In vitro antileishmanial activity of fisetin flavonoid via inhibition of glutathione biosynthesis and arginase activity in *Leishmania infantum*

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ABSTRACT

With the increasing emergence of drug resistant *Leishmania* sp. in recent years, combination therapy has been considered as a useful way to treat and control of Leishmaniasis. The present study was designed to evaluate the antileishmanial effects of the fisetin alone and combination of fisetin plus Meglumine antimoniate (Fi-MA) against *Leishmania infantum*. The IC₅₀ values for fisetin were obtained 0.283 and 0.102 μ M against promastigotes and amastigote forms, respectively. Meglumine antimoniate (MA, Glucantime) as control drug also revealed IC₅₀ values of 0.247 and 0.105 μ M for promastigotes and amastigotes of *L. infantum*, respectively. In order to determine the mode of action of fisetin and Meglumine antimoniate (MA, Glucantime), the activities of arginase (ARG), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured. Moreover, intracellular glutathione (GSH) and nitric oxide (NO) levels in *L. infantum*-infected macrophages and *L. infantum* promastigotes which were treated with IC₅₀ concentrations of fisetin, MA and Fi-MA were investigated. Our results showed that MA decreased CAT and SOD activity and increased NO levels in *L. infantum*-infected macrophages. In promastigotes, MA inhibited parasite SOD activity and reduced parasite NO production. The decreased levels of most of the antioxidant enzymes, accompanying by the raised level of NO in treated macrophages with MA, were observed to regain their normal profiles due to Fi-MA treatment. Furthermore, fisetin could prevent the growth of promastigotes by inhibition of ARG activity and reduction of GSH levels and NO production. In conclusion, these findings showed that fisetin improves MA side effects.

KEYWORDS

Leishmania infantum; fisetin; arginase; nitric oxide; glutathione; antioxidant enzymes

Introduction

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa and transmitted by a sand fly vector. Visceral Leishmaniasis (VL) caused by parasites of *Leishmania donovani* complex (*L. donovani infantum* in the Old World; *L. donovani chagasi* in the New World) is a severe human disease which often leads to death if left untreated [1].

L-arginine is one of the most versatile amino acids that is found in all tissues and microorganisms, and used as a precursor for the synthesis of protein, nitric oxide (NO), urea, ornithine, citrulline, creatinine, agmatine, glutamate, proline and polyamines [2]. In the macrophages, L-arginine based on the expression of inducible nitric oxide synthase (iNOS) and ARG enzymes can be catabolized in two different ways, which competitively controlled by Th1 and Th2 cytokines. Th1 cytokines induce iNOS expression and subsequently NO production, thus promoting parasite killing by macrophages, whereas Th2 cytokines determine the induction of host ARG, which are associated with parasite growth and proliferation

[3,4]. Hydrolysis of L-arginine amino acid by ARG leads to the production of L-ornithine and urea in the first step of polyamine biosynthesis (including putrescine, spermidine and spermine). Ornithine decarboxylase (ODC) is an enzyme in the polyamines pathway that catalyzes the conversion of L-ornithine to putrescine. The decarboxylated S-adenosylmethionine (dAdoMet) donates an aminopropyl group to putrescine to form spermidine. This reaction is catalyzed by spermidine synthase (SpdS) [5]. Polyamines have an important role in cell growth and differentiation [6]. Some lower eukaryotes like *Leishmania* also have a unique L-arginine metabolism pathway [7]. Generated spermidine in this pathway along with glutathione (GSH) is used for the synthesis of trypanothione (T(SH)₂), which plays an important role in the thiol redox balance, in the synthesis of deoxyribonucleotides, drug resistance and in the defense against chemical and oxidative stress [8–10]. ARG from *Leishmania* is the first enzyme in the polyamine pathway, and blocking it can lead to reduce production of T(SH)₂, redox imbalance and promote infection control [11–15]. Thus, ARG

seems to be a pharmacological target of interest for the development of new anti-leishmaniasis drugs.

Fisetin is one of the polyphenolic flavonoid, which is mainly can be found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber [12]. This flavonoid possesses a high potency in ARG inhibition of *Leishmania* while it doesn't have a significant effect on host ARG [16].

