Promising effects of formononetin, a natural isoflavone derived from herbs, against *Toxoplasma gondii*

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**A B S T R A C T**

**Introduction:** Formononetin (FMN) is a natural isoflavone found in many plants. This work examined the anti-*Toxoplasma* effects and cytotoxicity properties of FMN on *Toxoplasma gondii*.

**Methods:** Effects of FMN (2-64 µg/mL) on tachyzoites forms were measured by cell viability assay for 48 hours. The effects of different concentrations of FMN on infectivity rate, intracellular parasites, and nitric oxide (NO) in macrophage cells (J774-A1) were also evaluated.

**Results:** FMN markedly (*P* < 0.001) reduced the viability rate of tachyzoites forms with an IC$_{50}$ value of 9.85 µg/mL. FMN also declined the rate of intracellular tachyzoites whereas, FMN increased the FMN production in macrophage cells.

**Conclusion:** The results of the present *in vitro* study revealed the favorable anti-*Toxoplasma* effects of FMN against tachyzoites and intracellular forms of *T. gondii*. Although the accurate anti-*Toxoplasma* mechanisms of FMN are not clear, our results showed that triggering the NO production might be considered one of the main mechanism actions of FMN for controlling and eliminating *T. gondii*. However, further surveys are mandatory to assess the effects of FMN in animal models and to evaluate its accurate mechanism actions before its use in clinical phase.

**Implication for health policy/practice/research/medical education:**
In the present investigation we showed the high *in vitro* anti-*Toxoplasma* effects of FMN, a natural isoflavone, against tachyzoites and intracellular forms of *T. gondii* by triggering the NO production as one of the main mechanism actions of FMN. Nevertheless, further surveys are mandatory to assess the effects of FMN in animal models and to evaluate its accurate mechanism actions before use in clinical phase.


**Introduction**
*Toxoplasma gondii* as a protozoan parasite is seen in most warm-blood vertebrates such as humans (1). The parasite is transmitted to humans through ingesting contaminated undercooked meat, contaminated food, and congenital transmission (2).

Despite the fact that the infection is asymptomatic or mild in healthy people, transmission during pregnancy in the first exposure can be very disastrous leading to severe complications, e.g., hydrocephaly, miscarriage, and even death (3). Besides, it has been recognized as opportunistic and fatal in immunocompromised persons, e.g., organ transplant patients (4,5).

Today, pyrimethamine and sulfadiazine are selected as the principal treatment for toxoplasmosis. However, severe complications of these drugs, e.g., suppression of bone marrow, poorly tolerated, inhibition of folate synthesis, leukopenia, megaloblastic anemia, pancytopenia, and cardiac arrhythmia have been reported (6). Additionally, so far no active and efficient vaccine has been introduced commercially for widespread use in humans (7). Consequently, it is essential to discover a new agent with characteristics such as low toxicity and high efficacy against all stages of the parasite (7,8).
Among the polyphenolic compounds, isoflavones \((C_{20}H_{18}O_6)\) are a type of flavonoid compound whose chemical structure is analogous to estrogen (9). They can bind to estrogen receptors in the cell, which is why isoflavones are also called plant estrogens (9). These compounds have a beneficial effect on human health and in particular, preventing cancer and cardiovascular disease and reducing the symptoms of menopause. Therefore, it can be suggested that isoflavones are the active components in soybeans (10). Formononetin (FMN) is a natural isoflavone found in low concentrations in many herbs, mainly the Fabaceae family (11). The chemical name of the pheromone is Biochanin B and the IUPAC name is 7-Hydroxy-4'-methoxyisoflavone; its molecular formula is C16H12O4 (11). Isoflavonoids are found in large quantities in plants such as soybeans and clover. FMN was reported to have antimicrobial, antioxidant, anti-hyperlipidemic, anti-diabetic, anti-tumor, neuroprotective, and cardioprotective activities (12,13). This work was designed to examine the anti-Toxoplasma effects and cytotoxicity properties of FMN on \(T. gondii\).

### Materials and Methods

**Parasite**

Tachyzoites of \(T. gondii\) RH strain were preserved via intraperitoneal passages in mice. After 72 hours, tachyzoites were obtained and centrifuged at 21°C for discarding artifacts and peritoneal cells. By removing the supernatant, the remaining parasites were re-covered with PBS and by a hemocytometer slide and were adjusted into \(1\times10^6\) parasites per mL (14).

**Macrophage cells**

Macrophage cells (J774-A1) were cultured in RPMI1640 liquid media containing 10% inactivated FBS (Merck, Germany) and 1% pen/strep antibiotic at 37°C in the atmosphere; they were incubated in a condition containing 5% CO2 and 95% humidity.

**In vitro effects of FMN on tachyzoites**

To perform this assay, after adding 100 µL of \(T. gondii\) tachyzoites to each well of a 96-well plate containing various concentrations of FMN (purity >99%, 2-64 µg/mL), the plate was kept warm for 48 hours at 37°C. After adding MTT solution (5 mg/mL) to the tested wells, it was again incubated for 4 hours. Followed by adding dimethyl sulfoxide to the wells, their absorbance was evaluated at 570 nm using an ELISA reader (15).

