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Original papers

Investigating *in vitro* anti-leishmanial effects of silibinin and silymarin on *Leishmania major*

Roghiyeh Faridnia[1], Hamed Kalani[2], Mahdi Fakhar[1], Javad Akhtari[3]

[1]Department of Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Farah-Abad Road, P.O.Box 48175-866, Sari, Iran
[2]Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Hezar Jarib, P.O.Box 319, Isfahan, Iran
[3]Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Mazandaran University of Medical Sciences, Farah-Abad Road, P.O.Box 48175-866, Sari, Iran

Corresponding Author: Mahdi Fakhar; e-mail: mahdif53@yahoo.com

ABSTRACT. Cutaneous leishmaniosis is an important zoonotic disease caused by various *Leishmania* species. The aim of this study was to investigate the effects of silibinin and silymarin on the *in vitro* growth and proliferation of promastigotes and amastigotes of *Leishmania major* compared to glucantime-treated parasites. The promastigotes and amastigotes of this parasite were treated with the two drugs, silibinin and silymarin, in several concentrations (25–100 μM). The highest effect on promastigotes was for silymarin in concentration of 100 μM with 90% and 91% death rate at hours 48 and 72, respectively. Regarding amastigotes, the highest effect at 48 hours was for silibinin in concentration of 100 μM with 35% death rate. However, at 72 hours, silymarin showed the highest effect with 63% death rate in concentration of 100 μM. The highest observed maximal 50% lethal concentration (LC_{50}) for promastigotes was for silymarin with 19.34 μM at 48 hours and 18.22 μM at 72 hours. Likewise, maximal LC_{50} for amastigotes was for silymarin with 191 μM at 48 hours and 24.27 μM at 72 hours. Our findings demonstrated that both medications have suitable effects like Glucantime® on the parasite *in vitro*. Therefore, clinical assessment of the anti-leishmanial activity of silibinin and silymarin for treating the dermal lesions caused by *L. major* is recommended.

Key words: *Leishmania major*, silibinin, silymarin, *in vitro* anti-leishmanial agent

Introduction

Cutaneous leishmaniosis (CL) is an important zoonotic disease caused by various *Leishmania* (L.) species [1]. Sand flies belonging to the family Phlebotominae act as intermediate hosts and vectors for the transmission of the parasite to vertebrate hosts, including humans [2]. The incidence of CL ranges from 0.7 to 1.2 million new cases annually, of which about 70% are reported from 10 countries including Iran, Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, North Sudan, Peru, and the Syrian Arab Republic [3]. So far, no effective vaccine has been developed for the disease because the emergence of new strains and the development of drug resistance are important factors that make it difficult to prevent and control the disease [4]. There are currently a few drugs to treat CL, including sodium stibogluconate (Pentostam®), meglumine antimonate (Glucantime®), miltefosine, and amphotericin B [5]. These drugs have problems such as high toxicity, high cost, long-term treatment course, drug resistance, and occasionally low recovery rate [6]. Because of the drug resistances that have been reported in recent years for the treatment of CL, the World Health Organization (WHO) and recent systematic reviews strongly recommend the use of medicinal plants as complementary or alternative therapies [7,8]. One of the herbal compounds with anti-leishmanial effect is flavonoids [9]. Silibinin and silymarin are two types of flavonoids that prevent cell proliferation [10].

Recently, it has been illustrated that anti-cancer compounds can affect the growth of *Leishmania* parasites [11], and even miltefosine, which is an important therapeutic drug for the treatment of leishmaniosis, is an anti-cancer compound. Therefore, we focused on silibinin and silymarin as two anti-cancer compounds. Silymarin and silibinin showed to use as significant anti-neoplastic compounds in a wide variety of *in vitro* and *in vivo* cancer studies, including skin, breast, lung, colon, bladder, prostate and kidney carcinomas [12].

It has been demonstrated that silibinin can inhibit cancer cell signalling pathways, for example, growth inhibition, inhibition of angiogenesis, chemosensitization, and inhibition of invasion and metastasis. Additionally, silibinin is a potential agent to improve cancer chemoprevention and chemotherapy [13].

The aim of this study was to evaluate the effects of silibinin and silymarin on the *in vitro* growth and proliferation of promastigotes and amastigotes of *L. major* (Iranian strain MRHO/IR/75/ER).

