Usnic acid causes apoptotic-like death in *Leishmania major*, *L. infantum* and *L. tropica*

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Abstract

Leishmaniasis, a deadly parasitic infection, threatens many people worldwide. Since the high cost, toxicity, and resistance are drawbacks of current treatment options, it is necessary to find safer and more effective new antileishmanial drugs. The aim of this study was to determine the antileishmanial activity of usnic acid and its apoptotic mechanism on *Leishmania* spp. promastigotes. The antileishmanial activity was evaluated by MTT assay and apoptosis-related gene expression was investigated by qRT-PCR. Usnic acid was to be effective against *Leishmania major*, *L. infantum*, and *L. tropica* promastigotes at IC_{50} = 10.76 µg/ml, 13.34 µg/ml, and 21.06 µg/ml, respectively. We also demonstrated a novel mechanism by which usnic acid inhibited proliferation and caused apoptosis; usnic acid upregulated *p53*, *Bax*, *Casp-3*, and *Casp-9* gene expression and downregulated the level of *Bcl-2* gene expression. Accordingly, the expression level of the P53 gene increased in *L. major*, *L. infantum* and *L. tropica* by 14.4-, 11.8-, and 9.5-fold, respectively, and in contrast, the *Bcl-2* gene expression decreased in all three leishmaniasis by 0.8-, 0.8-, and 0.7-fold, respectively. The present study, therefore, revealed that usnic acid played a critical role in the usnic acid-induced apoptotic process in *Leishmania* species. Usnic acid is easily accessible and an inexpensive agent, and can be considered as an alternative therapeutic agent for *Leishmania* infections subject to further tests in animal models.

Keywords *Leishmania* · Lichen · Usnic acid · qRT-PCR · Apoptosis

Introduction

Leishmaniasis is an important health problem not only in the Mediterranean region but also all over the world (Dujardin et al. 2008). In Turkey, only visceral and cutaneous forms of this disease have been found (Ok et al. 2002; Ertabaklar et al. 2005). The main treatment choice of treating leishmaniasis was the pentavalent antimonial drugs, but nowadays, liposomal amphotericin B (L-AMB), paromomycin, miltefosine, aminosidine, pentamidine, alkylphosphocholines, metronidazole, ketoconazole, and fluconazole are

alternative options for treatment (Georgiadou et al. 2015; Copeland and Aronson 2015). There can be many disadvantages of using these compounds. For example, pentavalent antimonials, which are cheaper and can be administered IV or intramuscular (IM) ways, need patient hospitalization and monitoring due to the possibility of serious cardiotoxicity and pancreatitis (Georgiadou et al. 2015) side effects. In some cases, elevation of liver function functions and cytopenia can also be observed and IM usage can cause muscular fibrosis and abscess formation (Haldar et al. 2011; Georgiadou et al. 2015). L-AMB, can only be administered by intravenous (IV) route and is known to cause mild infusion reactions, back pain, and transient nephrotoxicity in 10–20% of patients (Georgiadou et al. 2015). Miltefosine has some mild side effects such as gastrointestinal disturbances and elevation of liver function but in addition is also a potential teratogen. Paromomycin is an uncommon treatment option especially for *Leishmania infantum*, since it has significant side effects such as ototoxicity, elevated liver function, and renal injury (Haldar et al. 2011; Georgiadou et al. 2015). Another potential concern for the currently used drugs is

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resistance and it has been reported that, in some geographical regions, almost 60% of cases cannot be treated with antimonials (Halder et al. 2011; Georgiadou et al. 2015). The recently introduced antileishmaniasis drugs which include aminosidine, pentamidine, alkylphosphocholines, metronidazole, ketoconazole, and fluconazole despite suffer from high costs (Copeland and Aronson 2015). Consequently, there is a need to identify drugs that are more effective, cheaper, and safer to use.

