Effects of Combined Therapy with Silymarin and Glucantime on Leishmaniasis Induced by *Leishmania major* in BALB/c Mice

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Abstract

Leishmania major is resistant to the traditional treatments in many parts of the world. PgpA, a member of (ABC) transporter superfamily, has been identified in *Leishmania* involved in antimony resistance. Silymarin can inhibit PgpA. The aim of this study was to determine the effect of combined therapy with glucantime and silymarin on Cutaneous Leishmaniasis. The effects of silymarin on response of *L. major* to glucantime were evaluated with amastigote macrophage and mice model of leishmaniasis. Immediately after injection in mice inoculated into footpads with *L. major* amastigote, systemic treatment was performed and the size of footpad swelling was measured twice a week. 4 and 8 weeks after the beginning of the treatment, splenic parasite burden was done. Silymarin showed no significant effect on the response of *L. major* promastigotes to glucantime. 2 formulations (glucantime 25 µm with silymarin 25 µm or 12.5 µm) reduced cell death in amastigote assays. The effect of silymarin on footpad swelling was detected when the combination of low-dose glucantime (20 mg/kg) with 25–50 mg/kg silymarin (especially 50 mg/kg) were used at day 22 of post infection ($P < 0.05$). According to the parasite burden data, use of silymarin in the presence of different doses of glucantime, did not show significant effect compared to glucantime alone. The results of this study suggest that silymarin in conjunction with glucantime may have benefit effects in murine model of cutaneous leishmaniasis.

Introduction

Cutaneous Leishmaniasis (CL) which demonstrates a wide spectrum of clinical features is caused by several species of protozoa of the genus *Leishmania* [1]. *Leishmania major*, an intracellular pathogen of the immune system, infecting macrophages, causes CL in man and mice [2]. The gold standard for the treatment of CL are intralesional or systemic antimony-containing compounds: Meglumine antimonite, (Glucantime®); Sodium stibogluconate, (Pentostam®) [3]. Unfortunately, there have been several reports of resistance to these first-line drugs in many sites of the world [4–7].

ATP-binding cassette (ABC) proteins, the largest family of transmembrane proteins, can confer resistance to antimonials in L. major [8,9]. PgpA (phosphorylated glycoprotein A), renamed as MRPA, a member of the superfamily proteins, is one of the first transporters identified in *Leishmania* involved in antimony resistance. This transporter confers resistance by sequestration of metal-thiol conjugates. It is localized in membrane vesicles that are close to the flagellar pocket, the site of endo- and exocytosis in the parasite. Overexpression of this transporter has been reported to decrease influx of antimony, and may play a major role in antimony resistance [9].

Silymarin, the active complex in milk thistle (Silybum marianum), has been used as a medical remedy from the time of ancient Greeks [10]. Silymarin combined with antiviral drugs may have therapeutic value in liver diseases [11]. It has been recently reported that silymarin can inhibit P-gp-mediated cellular efflux in multidrug resistant human cancer cell lines [12]. Silybin, the main component of silymarin, was shown to have synergism with doxorubicin in a doxorubicin-resistant cell line probably by inhibiting the function of P-gp [13].

Considering the resistance phenotype due to P-gp proteins in *Leishmania spp.* [14–16], and the effect of silymarin as an inhibitor of P-gp function, in this study, we assessed anti-*leishmania*

activity of glucantime in combination with silymarin on L. major parasite in vitro and also in susceptible BALB/c mice.

Materials and Methods

Chemicals

Meglumine Antimoniate (Glucantime®) purchased from Sanofi-Aventis, France; silymarin, RPMI-1640, NNN (Novy-MacNeal-Nicolle) medium obtained from Sigma-Aldrich, Germany; Alamar Blue®, penicillin and streptomycin from Gibco, USA; L-glutamine; fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) from Merck, Germany.

Parasites

Leishmania major (MRHO/75/ER) obtained from Center for Research and Training in Skin Disease and Leprosy, Tehran University of Medical Sciences Tehran, Iran. *L. major* was maintained by serial passages in BALB/c mice. Afterthat, amastigotes were isolated from infected spleens and were then cultured on NNN (Novy-MacNeal-Nicolle) medium and subcultured at 25 °C in RPMI -1640 containing 10 % FBS, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 g/ml of streptomycin sulfate.