Materials and Methods

Parasite. L. *major* promastigotes, Iranian strain MRHO/IR/75/ER, were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (Gibco, Paisley, Scotland) at 24°C with frequent passages every three days.

Drugs and drug supply. Silibinin and silymarin (Sigma, Lyon, France) were dissolved separately in dimethyl sulfoxide (DMSO) (1 mg/ml) up to the saturation point and kept as a stock solution at 4°C until use. The stock solution of Glucantime® (Rhône-Poulenc, France) was prepared in DMSO (1 mg/ml).

Assessment of anti-promastigote effect. The experiments were performed separately for 48 and 72 hours in a 96-well plate. Firstly, 100 µl RPMI-1640 medium containing 10^5 promastigotes was added to each well. Silibinin and silymarin were then added separately to the wells in triplicate at concentrations of 25, 50, 75, and 100 µM. Three wells were included as positive controls to which Glucantime® in a concentration of 12 µM was added. The DMSO concentration in the final volume of each well did not exceed 0.1%. The plate was then incubated at 24°C for 48 and 72 hours. At the end of incubation, 20 µl of each well was mixed with 20 µl of 2% formaldehyde solution in phosphate-buffered saline (PBS), and promastigotes/ml were counted using a haemocytometer under light microscope [14].

Assessment of anti-amastigote effect. The experiments were performed separately for 48 and 72 hours in a 96-well plate. The macrophage cell line J774A.1 was cultured by seeding 5×10^4 cells to each well in a volume of 100 µl RPMI-1640 medium. The plate was incubated at 37°C, 5% CO_2, for 24 hours. Afterwards, the supernatants were discarded, and 100 µl RPMI-1640 medium containing 10 stationary phase promastigotes was added to the wells for each cell (10:1). After 24 hours, the supernatants were discarded and the wells were washed gently with RPMI-1640 medium to remove free promastigotes. After washing, 100 µl of RPMI-1640 medium was added to each well. Then, silibinin and silymarin were added separately to the wells in triplicate at concentrations of 25, 50, 75, and 100 µM. The DMSO concentration in the final volume of each well did not exceed 0.1%. Three wells containing 100 µl of DMSO and three wells containing amastigote-infected cells without drug were included as blank and negative controls, respectively. Three wells were included as positive controls to which Glucantime® in a concentration of 12.5 µM was added. The plate was incubated at 37°C, 5% CO_2, for 48 and 72 hours. At the end of 48 and 72 hours, the supernatants were discarded gently, and 50 µl of thiazolyl blue tetrazolium bromide (MTT) solution (5 mg/ml) (Sigma, Lyon, France) was added to each well. The plate was then incubated at 37°C, 5% CO_2, for 4 hours. Then, MTT solution was discarded gently, the wells were washed with PBS gently and 100 µl of DMSO was added to each well. After 30 minutes under mild rotation, the optical absorbance of the wells was read using a scanning multiwell spectrophotometer (ELISA reader) at a wavelength of 570 nm. Cell death rate was determined by the formula: 1-[(AT-AB)/(AC-AB)]×100, where AT is the mean absorbance of treated wells for each concentration, AC is the mean absorbance of control wells, AB is the absorbance of blank wells.

Maximal 50% lethal concentration. Maximal 50% lethal concentration (LC_{50}) of each medication, silibinin or silymarin, on each of the parasite forms, promastigote or amastigote, was calculated according to the following formula:

$$ML = Min + [½(Max – Min)]$$

Where ML = maximal LC_{50}, Min = minimal death rate (%), Max = maximal death rate (%). The ML is the death rate (%) on the vertical axis of the logarithmic dose-response curve, on the basis of

