J Herbmed Pharmacol. 2023; 12(4): 592-596.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2023.44897

Journal of Herbmed Pharmacology

Antiparasitic and cytotoxicity effects of 7-hydroxy-4′-methoxy isoflavone against *Leishmania major*

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ARTICLE INFO

Article Type:
Short Communication

Article History:
Received: 17 January 2023
Accepted: 17 April 2023

Keywords:
Leishmaniasis
Promastigote
Amastigote
Nitric oxide
Macrophage

ABSTRACT

Introduction: Leishmaniasis caused by *Leishmania* spp. is observed in most parts of the world. Although, glucantime, a pentavalent antimony compound, and other synthetic drugs are broadly applied for leishmaniasis therapy; however, the use of these synthetic agents has some limitations. Hence, this study was designed to assess the inhibiting effects of 7-hydroxy-4′-methoxyisoflavone (7HMI) against promastigote and amastigote stages of *Leishmania major* in vitro.

Methods: The MTT assay was applied to study the antileishmanial activity of 7HMI against promastigotes and its cytotoxicity effects on macrophage cells. The nitric oxide (NO) produced by the treated macrophage cells with 7HMI was also assessed.

Results: 7HMI considerably ($P<0.05$) inhibited the growth rate of promastigotes and amastigotes stages. The 50% inhibitory concentrations of 7HMI and glucantim were 11.3 and 15.4 µg/mL for promastigote and amastigote, respectively. 7HMI, especially at 1/3 IC_{50} and 1/2 IC50 concentrations, considerably triggered the NO release.

Conclusion: The current research findings reported the favorable antileishmanial effects of 7HMI against *L. major* with possible mechanisms such as reducing the infectivity rate of macrophage cells and provoking NO creation. Nevertheless, more research must be performed to clear its efficacy in animal model and then in human.

Implication for health policy/practice/research/medical education:
We reported the favorable antileishmanial effects of 7HMI against L. major with possible mechanisms such as reducing the infectivity rate of macrophage cells and provoking NO creation. Hence, 7HMI might be considered for preparation of a new drug against L. major Nevertheless, more researches must be performed to determine its efficacy in vivo and clinical subjects.

Please cite this paper as: Aghaei M, Alizadegan F, Raziani Y. Kishore G, Saadatmand M, Kumar SA. Antiparasitic and cytotoxicity effects of 7-hydroxy-4′-methoxy isoflavone against *Leishmania major*. J Herbmed Pharmacol. 2023;12(4):592-596. doi: 10.34172/jhp.2023.44897.

Introduction

Leishmaniosis caused by *Leishmania* spp. is observed in most parts of the world (1,2). Leishmaniasis can be clinically separated into four categories: cutaneous, mucocutaneous, diffuse, and kala-azar (3). The cutaneous form is more frequent and is found in abundance in some countries such as Iran (4). At present, various methods, including local radiation therapy, burning the lesion site, cryotherapy, and local infiltration of drugs have been used to treat cutaneous leishmaniasis. Although, glucantime (meglumine antimoniate; MA), a pentavalent antimony compound, and other synthetic drugs are broadly applied for leishmaniasis therapy (5,6); however, the use of these synthetic agents has displayed some limitations, e.g., high cost, drug resistance, and adverse effects, resulting in increased efforts to discover the alternative therapies (7,8). Effective medications from herb derivatives or extracts are likely a valuable source of new therapeutic agents (8); whereas there are more than 200 000 pharmaceutical herb species worldwide (8). In recent tears, natural products and some constituents isolated from them have represented potent anti-leishmanial effects (9). Isoflavones as a

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subset of flavonoid constituents in herbs have displayed valuable pharmacological properties in modern medicine (9,10). 7-Hydroxy-4′-methoxyisoflavone (7HMI) or formononetin as a natural isoflavone, found in many herbs (11), has shown a wide range of pharmacological properties such as antioxidant, anticancer, anti-hyperlipidemic, anti-diabetic, and antimicrobial activities (12,13). Hence, this study was designed to assess the inhibiting effects of 7HMI against promastigote and amastigote stages of *Leishmania major* in vitro to find and introduce the novel antileishmanial agent.