In vitro experiments

Promastigote assay

The effect of silymarin on *Leishmania* promastigotes response to glucantime was estimated with oxidation-reduction indicator Alamar Blue (10 % v/v). Promastigotes were collected from the stationary phase of culture, seeded in 96-well microtitre plates at 72×10^5 promastigotes/ml in RPMI-1640 with 10 % FBS. All experiments were performed in triplicate and the experimental groups were: RPMI-1640 with 10 % FBS with or without DMSO as control groups; different concentrations of silymarin (6.25, 12.5, and 25 µm); different concentrations of glucantime (12.5, 25, 50, and 100 µm); mixture of silymarin and glucantime. Alamar Blue was also added to each well and the plates were incubated for 48 h under 5 % CO_2 atmosphere at 25 °C. Alamar Blue is reduced in living cells, changing its color from blue to red. After that, the absorbance, correlated to the number of promastigotes per well, was measured using a microplate reader at a test wavelength of 550 nm and a reference wavelength of 630 nm. Standard curve obtained using the results of different numbers of promastigotes treated with the Alamar Blue dye. The 50 % effective dose (ED_{50}) was calculated by the use of Litchfield-Wilcoxon method with PCS (version 4) software [17, 18].

Amastigote assay

Cells of J774 A.1 mouse macrophage cell line (Pasteur Institute, Tehran, Iran) were seeded into 8-well Lab-Tek (Nunc) chamber slides at a concentration of 5×10^4 macrophages/well and incubated at 37 °C in 5 % CO2 for 24 h to allow the adherence of the cells to the plate surface. Infection the cells were performed with *L. major* promastigotes at a ratio of 5 promastigotes per macrophage. The cultures were incubated for 24 h, in 5 % CO_2 at 37 °C to allow internalization of the parasites in the cells. Subsequently, cells were washed 3 times with PBS to remove excess amount of promastigotes, and then cultured in RPMI-1640 with 10 % FBS medium for an additional 24 h for establishment of the infection. The cells were then treated with RPMI-1640 with 10 % FBS with or without DMSO as control groups; different concentrations of silymarin (6.25, 12.5 and 25 µm); different concentrations of glucantime (12.5, 25, 50 and 100 µm); mixture of silymarin and glucantime. Each assay was performed in triplicate for 48 h. Subsequently, macrophages were fixed with methanol and stained with Giemsa. The percentages of infected cells were evaluated microscopically thereafter and the ED_{50} was calculated by the Litchfield-Wilcoxon method with PCS (version 4) software [17, 19].

In vivo experiments

Mouse infections

Animals: Female BALB/c mice (age: 6–8 weeks, weight 20 ± 5 g) provided by Animal House, School of Pharmacy, Mashhad University of Medical Sciences, Iran. They were housed in standard plastic cages with free access to water and standard rodent food under 12-h light/dark cycle, 22 ± 2 °C and 40–50 % humidity conditions in the colony room. All the experiments on the animals were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts.

Female BALB/c mice were randomly divided in to 8 groups of 6 animals each, and inoculated subcutaneously in the left hind footpad with 4×10^6 parasites (50 µL) at promastigote stage (stationary phase). The materials of interest (**Table 1**) were injected intraperitoneally to treatment groups from first day of infection for 30 days. The lesion size was determined twice a week for 8 consecutive weeks using a Vernier caliper (Mitutoyo Measuring Instruments, Japan) to measure 2 dimensions and defined as mean diameters [17, 20, 21].

Quantitative parasite burden: The number of viable parasites in the spleen of infected mice was determined by a limiting dilution assay [17, 20, 22]. The mice were sacrificed at 4 and 8 weeks after infection. The spleens were aseptically removed and was homogenized in 2 ml RPMI-1640 supplemented with 10 % FBS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate with a sterile syringe piston. The homogenates were diluted with the same media in 8 serial 10-fold dilutions and cultured in triplicate in sterile flat-bottom-96 well plates containing a solid layer of rabbit blood agar and incubated at 25 ± 1 °C for 10 days. The motile and non-motile parasites (positive and negetive wells, respectively) were determined with an inverted microscope (CETI, UK). For each spleen, the viable parasites were calculated as mean and standard error of the mean of the last positive well multiplied by the dilution factor [17, 21].

Statistical analysis

One-way Analysis of Variance (ANOVA) followed by Tukey post-hoc test was used to analyze the data using GraphPad InStat version 3.00 (GraphPad Software, San Diego, California, USA). *P*-values less than 0.05 were considered to be statistically significant.