For more than 60 years pentavalent antimonials (e.g. sodium stibogluconate and Meglumine antimoniate) have been used as the primary treatment of Leishmaniasis. Antimonials are less effective against various forms of Leishmaniasis and resistance is emerging [17,18]. In addition, antimonial-based therapy has disadvantages that include the long-term treatment requiring parenteral administration under close medical supervision due to high toxicity (arthralgia, nausea, abdominal pain; chemical pancreatitis and cardio toxicity associated with high doses) [19]. The appeal to a combined therapy has been suggested as a possible way to increase treatment efficacy, prevent the development of drug resistances and reduce the treatment's duration [20]. In our study, fisetin was selected for combination therapy with MA based on *in vitro* inhibitory activity against recombinant ARG of *L. amazonensis* [16]. Therefore, the aim of current study was to evaluate fisetin antileishmanial effect based on its role in inhibition of T (SH)₂ production by blocking parasite ARG activity, reduction of parasite defense capability against oxidative stress and perturbing the thiol redox potential of the parasite. In order to gain a better insight into the mechanism of fisetin and antimonial compounds, we determined inhibitory concentration (IC₅₀) of fisetin and MA using colorimetric assay (MTT). Then, the effect of fisetin and Fi-MA were evaluated on levels of ARG and SOD activity in promastigotes and levels of ARG, CAT, GPx and SOD activity in macrophages containing amastigotes of *L. infantum*. Moreover, NO and GSH levels were measured in promastigotes and *L. infantum*-infected macrophages, respectively.

Materials and methods

Reagents and equipment

Fisetin with 98% purity was purchased from Sigma-Aldrich and Meglumine antimoniate (MA, Glucantime Rhône-Poulenc, France) obtained from a commercial source. Fisetin was dissolved in dimethyl sulfoxide (DMSO) as a solvent. DMSO was obtained from Merck Company (Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Invitrogen Company (Scotland, UK). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Parasite culture

Promastigotes of standard strain of *L. infantum* (MCAN/IR/07/moheb-gh) were obtained from Medical Science University of Shiraz and subcultured in RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C, 30 min) FBS and 1% penicillin/streptomycin (P/S) mixture. The parasites were incubated at 26 °C and sub-passaged weekly.

Cell culture

Raw 264.7, a murine monocyte/macrophage cell line, was purchased from the Iranian biological resource center. Cells were cultured in RPMI-1640 supplemented 10% heat-inactivated (56 °C, 30 min) FBS and 1% P/S mixture. Culture mediums were changed three times per week. Subcultures were performed when monolayers completely covered the bottom of culture flasks.

Cell viability

The cytotoxicity effects of fisetin (0 to 40 μM) on raw 264.7 macrophage cell line were investigated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as previously described by Lyu et al [21]. Macrophages (10⁶ cells/well) were cultured in 96-well culture plates in the absence or presence of fisetin and incubated 24 h at 37 °C with 5% CO₂. Then, 50 μl MTT was added to each well and the plate was incubated for a further 4 h. Finally, DMSO was added to dissolve the formazan dye. The optical density was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular devices, Menlo Park, CA, U.S.A.) at 570 nm.

Determination of fisetin IC₅₀ against *L. infantum* promastigotes

MTT assay was used to evaluate IC₅₀ of fisetin against promastigotes. *L. infantum* promastigotes were cultured in 96-well plates (1 × 10⁶ parasites/well) in the presence of various concentrations of fisetin (final concentration in cultures: 0.028, 0.056, 0.112, 0.224, 0.443, 0.896 and 1.792 μM) and MA (final concentration in cultures: 0.021, 0.043, 0.087, 0.174, 0.349, 0.698 and 1.397 μM). Wells with medium and promastigotes were used as positive control and medium with no organism was considered as blank. Twenty-four hours after treatment, MTT was added to cells in culture and the plate was incubated for 4 h at 37 °C. Finally, DMSO was added to dissolve the formazan dye. The absorbance was measured at 570 nm. Viability percentage was calculated by:

$$\left[\frac{(AT - AB)}{(AC - AB)} \right] \times 100$$

Where, AB is OD of blank well, AC is OD of the negative control and AT is OD of treated cells.