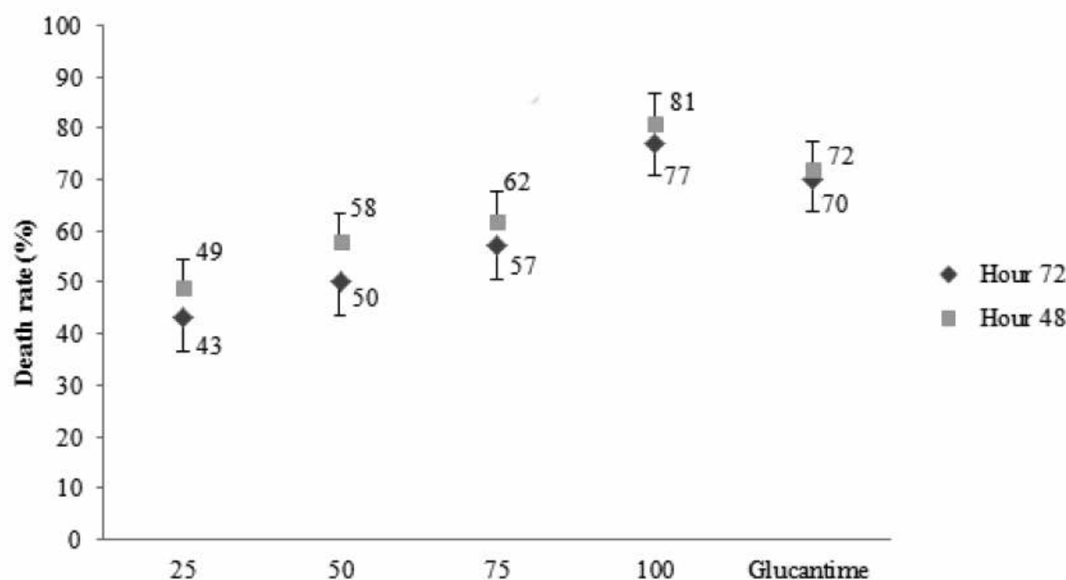

Fig. 1. Silibinin-treated promastigotes with different concentrations of the medication at 48 and 72 hours
Silibinin concentration (μM) and glucantime (12.5 μM)

which the number obtained on the horizontal axis shows the value of LC_{50}. It should be mentioned that two-fold serial dilutions of each medication were prepared to draw a logarithmic dose-response curve in order to calculate LC_{50}.

Cytotoxicity assessment and selectivity index. Cytotoxicity assay was performed similar to for LC_{50}, with this difference that the effect of each medication in different dilutions was evaluated on J774A.1 macrophages without amastigotes, and finally the concentration of each medication in which 50% of the macrophages were killed was considered as the cytotoxicity concentration 50 (CC_{50}). In addition, selectivity index (SI) was calculated by dividing CC_{50} by LC_{50} of amastigotes [15]. The SI>10 represents the safety of the medication.

Data analysis. Data were analysed by chi-square (χ^2) statistical test and two-tailed *t*-test using IBM SPSS v20 software (IBM Corp., NY, USA).

Results

Silibinin-treated promastigotes

The death rates in the positive control at 48 and 72 hours were 72% and 70%, respectively. The highest death rates at 48 h (81%) and 72 h (77%) were observed at the concentration of 100 μM (Fig. 1). On the other hand, the lowest death rates were observed at the concentration of 25 μM at both hours 48 (49%) and 72 (43%). However, there were no statistically significant differences in the death rates in all concentrations of silibinin and the positive control at 48 h (P=0.29) and at 72 h (P=0.1). Furthermore, the statistical analysis between the death rate of promastigotes at 48 h and 72 h was not significant (P=0.08). Moreover, no statistically significant difference was found between each concentration of silibinin and the positive control (P>0.05) at 48 h and 72 h. Maximal LC_{50} was equal to 57.1 μM at 48 h and 52.28 μM at 72 h.

Silymarin-treated promastigotes

Concerning the positive control, the death rate was the same as mentioned above. The highest death rates of 90% at 48 h and 91% at 72 h were observed at the concentration of 100 μM (Fig. 2). The lowest death rates were also observed at the concentration of 25 μM, being 87% at both 48 and 72 hours. However, no statistically significant differences were observed between all concentrations of the silymarin and the positive control (P=0.62) at 48 h (P=0.62) and at 72 h (P=0.46). In addition, there was no statistically significant difference (P=0.99) between the death rates at 48 and 72 hours. Moreover, there was no statistically significant difference between each concentration of silymarin and the positive control (P>0.05) at 48 and 72 hours. Maximal LC_{50} was equal to 19.34 μM at 48 h and 18.22 at 72 h.