Results

Effects of formulations on L. major promastigotes in vitro

No significant differences were seen between the activities of 12 formulations against *L. major* promastigotes (data not shown). In other words, silymarin showed no significant effect on the response of *L. major* promastigotes to glucantime.

Table 1 Lesion size comparison among groups that take glucantime alone or with silymarin and control group.

Control group	Glucantime alone-treated group	Combination formulation-treated group	Days after infections	P-values
No glucantime No silymarin	20 (mg/kg)	Glucantime (mg/kg) + silymarin (mg/kg) 20 + 25	22	$^{22}P_1 < 0.05$ $^{22}P_3 < 0.05$
	20 (mg/kg)	20 + 50	15, 22	$^{15}P_1 < 0.05$, $^{22}P_1 < 0.05$ $^{22}P_3 < 0.05$
	20 (mg/kg)	20 + 100	22	$^{22}P_1 < 0.05$
	100 (mg/kg)	100 + 25	15, 18, 22, 25	$^{15}P_1 < 0.05$, $^{18}P_1 < 0.05$, $^{22}P_1 < 0.05$, $^{25}P_1 < 0.05$, $^{15}P_2 < 0.05$, $^{18}P_2 < 0.05$, $^{22}P_2 < 0.05$, $^{25}P_2 < 0.05$
	100 (mg/kg)	100 + 50	11, 18, 22, 25, 50	$^{11}P_1 < 0.05$, $^{18}P_1 < 0.05$, $^{22}P_1 < 0.05$, $^{18}P_2 < 0.05$, $^{22}P_2 < 0.05$, $^{25}P_2 < 0.05$, $^{50}P_2 < 0.05$
	100 (mg/kg)	100 + 100	15, 18, 22, 25, 46	$^{15}P_1 < 0.01$, $^{18}P_1 < 0.05$, $^{22}P_1 < 0.001$ $^{15}P_2 < 0.05$, $^{18}P_2 < 0.05$, $^{22}P_2 < 0.05$, $^{25}P_2 < 0.05$, $^{46}P_2 < 0.05$

$^nP_1$: *P-value* of Lesion size between Combination formulation-treated group and control group

$^nP_2$: *P-value* of Lesion size between glucantime alone-treated group and control group

$^nP_3$: *P-value* of Lesion size between glucantime alone-treated group and Combination formulation-treated group

n: Days post-infection

P-values more than 0.05 are not shown

Table 2 Activities of formulations against *L. major* amastigotes in vitro.

Formulation	ED50 Range
Glucantime alone	19.697 (10.674–36.349)
Glucantime (25 µg/ml) + silymarin (12.5 µM)	16.96 (8.79–32.71)
Glucantime (25 µg/ml) + silymarin (25 µM)	15.926 (8.038–31.554)

ED50 values in µM with P95 confidence limits

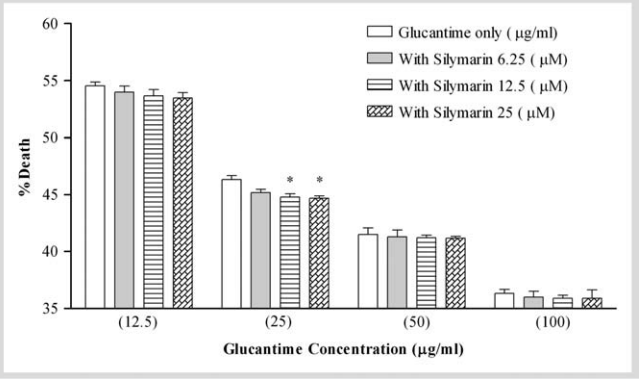

Fig. 1 Comparison the effect of different formulations of glucantime alone or with silymarin on the J774 A.1 mouse macrophage cell line infected with *L. major* amastigotes. Results are mean ± SEM, n = 6, $^*P < 0.05$, compared to glucantime alone as control.

Effects of formulations on L. major amastigotes in vitro

Out of the 12 combined formulations of glucantime and silymarin, the 2 formulations (glucantime 25 µg/ml + silymarin 25 µm or 12.5 µm) significantly reduced the cell death (**Table 2**, **Fig. 1**). There were no significant differences in the activities of the other formulations against *L. major* amastigotes.