Determination of fisetin IC₅₀ against *L. infantum* amastigotes

Infection of macrophages was performed in 12-well culture plates (10⁶ cells/well) as described by Méndez et al [22]. Briefly, raw 264.7 macrophages were flashed by phosphate-buffered saline (PBS) from culture flasks, centrifuged (1500g), resuspended in a complete culture medium, and counted in Neubauer chambers. Cell viability was determined by Trypan blue exclusion dye. One milliliter of cell suspensions with density (10⁶ cells/well) was added to culture chambers. Raw 264.7 macrophages were allowed to adhere for 3 h at 37 °C with air/5% CO₂ atmosphere. Stationary phase promastigotes were added to cultures (10 promastigotes: 1 macrophage) and kept overnight at 37 °C in the presence of 5% CO₂. Then, cultures were treated with various concentrations of fisetin (final concentration in cultures: 0.014, 0.028, 0.056, 0.112 and 0.224 µM) and MA (final concentration in cultures: 0.010, 0.021, 0.043, 0.087 and 0.174 µM) and incubated overnight at 37 °C with 5% CO₂. *L. infantum*-infected macrophages and uninfected macrophages were considered as positive and negative controls, respectively. The medium of culture chambers was removed, and adherent macrophages were flashed by PBS. Macrophages were fixed and stained with Giemsa (1:10 dilution), and Multiplication Index (MI) determined using the following formula:

$$MI = (\text{mean number of amastigotes per 100 treated macrophages} / \text{mean number of amastigotes per control 100 macrophages}) \times 100$$

Cultures grouping and drug treatment

After determining fisetin and MA IC₅₀ for amastigotes, five groups with three replications were considered for biochemical analysis: uninfected macrophages (M) and *L. infantum*-infected macrophages (MP) were considered as negative and positive controls, respectively.

L. infantum-infected macrophages (MP) which treated with fisetin, MA and Fi-MA IC₅₀ concentrations were considered as MP + F, MP + MA and MP + Fi-MA groups, respectively. Furthermore, promastigotes were divided into four groups with three replications, which are as follows: promastigotes treated with fisetin, MA and Fi-MA IC₅₀ concentrations were considered as P + F, P + MA and P + Fi-MA groups, respectively and promastigotes were used as control (C).

Cell lysis

Twenty-four hours after treatment, The culture medium of each well was removed and adherent macrophages were flashed with PBS. The cell suspension was transferred into a sterile microcentrifuge tube, centrifuged at

2000 rpm for 5 min, and removed supernatant. The cell pellet was washed three times in cold PBS and lysed in 500 µl of Radioimmunoprecipitation assay buffer (RIPA) on ice for 30 min. The resulting mixture was centrifuged at 10,000 rpm for 20 min, and the supernatant transferred into cryotubes for further analysis. Promastigotes lysis was conducted in the same manner. Protein concentration in the cell extracts was estimated by the Bradford method [23].

Biochemical analysis

Nitric oxide (NO) assay

NO levels in cell extract were determined using the protocol provided by the manufacturer of the kit (ZellBio GmbH NO assay kit (Cat.NO. ZB-NO-96A, V406)). NO levels were calculated from a standard curve and expressed as nmol/mg of protein.

Glutathione peroxidase assay

GPx was measured using a kit supplied by Randox Laboratories Ltd (Cat. No. RS505). This assay was modified for the microplate method. In summary, 200 µl RANSEL reagent (Reagent R1) and 8 µl cumene hydroperoxide (Cumene R2) were added to 40 µl of cell extract and initial absorbance of samples and blank (reaction mixture without cell extract) were measured at 340 nm after one minute and start timer simultaneously. GPx enzyme levels were expressed as mU/mg of protein.

Superoxide dismutase assay

SOD activity was measured according to the method described by Jain et al [24]. This method is based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium by superoxide. Briefly, the reaction mixture (250 µl) contained 130 µl of sodium carbonate buffer (50 mM), 30 µl of nitrobluetetrazolium (60 µM), 20 µl of Triton X-100 (0.6%) and 50 µl of hydroxylamine - HCl (20 mM). Subsequently, 20 µl of cell extract was added and absorbance (560 nm) was read for 5 min against blank (reaction mixture without cell extract). Change in absorbance per min (i.e. ΔA560) was calculated and used in the estimation of enzyme activity and results were reported as mU/mg of protein.