Lichens and other fungal and algal symbionts are known to synthesize low-molecular-weight bioactive compounds. Recently, it has been shown that lichen produces secondary bioactive anticancer metabolites (Dincsoy and Cansaran-Duman 2017; Kilic et al. 2018) in addition to antiviral, antibacterial, antifungal, and antiprotozoal compounds (Lauterwein et al. 1995; Muller 2001; Cansaran-Duman et al. 2006, 2007; Cansaran-Duman and Halici 2012). Usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bh)] [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bh)], a lichen secondary metabolite, has been extensively studied since its first isolation in 1844 (Ingolfssdottir 2002; Cocchietto et al. 2002; Molnar and Farkas 2010; Araujo et al. 2015) and has been shown to have antimicrobial, antifungal, antiprotozoal and anticancer properties (Cocchietto et al. 2002). Although usnic acid has been found to be effective as an antileishmanial compound, there are relatively few detailed reports. Here, we report the effects of usnic acid on several *Leishmania* spp and promastigotes which are commonly seen during leishmaniasis in Turkey as well as the Mediterranean region.

Materials and methods

Parasite culture

Promastigotes of strains of *Leishmania* spp. (*L. major*, *L. infantum*, and *L. tropica*) were purchased from the National Reference Parasitology Laboratory and cultured in T25 flasks (Nunc, Denmark) at 24 °C in fresh RPMI-1640 (Gibco, Denmark) medium supplemented with 10% Fetal Calf Serum (FCS) (HyClone, Utah, USA), and 200 U penicillin/ml and 0.2 mg streptomycin/ml (HyClone, Utah, USA). The proliferation of parasites was observed and fresh medium was added/replaced every 2–3 days to obtain 10 ml of promastigote-containing broth. The promastigotes were collected by centrifugation, washed 5 times with sterile saline and resuspended in fresh medium. The total number of cells was determined using a Thoma lamella. Following optimization studies, the appropriate number of parasites was determined and log phase promastigotes were subcultured every 72–96 h with an inoculum of 10^6 parasites/ml.

Preparation of usnic acid dilutions

Usnic acid (C₁₉H₁₄O₅; M_w , 322.31 Da) (Sigma-Aldrich) was freshly dissolved in dimethylsulfoxide (DMSO) as a stock solution and stored at 4 °C until used. When required, the stock solution of usnic acid was appropriately diluted to a final concentration of 0.1% DMSO (v/v) and subsequently serially diluted in RPMI medium to reach usnic acid concentrations of between 0.1 and 100 µg/ml. RPMI medium-containing DMSO at equivalent concentrations but lacking usnic acid was used as a negative control and to test the effect of DMSO on the promastigotes.

In vitro antileishmanial activity

To obtain the IC₅₀ (the half maximal inhibitory concentration) value of usnic acid on *Leishmania* spp., the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, Missouri, USA) assay was employed. Usnic acid dilutions (0.1–100 µg/ml), negative control, and MTT stain (1 mg/ml) were freshly prepared before use. Wells of 96-well flat-bottomed sterile plates were seeded to give 1×10^5 parasites/well in 100 µl of the RPMI medium. 100 µl RPMI medium-containing equivalent DMSO concentrations used as a solvent to prepare usnic acid but lacking usnic acid were used in control wells. Plates were incubated at 26 ± 1 °C under 5% CO₂ for 48 h and following which an increasing concentration of usnic acid (between 0.12 and 100 µg/ml) was added. At the end of incubation period (48 h), 50 µl of freshly prepared MTT solution prepared in DMSO was added to each well, and after 4 h, 100 µl acidified isopropanol was added to improve color development (Kılıç et al. 2018). Following gent shaking of the plates, the absorbance was read at 570 nm using a Victor Multilabel Plate Reader X3 (Perkin Elmer, Massachusetts, USA). The percent cell viability was determined by comparing with untreated control wells. OD values from plate-blank wells were subtracted from OD values of the test and control wells. Each measurement was performed in triplicate and data expressed as the mean of independent experiments ($n = 6$).

RNA isolation

2×10^6 cells of the three *Leishmania* species were seeded into 6-well culture plates and treated with IC₅₀ concentrations of usnic acid and control as determined above following incubation, Genezol Reagent was used to isolate total RNA according to the manufacturer's protocol. RNA concentrations were quantified and quality assessed

using standard procedures of 260 nm/280 nm ratio (Nanodrop 1000, Thermo Scientific, USA) and 1% agarose gel electrophoresis.