Effects of intraperitoneal (i.p.) formulations on the sizes of ulcers induced in BALB/c mice infected with L. major

Fig. 2 shows the lesion size comparison among groups that take glucantime (20 mg/kg) alone or with silymarin (25 mg/kg) and the group which is given no drug (control). According to **Fig. 2**, there was a significant difference in the lesion sizes between control group and the group which takes glucantime and silymarin together ($^{22}P_1 < 0.05$) at day 22 post-infection. However, there was no significant difference in the lesion sizes between control group and the group takes glucantime alone ($^{22}P_2 > 0.05$). There was also a significant difference between the 2 groups that had been given glucantime alone and glucantime combined with silymarin ($^{22}P_3 < 0.05$). The results for the other different formulations are summarized in **Table 1**. The *P*-values more than 0.05 are not shown in the table.

Effects of i.p. formulations on splenic parasite burden

The number of viable L. major was quantified in the spleen of different groups of mice at 4 and 8 weeks after infections (**Fig. 3a, b**). According to **Fig. 3a**, at week 4 post-infection, significant differences were seen between the groups of the mice treated with glucantime without or with silymarin and control group ($P < 0.05$). However, no significant differences were seen between the groups treated with glucantime in combination with silymarin and glucantime alone ($P > 0.05$). According to **Fig. 3b**, at week 8 post-infection, the mice treated with glucantime alone ($P < 0.05$) and those treated with glucantime plus silymarin ($P < 0.05$) showed significantly lower parasite burden than control group. However, no statistically significant differences were seen between the parasite burden of the mice treated with glucantime alone and those that received glucantime plus silymarin ($P > 0.05$). Unlike the animals received glucantime plus silymarin, no relapse was seen in the mice treated with glucantime alone.

Discussion

In this study, we assessed the effect of silymarin and its combination therapy in the course of *Leishmania major* infection in susceptible BALB/c Mice. *Leishmania major* is one of the several species of *Leishmania* parasite that caused Cutaneous Leishmaniasis (CL) which is endemic in many parts of the world [23]. Although pentavalent antimonials are the first-line treatment for CL, they are not very efficient because of increased appearance of drug resistance [17].

Silybum marianum (Milk thistle) is a herbal drug which its protective effects have been shown in many field of diseases, such