Catalase assay

CAT activity was measured by the method described by Korolyuk et al [25]. Briefly, the reaction mixture (205 µl) including 50 µl Tris HCl buffer (0.05 mM), 100 µl H₂O₂ (10 mM) in Tris HCl and 5 µl cell extract were added to 96 well plate. After 10 min at room temperature, 50 µl ammonium molybdate 4% was added to each well and

absorbance (410 nm) was read against blank (reaction mixture without cell extract). Then the activity of CAT is calculated using this formula:

CAT activity ($\mu\text{mol}/\text{min}$)

$$= (\text{Control absorbance} - \text{Sample absorbance}) / \text{Control absorbance} \times \text{Control concentration} / 10$$

CAT activity was expressed as IU/mg of protein.

Arginase assay

This method was based upon the colorimetric determination of released urea nitrogen using a commercial kit (Pars Azmoon Co., Tehran, Iran) [26]. In summary, 20 μl MnSO_4 (0.05 M) and Maleic acid (0.05 M), PH 7.0 were added to 20 μl of cell lysis and the enzyme was then activated for 4 h at 37 °C. Arginine hydrolysis was performed by the addition 20 μl of L-arginine (0.5 M), pH 9.5 to a 20 μl aliquot of the previously activated lysate. The mixture was incubated at 37 °C for 12 min and released urea was evaluated using a commercial kit (Pars Azmoon Co., Tehran, Iran). ARG activity was expressed as $\mu\text{mol}/\mu\text{g}$ of protein.

Glutathione assay

GSH was assayed according to the method of Ellman [27], with slight modification. Briefly, 260 μL of reaction buffer containing sodium phosphate (0.1 M) and EDTA (1 mM), pH 8.0 was added to 96 well plate, followed by 15 μL cell extract. 5 μL Ellman reagent solution including 4 mg Ellman reagent (5_5_dithio-bis) (2-nitrobenzoic acid) (DTNB) in 1 ml reaction buffer was then added to the mixture. Blanks were prepared as above, with the exception that there was no DTNB in the reaction buffer. Standard stock (1.5 mM) was made by dissolving cysteine hydrochloride monohydrate in reaction buffer. A standard curve was obtained with increasing amount of standard stock between 5 to 25 μl . Following incubation for 15 min at room temperature, sample absorbance was read at 412 nm on an ELIZA reader. The concentration of GSH was evaluated using standard curve and results were reported as nmol/mg of protein.

Statistical analysis

One-way ANOVA and followed by the Sidak's multiple comparisons test was employed to analyze the biochemical results and the difference at $p < 0.05$ was considered as significant. IC50 determination and data analysis were calculated by GraphPad prism software for Windows.

Results

Antileishmanial effects

Growth inhibitory effect of different concentrations of fisetin and MA on the promastigotes were determined

after 48 h, and cell viability evaluated using MTT assay. The finding indicated that fisetin and MA had potent antileishmanial activity against the promastigote stage based on a dose-dependent response ($p < 0.05$) (Figure 1). The IC50 value for fisetin was obtained 0.283 μM against promastigotes of *L. infantum*, whereas this value was 0.247 μM for MA.

The results showed that fisetin and MA inhibited the multiplication of intra-macrophage amastigotes in a dose-dependent manner ($p < 0.05$) (Figure 1). The IC50 value for fisetin and MA against amastigotes of *L. infantum* were evaluated 0.102 and 0.105 μM , respectively. The IC50 value of fisetin in raw 264.7 uninfected macrophages was obtained 24.38 μM (Figure 1).

Evaluation of biochemical parameters in *L. infantum*-infected macrophages and promastigotes

There was no significant change in GSH levels in positive control with respect to negative control ($p > 0.05$), while a significant increase in GSH level was seen in *L. infantum*-infected macrophages treated with fisetin ($p < 0.0001$) and Fi-MA ($p < 0.05$) as compared to positive control (Figure 2(a)). GSH levels in *L. infantum*-infected macrophages treated with MA remained constant with respect to positive control ($p > 0.05$).