Amastigote-infected macrophages treated with silibinin

The death rates were 62% at 48 h and 64% at 72 h for the positive control. The highest death rates of 35% at 48 h and 61% at 72 h were observed at the

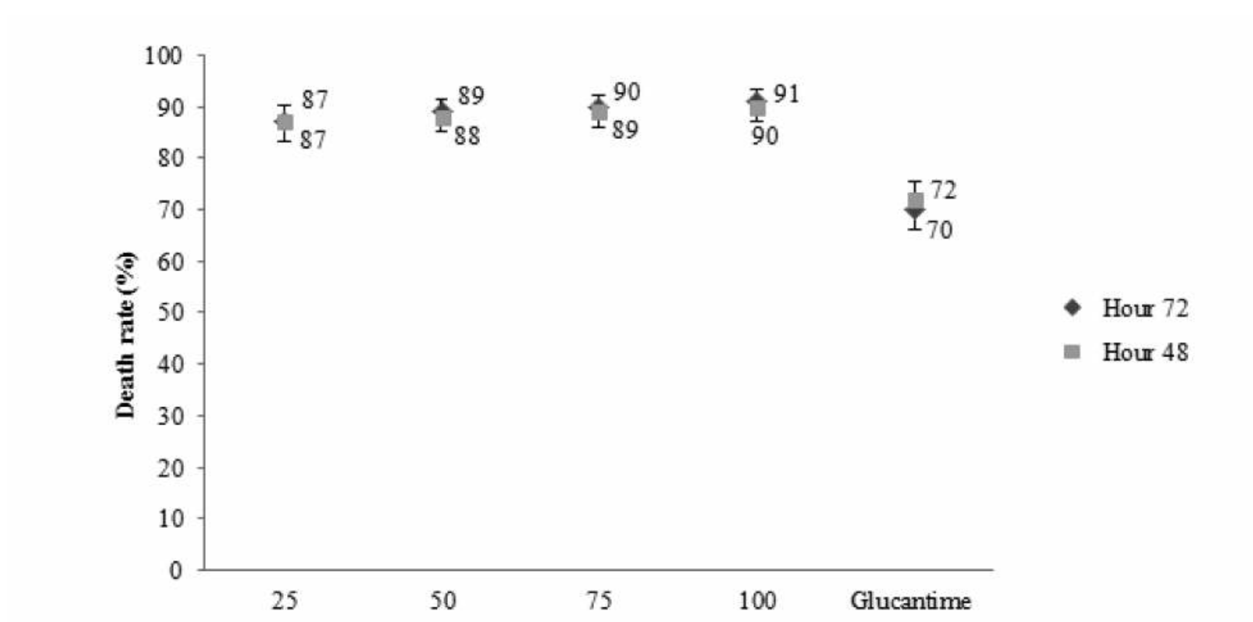

Fig. 2. Silymarin-treated promastigotes with different concentrations of the medication at 48 and 72 hours
Silymarin concentration (μM) and glucantime (12.5 μM)

concentration of 100 μM (Fig. 3). Furthermore, the lowest death rates were observed at 48 h (24%) and 72 h (53%) at the concentration of 25 μM. There was a statistically significant difference between all concentrations of the silibinin and the positive control at 48 h (P=0.0001) and 72 h (P=0.0001). Moreover, there was a statistically significant difference in the death rates at 48 and 72 hours (P=0.001). Additionally, there was a statistically significant difference ($P<0.05$) between the positive control and each of the concentrations of the silibinin (25–100 μM) at 48 h. However, there was no statistically significant difference between the positive control and each of the concentrations of the silibinin (25–100 μM) at 72 h ($P>0.05$). Maximal LC_{50} was equal to 204.8 μM at 48 h and 66.12 at 72 h.

Amastigote-infected macrophages treated with silymarin

The death rates for the positive control were the same as mentioned above. At 48 and 72 hours, the highest death rates were 30% and 63%, respectively,

