and ex vivo. This effect was accompanied by a significant increase in caspase-3 activity ($p<0.001$) and increased membrane permeability of protoscolices. In addition, in the study of Mahmoudvand et al. (2023), it was also shown that formononetin significantly ($p<0.001$) reduced the viability of *Toxoplasma gondii* tachyzoites, and its IC_{50} value was reported to be 9.85 µg/mL.

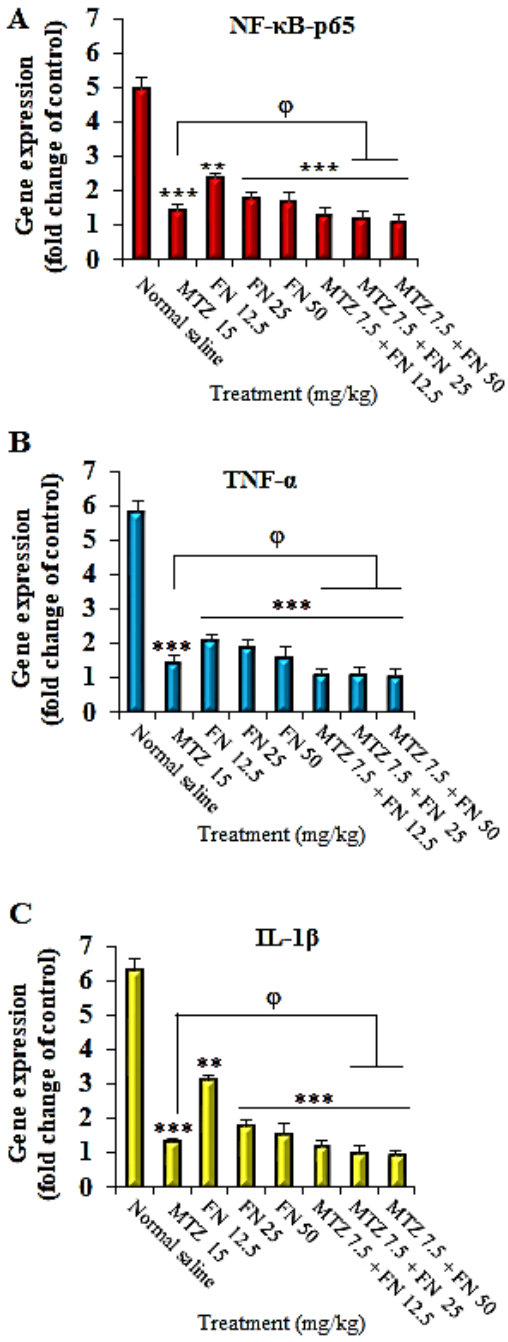

Figure 2. The in vivo effect of different doses of formononetin (FN) on the gene expression level of A: nuclear factor κB p65 (NF-κB p65); B: tumor necrosis factor-alpha (TNF-α), and C: interleukin-1 beta (IL-1β), in mice with giardiasis in comparison to metronidazole (MTZ); n= 8 mice/group; *p<0.05; ** p<0.01; *** p<0.001 compared to normal saline; φ p<0.001 compared to MTZ by post hoc analysis

The treatment under study reduced the number of intracellular protozoa and simultaneously increased nitric oxide production in macrophages [25]. Studies by Mahmoudvand et al. [26] also showed that formononetin significantly reduced the survival rate and number of promastigote and amastigotes of *Leishmania* ($p<0.001$), with IC_{50} values of 9.3 μM for promastigotes and 14.3 μM for amastigotes. Also, macrophages treated with formononetin showed a significant increase in NO secretion and mRNA expression of inflammatory factors such as IFN-γ and inducible nitric oxide synthase (iNOS).

A comprehensive review of the available literature suggests that flavonoids exert their antimicrobial activities through a variety of mechanisms, including induction of apoptosis, generation of oxidative stress, release of nitric oxide, inhibition of virulence factors, disruption of biofilm formation, impairment of cell membrane integrity, interference with cell envelope synthesis, inhibition of nucleic acid synthesis, and reduction of DNA replication [19,20,22].Numerous studies have examined the traditional use of medicinal plants for treating diarrhea by assessing the biological effects of their extracts [29]. These extracts usually contain phytochemicals such as flavonoids, alkaloids, tannins, and terpenes, which are thought to play a major role in their antidiarrheal effects [30]. It has been hypothesized that formononetin may reduce the symptoms of giardiasis by modulating serum electrolyte levels in infected mice. Evidence from human studies and in vitro data suggests that *Giardia* infections can induce pro-inflammatory responses in the gastrointestinal tract [31]. Secretory and excretory products of *Giardia* trophozoites activate inflammatory signaling pathways, particularly the MAPK and NF-κB pathways, in the intestinal epithelial layers [31], which in turn leads to the production of pro-inflammatory cytokines and chemokines, including TNF-α. Our findings showed that formononetin administration, especially in combination with metronidazole, significantly reduced ($p<0.001$) the expression levels of IL-1β, TNF-α, and NF-κB-p65 compared to the saline-treated infected group. These results suggest that formononetin likely exerts its anti-inflammatory and anti-giardial effects in mouse models by inhibiting NF-κB signaling pathways and suppressing some inflammatory cytokines.

Investigation of the cytotoxic effects of formononetin showed that this compound, metronidazole, and their combination dose-dependently reduced cell survival. The calculated CC_{50} values for formononetin, metronidazole, and their combination on normal NCM460 cells were 382.3, 251.8, and 237.8 μg/mL, respectively. These results indicate that while the metronidazole + formononetin combination exhibited the greatest anti-giardial effect in vitro, it was not more cytotoxic than metronidazole alone. Also, Mahmoudvand et al. [26] reported CC_{50} values of 159.3 μg/mL for formononetin and 874.6 μg/mL for glucantime on normal macrophages. Therefore, a selectivity index (SI) of greater than 10 for formononetin and MA indicates their specificity towards *L. tropica* amastigotes and minimal toxicity to macrophages.

The findings of this study indicate that formononetin may possess antigiardial properties; however, there are some limitations. Additional research is necessary to explore the pharmacodynamic effects of formononetin on different therapeutic targets. Moreover, a more detailed investigation into the absorption, bioavailability, and metabolic processes of this compound is required. To validate the clinical use of formononetin, clinical trials assessing its toxicity and therapeutic effectiveness are highly recommended.

Conclusion

The findings of this study indicate that the combination of formononetin and metronidazole is highly effective in treating giardiasis, as shown in both laboratory and mouse models. This effectiveness seems to be linked to the regulation of electrolyte balance and inflammatory reactions. Nonetheless, additional studies are needed to better understand the exact mechanisms and the treatment's effectiveness against *G. lamblia* infections.

AI tools

The author(s) declare that no generative AI was used in the writing of manuscript, production of images or graphical elements of the paper, or in the collection and analysis of data.

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Author contributions

Javad Ghasemian Yadegari and Hossein Mahmoudvand planned the tests and supervised the study; Fatemeh Ataie, Fatemeh Ezzatkhah, and Zeinab Kavandi performed tests and collected data; Amal Khudair Khalaf, and Hossein Mahmudvand, prepared the draft and edited the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

IC_{50}: the 50% inhibitory concentration; CC_{50}: 50% cytotoxic concentration; FICI: fractional inhibitory concentration index