A significant decrease in NO level was seen after parasite infection in positive control as compared to negative control ($p < 0.01$). However, the decreased NO levels in positive control significantly enhanced after MA treatment (MP + MA) ($p < 0.005$). Treatment of *L. infantum*-infected macrophages with fisetin (MP + F) and Fi-MA (MP + Fi-MA) had no significant effect on NO levels as compared to positive control ($p > 0.05$) (Figure 2(a)).

According to Figure 2(b), GSH levels significantly decreased in promastigotes treated with fisetin ($p < 0.0001$) and Fi-MA ($p < 0.001$) with respect to Control. However, treatment of promastigotes with MA had no significant effect on intracellular GSH levels ($p > 0.05$).

Treatment of promastigotes with MA (P + MA), fisetin (P + F) and Fi-MA (P + Fi-MA) resulted in a significant decrease in NO levels as compared to control group ($p < 0.0001$) (Figure 2(b)).

The CAT activity significantly increased after parasite infection in positive control as compared to negative control ($p < 0.01$), but treatment of *L. infantum*-infected macrophages with MA (MP + MA) lead to a significant decrease in CAT activity ($p < 0.01$). In contrast, the CAT activity in *L. infantum*-infected macrophages treated with fisetin (MP + F) significantly increased relative to positive control ($p < 0.0001$). Treatment of *L. infantum*-infected macrophages with Fi-MA (MP + Fi-MA) had no significant effect on CAT activity with respect to positive control ($p > 0.05$) (Figure 3(a)).

Figure 1. Effects of different concentrations of fisetin and meglumine antimoniate (MA) on the multiplication index (MI) of amastigotes and viability of *Leishmania infantum* promastigotes in comparison with corresponding control groups.

Notes: Values have been represented as mean $\pm$ SD, where $n = 3$ and each experiment is done in triplicate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ and **** $p < 0.0001$ vs. control group according to one-way ANOVA followed by the Sidak's multiple comparisons test (a). The effect of fisetin on cell viability of raw 264.7 cells (b).

It is noteworthy to mention that the treatment of *L. infantum*-infected macrophages with MA, fisetin and Fi-MA had no significant effect on GPx and ARG activity in any of the examined samples relative to corresponding control groups ($p > 0.05$) (Figure 3(a)).

The SOD activity remained unchanged in positive [control ($p > 0.05$), while a significant decrease in SOD activity was observed in *L. infantum*-infected macrophages treated with MA (MP + MA) relative to positive control ($p < 0.01$). The SOD activity levels in *L. infantum*-infected macrophages treated with fisetin (MP + F) were significantly increased with respect to positive control

($p < 0.0005$). Moreover, no significant changes in SOD activity levels could be observed in *L. infantum*-infected macrophages treated with Fi-MA (MP + Fi-MA) relative to positive control ($p > 0.05$) (Figure 3(a)).

A significant decrease in ARG activity was seen in promastigotes treated with fisetin ($p < 0.001$) and Fi-MA ($p < 0.05$) relative to control, while treatment of promastigotes with MA have not shown any changes in ARG activity as compared to control ($p > 0.05$) (Figure 3(b)).

There was a significant decrease in SOD activity levels in promastigotes treated with MA ($p < 0.01$) and Fi-MA ($p < 0.0005$) with respect to control, but the treatment

Figure 2. Effects of inhibitory concentration (IC₅₀) of fisetin (F), Meglumine antimoniate (MA) and fisetin in combination with MA (Fi-MA) on GSH and NO levels in *Leishmania infantum*-infected macrophages and promastigotes are shown in Figure 2(a) and (b), respectively.

Notes: Values have been represented as mean $\pm$ SD, where $n = 3$ and each experiment is done in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ and ***** $p < 0.0001$ vs. control group according to one-way ANOVA followed by the Sidak's multiple comparisons test.

of promastigotes with fisetin did not cause a significant change in SOD activity ($p > 0.05$) (Figure 3(b)).

Discussion

Combination therapy has been considered as a way to discover more effective treatment, decreasing toxicity and resistance [28]. In the present study, the effect of fisetin as ARG inhibitor and MA on the growth of promastigote and amastigote stages of *L. infantum* has been evaluated.