Primers

Bax, *P53*, *Bcl-2*, *Casp-3*, and *Casp-9* genes play a central role in the events of apoptosis, and the level of expression of these genes also greatly influences of apoptosis. The primers for *Bax*, *P53*, *Bcl-2*, *Casp-3*, and *Casp-9* genes were designed using Primer3. These primers and GAPDH, a house-keeping gene, were synthesised by Sentegen Biotechnology. The sequences of the primers used qRT-PCR are listed in Table 1.

cDNAs syntheses and qRT-PCR

The First Strand cDNA Synthesis Kit (Roche, Switzerland) was used for cDNA synthesis from total RNA using the manufacturer's protocol. Briefly, for cDNA synthesis RNA (2 µg), anchorage-oligo (dT)₁₈ (2.5 µM), protective RNase inhibitor (20 U), 1× transcriptor high-fidelity reverse transcriptase reaction buffer, 1 mM deoxynucleotide mix, and transcript high-fidelity reverse transcriptase (10 U) were mixed and incubated at 65 °C for 10 min, than at 55 °C for 30 min, and, finally, at 85 °C for 5 min.

Real-time quantitative PCR (qRT-PCR) was performed using a Roche Light Cycler 480 Real Time (Roche, Switzerland) using SYBR Green I Master. After pre-denaturation for 10 min at 95 °C, 45 cycles of 15 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C were applied. Melting curve analysis was performed to determine the efficacy of qRT-PCR and to monitor for dimer formation. *P53*, *Bcl-2*, *Bax*, *Casp-3*, *Casp-9*, and the reference house-keeping gene (GAPDH) were optimized by the qRT-PCR assay.

Table 1 The Sequence of primers used in the gene expression assay

Primers	Sequences (5' → 3')	PI
<i>Bax</i> -F	TTT CTG ACG GCA ACT TCA ACT GGGT	24
<i>Bax</i> -R	TGT CCA GCC CAT GAT GGT TCT GAT	24
<i>P53</i> -F	TAC CAC CAT CCA CTA CAA CT	20
<i>P53</i> -R	CAG AGG CAC AAA CAC GCA C	19
<i>Bcl-2</i> -F	ATG TGT GTG GAG AGC GTC AACC	22
<i>Bcl-2</i> -R	TGA GCA GAG TCT TCA GAG ACA GCC	24
<i>Casp-3</i> -F	TGT TTG TGT GCT TCT GAG CC	20
<i>Casp-3</i> -R	CAC GCC ATG TCA TCA TCA AC	20
<i>Casp-9</i> -F	CAT TTC ATG GTG GAG GTG AAG	21
<i>Casp-9</i> -R	GGG AAC TGC AGG TGG CTG	18
<i>GAPDH</i> -F	GAA GGT GAA GGT CGG AGT C	19
<i>GAPDH</i> -R	GAA AGA TGG TGA TGG GAT TT	20

F forward, R reverse, PI product length

Effects of Usnic Acid on *Leishmania* Species

Fig. 1 Comparison of the effects of usnic acid concentrations on viability of *Leishmania* species compared to control. The results of IC₅₀ values of usnic acid were calculated after 48 h incubation. The IC₅₀ values were 10.76 µg/ml, 13.34 µg/ml, and 21.06 µg/ml for *L. major*, *L. tropica*, and *L. infantum*, respectively. All the experiments were performed in triplicate and data are expressed as the mean value

Statistical analysis

Statistical analyses were performed by non-linear regression using the Sigma Plot 11 program to obtain the IC₅₀. The differences between treated and control groups were evaluated using one-way ANOVA (Analysis of variance) with post hoc Tukey tests and differences were considered significant when the *p* value was less than 0.05. All tests were performed in triplicate in six independent experiments.

Relative mRNA expression profiles of the five targeted genes were normalized with reference gene (GAPDH) and calculated by the 2^{-ΔΔCT} method (Livak and Schmittgen 2001). Changes in expression levels of the examined genes were calculated for statistical significance with one-way ANOVA. Fisher's Least Significant Difference (LSD) test was applied with *p* < 0.05.