**Effect of FMN on infectivity rate and intracellular parasites**

To evaluate the effect of FMN on infectivity rate and intracellular parasites, at first J774-A1 cells (\(10^5\) cells/mL) were put in the 24-well plate and kept warm at 37°C for 24 hours. After exposing the cells to parasites (\(1 \times 10^5\)/mL) they were again incubated at 37°C for 24 hours. After this time the infected cells were exposed to FMN (2-64 µg/mL) for 48 hours. The treated cells were stained with Giemsa and examined by light microscopy to assess the infectivity and the number of intracellular parasites through the examination of 100 cells (16).

**Nitric oxide (NO) production**

The effects of FMN on NO production in J774-A1 macrophage cells were studied by means of the Nitrite Assay Kit (Griess Reagent, Sigma-Aldrich, Germany) based on the producer’s instructions. Lipopolysaccharides (LPS)+IFN-γ (10 U/mL) were measured as the positive control (17).

**Statistical analysis**

The results were evaluated by means of SPSS software (ver. 26.0). The 50% inhibitory concentrations \((IC_{50})\) were assessed through the probit test. \(P<0.05\) reflected a significant difference.

### Results

**Effects on \(T. gondii\) tachyzoites**

As depicted in Figure 1, FMN significantly \((P<0.001)\) declined the viability rate of tachyzoites compared to the control group. The \(IC_{50}\) values for FMN and pyrimethamine were reported as 9.85 µg/mL and 5.76 µg/mL, respectively.

**Effect on the infectivity of macrophages**

The best inhibitory action of FMN was observed at 64 µg/mL, whereas it significantly reduced the level of infection in macrophages \((P<0.001)\) by 22.1%. FMN in doses of 16 and 32 µg/mL also reduced the infection by 68.1% and 39.4%, respectively \((P<0.001)\) (Figure 2).

**Effect of FMN on intracellular parasites**

As exhibited in Figure 3, FMN mainly at 16, 32, and 64 µg/mL significantly declined the average number of...
Effect of FMN on the NO production in macrophage cells

The findings of Nitrite assay showed that FMN mainly at ½ IC_{50} and IC_{50} markedly elevated (P < 0.001) the NO production in macrophage cells compared with the non-treated cells (Table 1).

Discussion

Today, pyrimethamine and sulfadiazine are selected as the principal treatment of toxoplasmosis. However, severe complications of these drugs, e.g. suppression of bone marrow, poorly tolerated, inhibition of folate synthesis, leukopenia, megaloblastic anemia, pancytopenia, and cardiac arrhythmia have been reported. This work was designed to examine the anti-Toxoplasma effects and cytotoxicity properties of FMN on *T. gondii*. FMN significantly (P < 0.001) declined the viability rate of tachyzoites and the level of infection in macrophages (P < 0.001), whereas significantly declined the average number of parasites inside macrophages.

Numerous investigations have reported the promising antimicrobial properties of FMN on some pathogenic bacterial (*Staphylococcus aureus*, *S. aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa*) and fungal (*Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans*) strains with IC_{50} values from 25 to 200 μg/mL (19). Yang et al showed the antibacterial effects of FMN against *Enterobacter cloacae*, *Escherichia coli*, and *P. aeruginosa* with MICs varying from 0.78 to 50 μg/mL (20). Wang et al reported potent antiviral effects against coxsackievirus B2 (CVB2), CVB3, CVB6, and enterovirus 71 (EV71) in SK-N-SH cells (21). FMN inhibited the adhesion and motility of *Giardia lamblia* trophozoites and reduced the parasite load in mice (22). Even though the exact antimicrobial mechanism action of FMN has not yet been evaluated; however, previous studies reported that isoflavones had antimicrobial mechanisms mainly through the disruption of the plasma membranes, altering cell permeability, increasing leakage of ions and vital materials, reducing the membrane's electrical power, and induction of cellular apoptosis (23,24).

Macrophage cells, through the triggering of NO synthesis and then the release of NO, lead to the elimination and control of the intracellular parasite (25). It has been previously proven that *T. gondii* replication in macrophages inhibits NO production in these cells (26). Our findings revealed that FMN mainly at ½ IC_{50} and IC_{50} markedly elevated (P < 0.001) the NO production in macrophage cells in comparison with the control group. These findings indicated that FMN by increasing NO production could be probably considered as one of the possible mechanisms for controlling and eliminating *T. gondii* parasites.

Conclusion

The present in vitro study as the first step to find new anti-Toxoplasma agents revealed the promising anti-Toxoplasma effects of FMN, a natural isoflavone, against tachyzoites and intracellular forms of *T. gondii*. The accurate anti-Toxoplasma mechanisms of FMN are not clear; however, our results showed that triggering the NO production might be considered one of the main mechanism actions of FMN for controlling and eliminating *T. gondii*. However, further surveys are needed.
mandatory to assess the effects of FMN in animal models and to evaluate its accurate mechanism actions before use in the clinical phase.

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Authors’ contribution
JGY supervised the study, HM, AKK, NK, PZR, and LM reviewed and contributed to data collection and preparation of the manuscript. The first draft was prepared by HM and MS. All authors read the final version and confirmed it for publication.

Conflict of interests
The authors declare that there are no conflicts of interest.

Ethical considerations
The authors confirm it for publication.

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