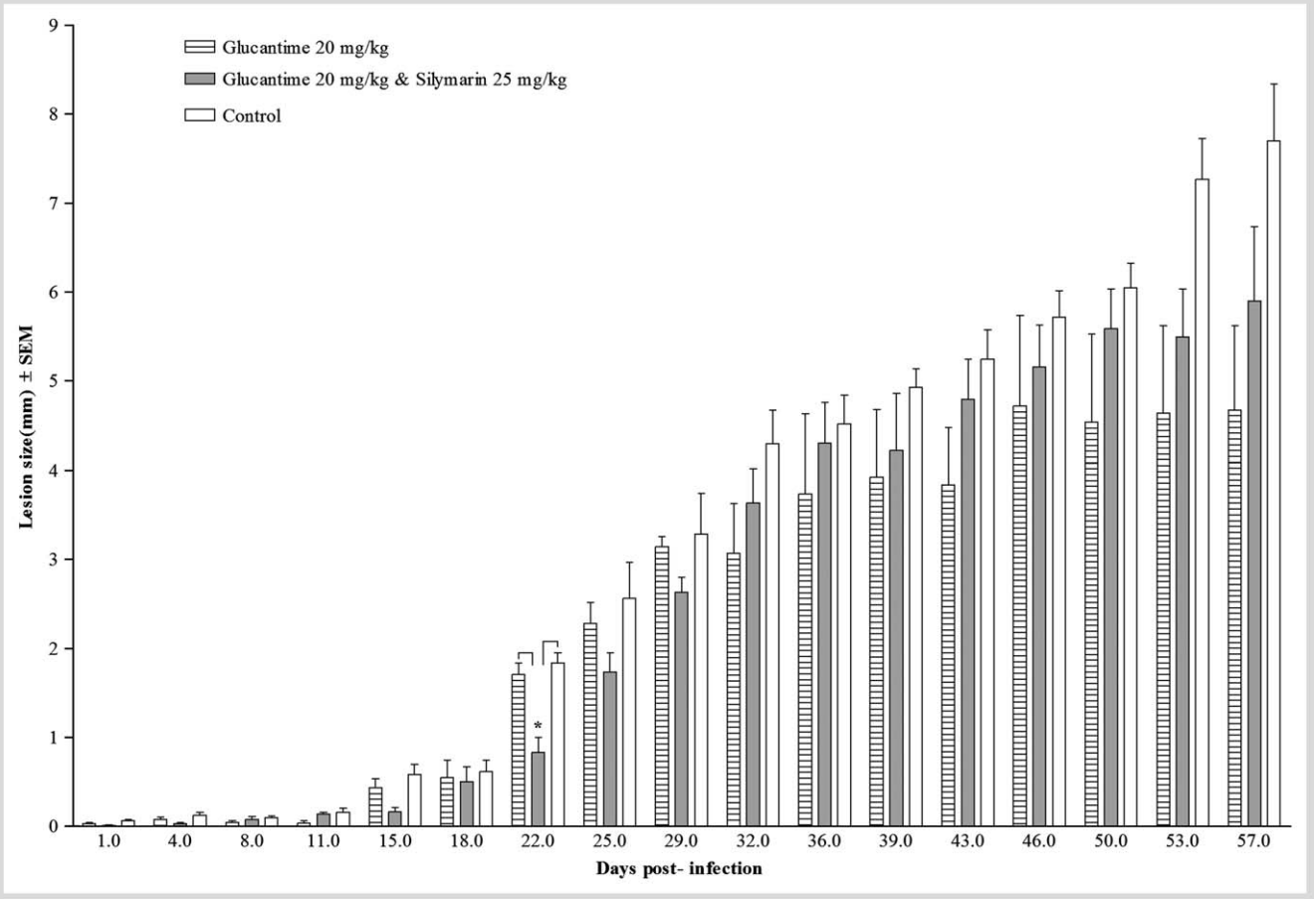

Fig. 2 Lesion size comparison among groups that takes glucantime (20 mg/kg) alone or with silymarin (25 mg/kg) and the group which is given no drug (control). (Mean ± SEM, n = 6).

as; hepatotoxicity, viral hepatitis, cancer, nephrotoxicity, etc [24]. Silymarin, the active component in milk thistle extract, is mainly composed of 3 isomer flavonolignans: silybin, silychristin and silydianin [24].

Pgp belongs to the ABC transporter family. It is an ATP-dependent pump that exports many drugs from cells and causing decrease in intracellular drug concentrations and their activity [10]. There are several studies demonstrated the over expression of Pgp protein occurs in drug-resistant *Leishmania spp*. Line. [14–16, 25]. Silymarin is found to be an inhibitor of Pgp function [26]. In this study, we investigated possible protective effect of silymarin in combination with glucantime against *L. major* promastigotes and amastigotes in vitro and also in BALB/c mice infected with *L. major*. Ephros et al. 1999 have reported that promastigote form of *Leishmania* were 73–271 time less susceptible to SbV than were amastigotes in vitro [27]. Ashutosh et al. 2007 demonstrated that pentavalent antimony compounds had low activity against *Leishmania spp*. Promastigotes [28]. In addition to this finding, we have found that simultaneous administration of silymarin with glucantime did not affect the in vitro response of *L. major* promastigote to glucantime. The leishmanicidal activities of the formulations were analyzed against the intracellular amastigote form of the parasite. According to the ED50s (Effective Dose 50%) of the formulations (**Table 2**), significant enhancement of leishmanicidal activity was seen after the addition of silymarin to low-dose glucantime (25 µg/ml). However, addition of silymarin to high-dose glucantime (100 µg/ml), showed no significant effect on amastigote forms. Maybe this result is due to the interference of silymarin with high-dose glucantime during the incubation time. Perhaps the high-dose glucantime lead to activation of other resistance mechanisms other than Pgp inhibition [28, 29]. The result of in vitro studies was confirmed by some in vivo experiments, in BALB/c mice infected with *L. major*. The result of in vivo study demonstrated that, there was generally a progressive increase in footpad swelling during and after treatment.

In part 3 of the study, which we compared the size of the footpad swelling in the groups (glucantime alone-treated, combined formulation-treated and control groups which is given no drug), we were looking for the combined formulations that significantly reduced the size of footpad swelling compared with control group (${}^{n}P_1 < 0.05$) and also with glucantime alone-treated group (${}^{n}P_3 < 0.05$). Comparing the size of footpad swelling in the groups receiving glucantime alone (20 mg/kg and 100 mg/kg) or with silymarin to control group demonstrated that the significant effect of silymarin on footpad swelling was detected when the combination of low-dose glucantime (20 mg/kg) with 25–50 mg/kg silymarin were used. The application of these combined formulations resulted in significant footpad swelling reduction, compared to glucantime alone or control group at day 22 (${}^{22}P_1$, ${}^{22}P_3$ in **Table 1**). Unlike low dose glucantime (20 mg/kg) with silymarin treated groups, high dose glucantime (100 mg/kg) with silymarin treated groups showed no significant differences in lesion size compare to control group (**Table 1** $P_3 > 0.05$, data not shown). It could be argued that silymarin and glucantime compete for binding to the Pgp active site. With