Results

Antileishmanial activity and IC₅₀ values of usnic acid

The effects of usnic acid on *L. major*, *L. infantum*, and *L. tropica* survival and IC₅₀ values were demonstrated by the MTT cell viability assay. Usnic acid reduced the viability of the three parasites at different levels in a dose-dependent manner compared to the controls which had no effect. IC₅₀ values of usnic acid for *L. major*, *L. tropica*, and *L. infantum* were determined to be 10.76 µg/ml, 13.34 µg/ml, and 21.06 µg/ml, respectively. It was interest to see that *L. major*

viability was totally lost after 48 h incubation with 100 μM of usnic acid (Fig. 1).

The change of apoptotic genes on *Leishmania* spp.

The change of mRNA expression profiles of antiapoptotic (*Bcl-2*) and pro-apoptotic genes [*P53*, *Bax*, and Caspase family (*Casp-3* and *Casp-9*)] of *Leishmania* spp. with usnic acid was determined by qRT-PCR (Fig. 2).

When usnic acid has been applied to *L. major* at the dose of IC_{50} , 14- and 12-fold increases have been found in *P53* and *Bax*, respectively, whereas 0.5-fold decrease in *Bcl-2* was obtained compared to the control cells ($p < 0.05$). There was eightfold increase in *Casp-3* ($p < 0.05$) and *Casp-9* ($p < 0.01$) compared to the untreated cells (Fig. 2a).

The exposure of usnic acid at the indicated IC_{50} value to *L. infantum* led to an approximately 12- and 11-fold increase in tumor suppressor genes like *P53*, antiapoptotic genes *Bax* ($p < 0.01$), respectively, but a 0.3-fold decrease in *Bcl-2* ($p < 0.05$) compared to the control cells. There was a five and sixfold increase in *Casp-3* and *Casp-9* ($p < 0.01$) (Fig. 2b).

The expression changes of suppressor genes and pro-apoptotic genes of *L. tropica* after the application of usnic acid have been observed as a ninefold increase in *P53* compared to the control cells, and nearly an eightfold increase in *Bax* ($p < 0.05$), although 0.2-fold decrease was shown in *Bcl-2* ($p < 0.05$). Approximately threefold increase was found in *Casp-3* ($p < 0.01$), and sixfold increase was observed in *Casp-9* compared to the control cells ($p < 0.05$) (Fig. 2c).

Discussion

Leishmaniasis is common vector-borne disease in Europe and in Turkey; visceral and cutaneous forms are seen (Ok et al. 2002; Djuardin et al. 2008; Oztoprak et al. 2010; Dincer et al. 2012) as well as the visceral form of the disease can cause even death, especially for the children and immune-suppressed patients (Tanir et al. 2006; Ozkan et al.

2008; Arik-Yilmaz et al. 2009). There are a few options for the treatment of leishmaniasis. The resistance, side effects, and cost of these agents are the main concerns. New treatment choices such as plant extracts or fungus-derived compounds are being investigated for solving these problems (Schemeda-Hirschmann et al. 2008; Albakhit et al. 2016). Lichens have been used for various purposes, such as dyes, perfumes, cosmetic, folk medicines, and mainly pharmaceutical use (Muller 2001). Secondary metabolites of lichen and especially usnic acid which is efficiency where analyzed recently could be alternative for the treatment of leishmaniasis (Luz et al. 2015).

MTT is the most commonly used indicator for the detection of viability and IC_{50} values and functions with the principle of tetrazolium–formazan conversion by means of the living cell mitochondrial enzymes. In our study, the antileishmanial activity of usnic acid was studied in three different *Leishmania* species, and the IC_{50} values at 48 h were found as 10.70 $\mu\text{g}/\text{ml}$ for *L. major*, 13.34 $\mu\text{g}/\text{ml}$ for *L. tropica*, and 21.06 $\mu\text{g}/\text{ml}$ for *L. infantum*. These values are the first in the literature to show the effectiveness of usnic acid on these *Leishmania* species which are commonly seen in Mediterranean region. These findings were parallel that obtained by Luz et al. ($\text{IC}_{50} = 18.30 \mu\text{g}/\text{ml}$) for *L. infantum chagasi* (Luz et al. 2015) and Carvalho et al. ($\text{IC}_{50} = 20 \mu\text{g}/\text{ml}$) for another similar parasite *Trypanosoma cruzi* (Carvalho et al. 2005). Our findings show that *L. major* and *L. tropica* species are more susceptible to cytotoxicity of usnic acid in in vitro conditions than *L. infantum*.