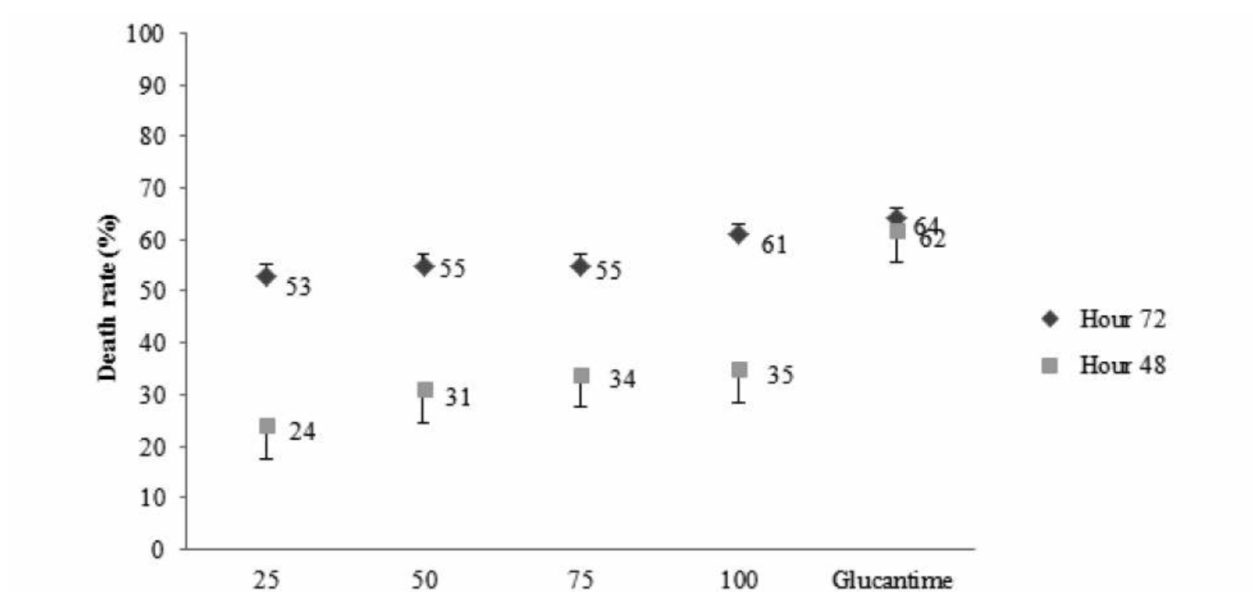

Fig. 3. Amastigote-infected macrophages treated with silibinin with different concentrations of the medication at 48 and 72 hours
Silibinin concentration (μM) and glucantime (12.5 μM)

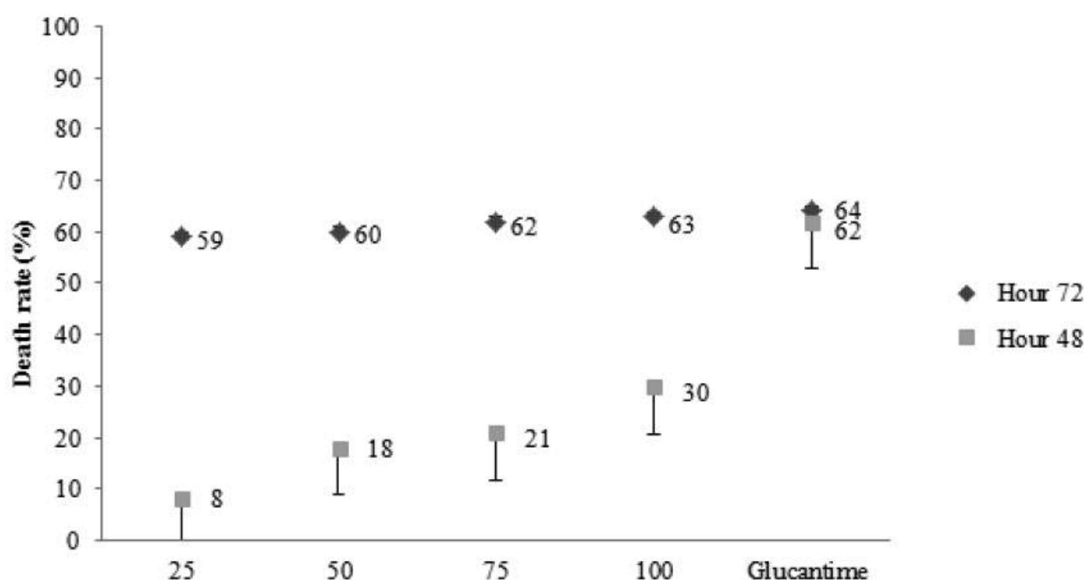

Fig. 4. Amastigote-infected macrophages treated with silymarin with different concentrations of the medication at 48 and 72 hours
Silymarin concentration (µM) and glucantime (12.5 µM)

at the concentration of 100 µM (Fig. 4). Moreover, the lowest death rates were observed at 48 h (8%) and 72 h (59%) at the concentration of 25 µM. At 48 h, there was a statistically significant difference between all concentrations of the silymarin and the positive control (P=0.0001). However, at 72 h, no statistically significant difference was observed (P=0.99). The analysis of the death rates between 48 and 72 hours showed a statistically significant difference (P=0.005). Additionally, a statistically significant difference (P<0.05) was observed between the positive control and each of the concentrations of the silymarin (25–100 µM) at 48 h. However, at 72 h, there was no statistical significant difference (P>0.05) between the positive control and each of the concentrations of the silymarin (25–100 µM). Moreover, maximal LC_{50} was equal to 191 µM at 48 h and 24.27 µM at 72 h.