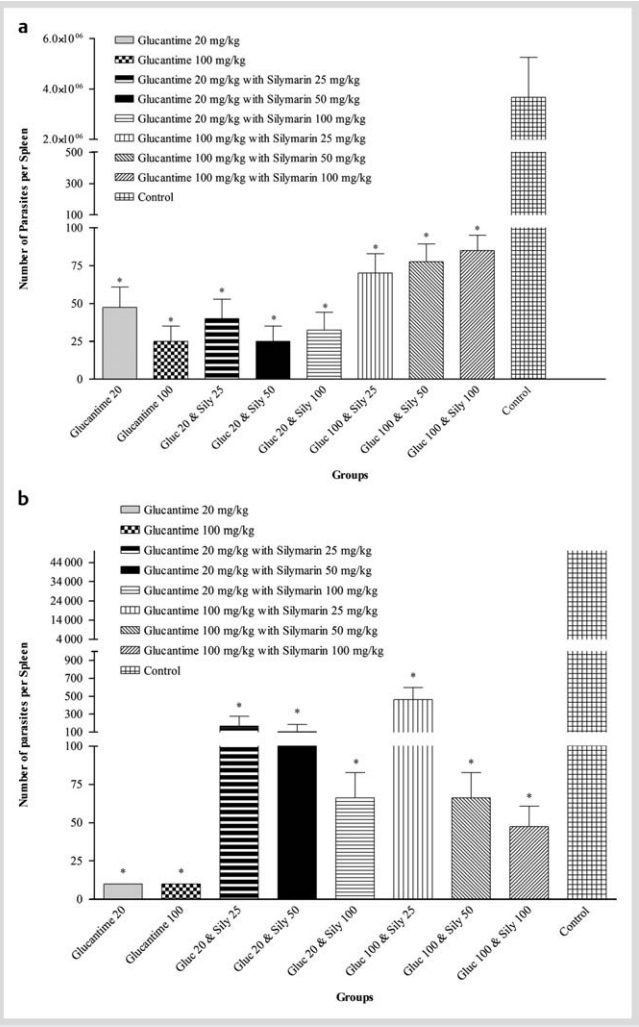

Fig. 3 **a, b** splenic parasite burdens in BALB/c mice treated with intraperitoneal glucantime plus silymarin treated groups. Untreated infected mice were considered controls. The numbers of viable *L. major* parasites in glucantime plus silymarin treated groups were compared with the groups treated with glucantime alone at 4 weeks **a** and 8 weeks **b** after infection with *L. major* promastigotes. Data represent the mean ± SEM for 6 animals per group. There were statistically significant differences ($P<0.05$) between glucantime with or without silymarin treated and control groups. There were not significant differences between glucantime-treated and glucantime plus silymarin treated groups ($P>0.05$). $^{*}P<0.05$

increasing glucantime concentration as a substrate, silymarin cannot bind to the Pgp active sites [12]. The increase in footpad swelling size which was seen on the days after 22 in mice treated with combination formula is maybe due to the large numbers of parasites released by infected macrophages, so that silymarin showed limited effectiveness.

Our results showed that the mice taken glucantime (alone or plus silymarin) had significantly lower parasite burden in the spleen than the control mice 4 and 8 weeks post infection (**Fig. 3a, b**). According to the parasite burden data, use of silymarin in the presence of different doses of glucantime, did not show significant effect compared to glucantime alone. This result is maybe due to the lack of sensitivity of laboratory methods for counting parasites in the spleen of mice, and the small number of mice in each group.

In summary, the data of in vitro studies on *L. major* amastigote forms showed that silymarin in combination with low-dose glucantime caused a significant reduction in cell death whereas no effect was seen on promastigote assays. Combined formulation of silymarin (25–50 mg/kg) and low-dose glucantime (20 mg/kg), also reduced the footpad swelling sizes especially at early days after the onset of inflation. According to the overall results obtained in this study, silymarin combined with glucantime may have benefit effect against *L. major*. It is recommended that further research be undertaken in this area.

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Conflict of Interest

The authors have declared no conflict of interest.

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