Materials and Methods

Cell and parasite

Leishmania major (MRHO/IR/75/ER) and J774-A1 macrophage cell lines (Pasteur Institute, Iran) were cultured in 1640 RPMI medium (Sigma-Aldrich, Germany) with fetal bovine serum (10%), penicillin/streptomycin (100 mL/IU) at 24 ± 1 and 37°C, respectively (14).

Inhibitory effects on promastigotes forms

The inhibitory effects of 7HMI on promastigote forms of *Leishmania* were performed by the MTT assay based on a previous study (15). Promastigotes (1×10^6) were exposed to 7HMI (Sigma-Aldrich, Germany, at 1.56-25 µg/mL) and amphotericin B at 24°C for 48 hours. Followed by adding MTT solution (0.5 mg/mL), the optical density of the mixture was measured at 570 nm by an ELISA plate reader.

Inhibitory effects on amastigotes forms

The inhibitory effects of 7HMI on amastigote forms of *Leishmania* was performed by the macrophage model based on a previous study (16). Briefly, promastigotes (1×10^6/mL) in stationary phase (at ratio of 10:1) were exposed to macrophage cells (1×10^5/mL) at 37°C in 5% CO_2 for 24 hours. Then, macrophages were exposed to 7HMI (6.25-200 µg/mL) and MA for 48 hours and then the number of amastigotes were recorded through preparing smears.

Effect of 7HMI on the infectivity rate

The effect of 7HMI on the infectivity rate of macrophages was assessed based on the method explained by Mahmoudvand et al (17) through exposing the promastigotes to 7HMI for 2 hours and then exposing to macrophages for 24 hours. The number of infected macrophages were recorded through preparing smears.

Cytotoxicity against macrophages cells

The cytotoxic effects of 7HMI on macrophage cells (1×10^5/mL) were performed by the MTT assay based on a previous study and in the same conditions of cytotoxic effects on promastigotes forms (18).

The selectivity index (SI) measurement

The SI of 7HMI was measured by dividing the CC_{50} value of macrophage cells on IC_{50} value of amastigote forms; whereas SI value more than 10 indicated promising antileishmanial effects of 7HMI on intracellular amastigotes with no cytotoxic effects on host macrophage cells (19).

Effect on nitric oxide (NO) generation

The effect of 7HMI on NO production in macrophage cells was studied by Greiss reagent assay using the commercial kit (Sigma-Aldrich, Germany) based on the producer instructions. Lipopolysaccharide (10 ng/mL) + IFN-γ (10 U/mL) was considered as the positive control (20).

Statistical analysis

SPSS software version 25.0 was applied to data analysis and one-way analysis of variance (ANOVA) was utilized for the comparison of groups. The significance level was considered as $P<0.05$.

Results

The 7HMI and AmB markedly ($P<0.05$) inhibited the

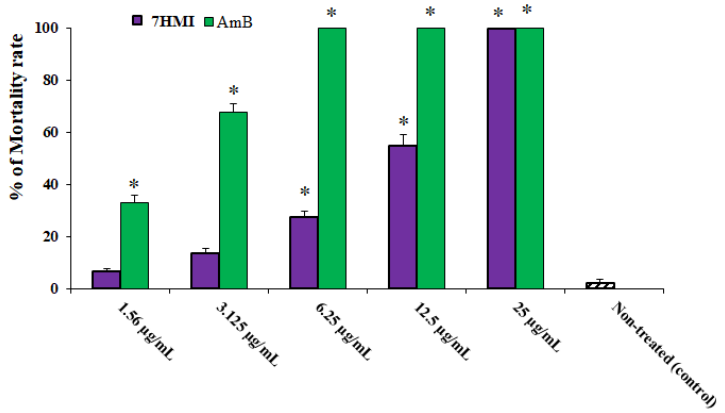

Figure 1. Anti-promastigote activity of 7-hydroxy-4′-methoxyisoflavone (7HMI) and amphotericin B (AmB) on the mortality rate of promastigotes of Leishmania major. *$P<0.05$ compared to the non-treated promastigotes.

growth of *L. major* promastigotes (Figure 1) with the IC_{50} value of 11.4 µg/mL and 2.31 µg/mL, respectively (Table 1).