Cytotoxicity and selectivity index

The cytotoxicity of silymarin and silibinin was significantly lower than Glucantime® (P<0.001). The selectivity index of silibinin for LC_{50} of 66.12 µM was 3.19 and that of silymarin for LC_{50} of 24.27 µM was 17.51.

Discussion

The use of the plant milk thistle (*Silybum marianum*) goes back to a few thousand years [16]. This plant is native to the Mediterranean region, North America, Europe, China, India, Africa, and Australia. It has medical importance and has been used for centuries to treat liver diseases [17]. The most important active compound of this plant is silymarin that is rich in flavonolignans such as silibinin A, silibinin B, isosilibinin A, isosilibinin B, silicristin, and silidianin, among which silibinin is the most active [18]. These compounds have anti-angiogenic, anti-metastatic, antioxidant, anti-inflammatory and wound healing activities and can also influence the cell cycle and apoptosis of cancer cells [18–20]. A study evaluated the effect of cisplatin in combination with some antioxidants such as silibinin in treatment of *Leishmania donovani*-infected mice and showed that this combination improved liver and kidney functions as well as immune responses [21]. On the other hand, Glucantime® was used in combination with silymarin for the treatment of CL caused by *L. major* in mice, where the combination had no statistically significant effect over treatment with Glucantime® alone [22]. The present study showed a higher effect of silymarin on promastigote than Glucantime®; however, it was not statistically significant (P>0.05). Furthermore, both silibinin and silymarin had notable effects close to Glucantime® on amastigotes at hour 72. The cytotoxicity of silymarin and silibinin was also considerably lower than Glucantime® (P<0.001), indicating the safety of these medications compared

to Glucantime®. Moreover, the selectivity index of silibinin (LC_{50} of 66.12 μM) was 3.19, indicating that it affects both the cells and the parasites. However, the selectivity index of silymarin was 17.51 (LC_{50} of 24.27 μM), indicating that it has a selective effect on the parasite but a tiny effect on the cell. However, in the present study, in vivo testing was not performed.

A number of studies were performed on the antiparasitic, antifungal, antiviral, and antibacterial effects of silymarin and silibinin. For instance, the effect of silymarin on mice experimentally infected with *Schistosoma mansoni* showed that interferon gamma (IFN-γ), interleukin 4 (IL-4), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β1) levels were higher in mice treated with silymarin compared to the controls [23]. Additionally, silymarin seems to play an important role in reducing the liver fibrosis caused by *S. mansoni* and significantly increasing the level of IFN-γ [24,25]. In addition to reducing liver fibrosis, silymarin was found to reduce helminth burden, IgG and IgM serum level, liver enzymes function, and granuloma diameter in mice experimentally infected with *S. mansoni* [26]. On the other hand, silymarin at the concentration of 50 mg/ml was found to have a complete inhibitory effect on the growth of *Cryptosporidium parvum* and to exhibit no toxic effect on the host cell [27]. Its combination with praziquantel was found to lead to a greater effect on the cestode *Mesocestoides vogae*, increasing the proliferation of hepatocytes and reducing the production of harmful substances in the liver such as superoxide anions [28]. Silymarin can improve the efficacy of praziquantel and prevent fibrogenesis in liver and also protect hepatocytes from oxidative damage [29]. On the other hand, silymarin was found to remarkably reduce the liver damage caused by the rodent malaria parasite *Plasmodium berghei*, but has no effect on the parasitemia [30].

As a whole, the above-mentioned studies show that silibinin and silymarin are often used as an adjunctive therapy along with the main antiparasitic drugs. However, the present study shows that these two compounds are as efficacious as Glucantime® on *L. major in vitro*. Therefore, it is recommended to examine their *in vivo* effect on the lesions caused by *L. major*.

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