Oxidative stress in mammalian cells is prevented by antioxidant enzymes. For example, SOD enzyme is responsible for the conversion of superoxide anions (O_2^-) produced by phagocytes to hydrogen peroxide (H_2O_2). Generated H_2O_2 in this process can be reduced into water and oxygen by CAT. Moreover, GPx enzyme responsible

for the reduction of H_2O_2 and hydroperoxides (ROOH) utilizing reduced glutathione as hydrogen donor [29]. In the present study, elevation of CAT activity in macrophages after parasite infection is due to the compensatory up-regulation of this antioxidant enzyme after the initial generation of reactive oxygen species (ROS) [30]. In addition, *Leishmania* possesses an antioxidant defense system for detoxification of ROS and reactive nitrogen species (RNOS). These parasites are able to scavenge H_2O_2 , peroxynitrite ($ONOO^-$) and radiation-induced radicals by trypanothione (T (SH)₂)/Tryparedoxin (TXN) coupled with high efficiency [31]. The reducing equivalents transfer to TXN by T (SH)₂. Tryparedoxin peroxidase (TXNPs) and Peroxiredoxin (PRX) catalyze the reduction of H_2O_2 , hydroperoxide (ROOH) and $ONOO^-$ using TXN as the electron donor and thus overcome the oxidative stress induced by the host defense mechanisms [32].

Figure 3. Effects of inhibitory concentration (IC₅₀) of fisetin, Meglumine antimoniate (MA) and fisetin plus MA on catalase (CAT), glutathione peroxidase (GPx), arginase (ARG) and superoxide dismutase (SOD) activity levels in *Leishmania infantum*-infected macrophages and ARG and SOD activity levels in promastigotes are shown in Figure 3(a) and (b), respectively.

Notes: Values have been represented as mean $\pm$ SD, where $n = 3$ and each experiment is done in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ and ***** $p < 0.0001$ vs. control group according to one-way ANOVA followed by the Sidak's multiple comparisons test.

Therefore, NO levels reduction in *Leishmania*-infected macrophages could be related to activation of T (SH)₂/TXN system, which acts as parasite antioxidant defense. However, MA treatment decreased SOD and CAT activity and raised NO levels in macrophages containing amastigotes that could be the result of inactivation of

these enzymes by interaction with oxygen radicals [33]. Moreover, MA treatment via activation of PI3 K and p38 MAPK pathways induce NO production in *Leishmania*-infected macrophages [34]. The production of highly ROS and RNOS induced by MA is involved in the progression of *Leishmaniasis* and some of its toxic adverse effects [35].

Figure 4. The activities of the enzymes arginase (ARG) and inducible nitric oxide synthase (iNOS) are explained in parasite and macrophage. L-Arginine is a precursor for the synthesis of urea and L-ornithine by the ARG enzyme and for the synthesis of nitric oxide (NO) and L-citrulline by the enzyme iNOS. L-ornithine is metabolized by the enzymes ornithine decarboxylase (ODC) and ornithine aminotransferase (OAT) to produce polyamines (including putrescine, spermidine and spermine) and L-proline, respectively. Superoxide anions (O_2^-) can combine with nitric oxide (NO) to generate peroxynitrites ($ONOO^-$) or with water (H_2O) to produce hydrogen peroxide (H_2O_2). The dismutation of superoxide to oxygen (O_2) and H_2O_2 catalyzes by superoxide dismutase (SOD). Both the H_2O_2 from O_2^- disproportionation and the $ONOO^-$ may be reduced at the expense of glutathione (GSH) in reactions that are catalyzed by glutathione peroxidase (GPx) and catalase (CAT). GSH is formed from glutamate (Glu), cysteine (Cys) and glycine (Gly) in two ATP-dependant steps catalyzed by γ -glutamylcysteine synthetase (γ -GCS) and glutathione synthetase (GluS). The generated spermidine by spermidine synthase (SpS) accompanying with two molecules of GSH produce glutathionylspermidine (GSP) as a precursor of trypanothione ($T(SH)_2$). GSP is then converted into $T(SH)_2$ by trypanothione synthase (TryS). Dithiol trypanothione ($T(S)_2$) is reduced to $T(SH)_2$ by trypanothione reductase (TryR) with the consumption of NADPH. Tryparedoxin-dependent peroxidases (TXNPx) and peroxiredoxin (PRX) are peroxidases in trypanosomatids that obtain their reducing equivalents from NADPH via the $T(SH)_2$ /TXN couple and detoxify $ONOO^-$, H_2O_2 and radiation-induced radicals produced by host defense mechanisms. CAT-2B and AAP3 are transporters of L-arginine in Macrophage and promastigote membrane, respectively. PV: parasitophorous vacuole.