By anti-amastigote assay, 7HMI and MA displayed significant antileishmanial activities on amastigote forms with a dose-dependent response (Figure 2) with the IC_{50} values of 18.9 and 21.4 µg/mL, respectively (Table 1).

Figure 3 shows the cytotoxicity effects of 7HNI and MA against macrophage cells. The CC_{50} levels of the 7HMI and MA were 159.3 and 874.6 µg/mL, respectively (Table 1). The measured SI values for 7HMI and MA were 10.3 and 40.8, respectively.

Effect of 7HMI on the infectivity rate

The exposure of promastigotes to the 7HMI and MA declined the rate of infected macrophages from 77.4 ± 5.26 to 34.6 ± 3.21 and 32.3 ± 3.21, representing the infection rate by 44.7% and 55.2%, respectively ($P < 0.05$).

Effect on NO production

Followed by the exposure of the macrophages to 7HMI, the NO release was significantly increased ($P < 0.001$). Table 2 shows the level of NO production in the treated macrophages.

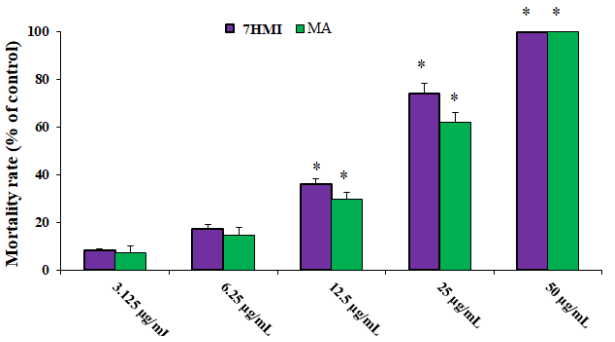

Figure 2. Anti-amastigote activities of 7-Hydroxy-4′-methoxyisoflavone and meglumine antimoniate on the mortality rate of amastigotes of Leishmania major. * $P < 0.05$ compared to non-treated promastigotes (control).

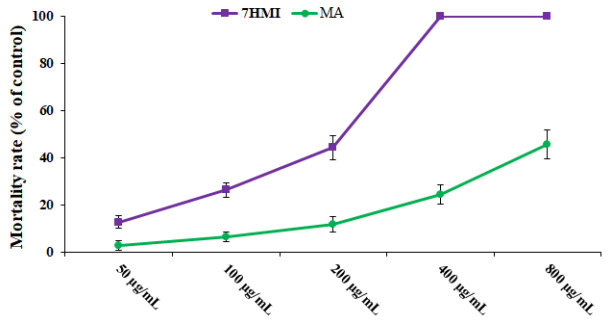

Figure 3. Cytotoxic activity of 7-Hydroxy-4′-methoxyisoflavone (7HMI) and meglumine antimoniate (MA) on the mortality rate of macrophage cells.

Discussion

Chemical and synthetic medications, which are widely utilized for cutaneous leishmaniasis therapy, have displayed several limitations, e.g., high cost, drug resistance, and adverse effects resulting in increased efforts to discover the alternative therapies (7,8). Natural products and some constituents isolated from them have represented the potent anti-leishmanial effects (9). Thus, this work aimed to assess the inhibiting effects of 7HMI against promastigote and amastigote stages of *L. major* in vitro to find and introduce a novel antileishmanial agent. We found that 7HMI markedly ($P<0.001$) inhibited the growth rate of promastigote and amastigote forms of *L. tropica*.

In recent years, the antileishmanial effects of a number of flavones-rich compounds, e.g., luteolin, 7,8-dihydroxyflavone, rhamnetin, 3-hydroxyflavone, catechol, 7,8,3′,4′-tetrahydroxyflavone, and apigenin against *L. amazonensis L. donovani,* and *L. tropica* have been reported (21,22). A review reported the potent antimicrobial effects of 7HMI against *Staphylococcus aureus, S. aureus, S. epidermidis, Pseudomonas aeruginosa*, *Candida albicans, C. tropicalis, Cryptococcus neoformans,* and enterovirus-51 viruses (23-25). A previous stud also reported that 7HMI significantly repressed the attachment, flagellar motility, and viability of *Giardia* trophozoites in mice (26). The precise antimicrobial mechanism action of isoflavonoids has not yet been reported; however, previous studies have reported that isoflavonoids act mainly through the membrane, disrupting the cell permeability, which subsequently cause the leakage of vital metabolites, minerals, and contents, e.g., amino acids, ions, and

Table 1. Antileishmanial and cytotoxic effects of 7HMI, amphotericin B, and glucantime by determining IC_{50} and () values and SI

Material	IC_{50} (µg/mL)		CC_{50} (µg/mL)	SI
	Promastigote	Amastigote		
7HMI	11.4 ± 1.56	18.9 ± 2.16	195.3± 6.45	10.3
Glucantime	-	21.4 ± 3.12	874.6± 14.2	40.8
Amphotericin B	2.31 ± 0.087	-	-	-

7HMI, 7-Hydroxy-4′-methoxyisoflavone; IC_{50}, 50% inhibitory concentration; SI, selectivity index; CC_{50}, 50% cytotoxic concentration.
Data are expressed as mean ± standard deviation (n=3).

Table 2. The effect of 7HMI on NO generation in J774-A1 macrophage cells in comparison with the control groups (Mean ± SD)

Material	NO production (nM)
4.72 µg/mL	3.12 ± 0.24
6.3 µg/mL	4.24 ± 0.85 **
9.45 µg/mL	12.3 ± 1.42 **
Non-treated	2.49 ± 0.31
IFN-γ+LPS	30.24± 4.45

IFN-γ: Gamma interferon; LPS: Lipopolysaccharide; 7HMI, 7-Hydroxy-4′-methoxyisoflavone; NO, nitric oxide.
*** $P < 0.001$ compared with the non-treated cells.

calcium (9-12,27,28).

Host immune cells, such as macrophage cells, play an important role in eliminating the intracellular parasites such as *Leishmania* through provoking NO production (29). Additionally, the inhibition of infection in macrophage cells is considered a principle mechanism developing new agents (20). The results of this work showed that the exposure of promastigotes to 7HMI and MA significantly declined the rate of infected macrophages, while the NO production was significantly increased. By the cytotoxicity activity of 7HMI, the CC_{50} levels of 7HMI and MA were 159.3 and 874.6 µg/mL, respectively. The measured SI values for 7HMI and MA were 10.3 and 40.8, respectively. The SI>10 indicated their specificity to *L. major* amastigotes with the minimum cytotoxicity on macrophage cells.

Conclusion

The current research findings suggested the favorable antileishmanial effects of 7HMI against *L. major* with possible mechanisms such as reducing the infectivity rate of macrophage cells and provoking NO creation. Nevertheless, more clinical researches must be performed to clear its efficacy.

Author contribution

MA and GK designed the experiments; FA, MA, and MA performed experiments and collected data; FA, YR, and SAK discussed the results and strategy; MA supervised, directed, and managed the study; all authors approved the final version to be published.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The work was permitted by the Ethics Committee of Visveswarapura Institute of Pharmaceutical Sciences, Rajiv Gandhi University of Health Sciences, Bangalore, India (14Q0028).

Funding/Support

The authors declare that they have not received any financial support.

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