In the present context also, Fi-MA treatment was found to reverse the oxidative stress induced by MA in macrophages. Fisetin enhanced levels of SOD and CAT activity hence there is a significant elevation in the antioxidant enzymes in the fisetin group (Figure 4).

Although the parasite lacks CAT and GPx enzymes, but can maintain redox equilibrium by TXN, TXNPxs, PRX and ascorbate peroxidase (APX) enzymes. Moreover, the activity of these enzymes is associated with the function of $T(SH)_2$ system for defense against oxidative stress induced by host macrophages [36,37]. $T(SH)_2$ is synthesized by trypanothione synthetase in two ATP-dependent steps that involve two molecules of GSH and one molecule of spermidine [38]. In the present study, fisetin reduced the ARG activity and GSH levels of promastigotes. Reduction of ARG activity leads to decrease the production of polyamines (including putrescine, spermidine and spermine). Since the cellular

$T(SH)_2$ content depends on the availability of spermidine and GSH, fisetin causes spermidine and GSH levels to decrease and possibly $T(SH)_2$ levels to be depleted. Reduction in levels of $T(SH)_2$ impairs the trypanosomatid redox system and lead to the ultimate death of the parasite (Figure 4). Hence, the viability of promastigotes and multiplication index of amastigotes with increasing concentrations of fisetin significantly decreased as compared to corresponding control groups (Figure 1). Treatment of *L. infantum*-infected macrophages with MA had no effect on cellular GSH levels. However, fisetin and Fi-MA treatment increased GSH levels in macrophages containing amastigotes (Figure 4).

Pentavalent antimony (Sb (V)) is the first-line treatment for *Leishmania* infections in most parts of the world. However, Sb (V) for biological activation against *Leishmania* parasites must be reduced to much more leishmanicidal trivalent antimony (Sb (III)) within

macrophages [39]. Frézard et al [40]. demonstrated that GSH promotes the reduction of Sb (V) to Sb (III) and characterized the oxidation-reduction reaction. Thus, fisetin by increasing intracellular GSH levels in macrophages may be facilitated the transformation of antimony in Sb (V) to its more toxic Sb (III), thereby elevate efficacy MA treatment against *Leishmania*.

Leishmania and *Trypanosoma* possess unique iron-containing SOD, FeSOD-A, that seems to be the first line of defense against ROS. This enzyme can convert O_2^- to oxygen (O_2) and hydrogen peroxide (H_2O_2) and increase parasite survival within macrophages [41]. Interestingly, this study demonstrated that MA can impair the parasite's antioxidant defense against ROS and RNOS by inhibiting the activity of SOD. Fisetin alone had no significant effect on SOD activity of parasite, but Fi-MA could strongly inhibit the SOD activity of parasite.

Our result showed that MA, fisetin and Fi-MA treatment decrease parasite survival by reducing production of parasite NO levels. It was expected that reduction of ARG activity in parasites leads to increase the levels of NO, but decrease levels of NO after fisetin, MA and Fi-MA treatment could be attributed to reducing expression of AAP3, a transporter of L-arginine across the membrane of *Leishmania* promastigotes and amastigotes, and subsequently decrease levels of L-arginine within parasites.

In conclusion, in the present study, a high antileishmanial effect of fisetin against intracellular amastigotes of *L. infantum* has been demonstrated. In addition, fisetin in combination with MA was able to inhibit the growth of promastigotes by reducing of SOD and ARG activity and decreasing of the GSH levels in parasites.

The incidence of resistant *Leishmania* sp. and low susceptibility of them to current treatments (miltefosine, sodium stibogluconate and Meglumine antimoniate) highlight the importance of using combined therapy in order to reduce or to avoid the potential emergence and spread of acquired drug resistance. Further studies are required to investigate fisetin effect on antimony-resistant *Leishmania* sp. and animal models or volunteer human subjects.

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Declaration of interest

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