In a study by Fournet and Munoz, the effects of lichen compounds on *Leishmania braziliensis*, *Leishmania amazonensis* and *Leishmania donovani* were investigated (Fournet and Munoz 2002). In this study, similar to our findings in *L. major*, it was demonstrated that application of usnic acid at 10 $\mu\text{g}/\text{ml}$ on *L. braziliensis* and *L. amazonensis* strains revealed 80–90% lysis of parasites and 100% lysis of *L. donovani*. The IC_{50} values of glucantime of *L. major*, which is clinically used in the treatment of leishmaniasis, are ranging from 12 to 22 $\mu\text{g}/\text{ml}$ in the literature

Fig. 2 Effect of usnic acid on mRNA expression patterns of *P53*, *Bcl-2*, *Bax*, *Casp-3*, and *Casp-9* genes of *Leishmania* species. Expression level of mRNA was determined using RT-PCR that repeated three

times after optimization. The results were calculated as mean $\pm$ SD from three independent experiments ($n = 3$); ANOVA. Values are statistically significant at $*p < 0.05$ respective control

(Schmeda-Hirschmann et al. 2008; Eskandari et al. 2016; Foroutan-Rad et al. 2016). Our study is also valuable that usnic acid is at least as effective as glucantime on *L. major*.

Many scientific investigations have been designed with the aim of examining the apoptotic pathways of association between usnic acid and cancer cells (Dincsoy and Cansaran-Duman 2017; Kilic et al. 2018). On the other hand, there are some studies on the effect of usnic acid to different *Leishmania* species (Fournet and Munoz 2002; Luz et al. 2015; Araujo et al. 2015), but there are no available data that show the usnic acid's apoptotic pathway on *Leishmania* spp.

Bcl-2, Bax, Fas proteins, and caspase-3 and caspase-9 enzymes are the major determinants of apoptosis which is a form of programmed cell death (Zhao et al. 2010; Chu et al. 2011). The demonstration of the apoptotic pathways and related signal mechanisms has supported the new drug discovery which was focused on these highly popular topic. Thus, research on apoptotic processes has become increasingly important. First and foremost, the investigation of the mechanism of apoptosis is necessary and meaningful; it will go deep into clinical care and drug guidance to guide drug design. Therefore, we focused that apoptosis could be the mode of cell death in *Leishmania* species through either nuclear (*P53*, *Bcl-2*, and *Bax*) or mitochondrial (Caspases) pathway.

The previous studies have shown that some novel drug candidate molecules induce apoptosis-associated inhibition by initiating flow cytometry assays (Tolomeo et al. 2013; Varela et al. 2014; Foroutan-Rad et al. 2016), TUNEL assay (Dutta et al. 2007; Sardar et al. 2013; Marinho et al. 2014) and caspase assay (Saha et al. 2009; Sardar et al. 2013). Previously mentioned studies performed DNA-based molecular techniques, but our study shows apoptosis of *Leishmania* after usnic acid treatment by qRT-PCR at RNA level in the first time. In the light of the knowledge about *Leishmania* spp. are able to undergo apoptosis, our study suggests that the usnic acid regulated target for apoptosis may depend upon the *Leishmania* spp. via apoptosis-related gene expression.

In recent years, antileishmanial activity of some natural and synthetic natural compounds such as in vitro and stilbene derivatives has been shown in studies conducted (Tolomeo et al. 2013). It has been observed that all of the compounds taken to work reduce parasite viability in a dose-dependent manner. It has been suggested that the mechanism of action of these compounds on apoptosis in parasites may disrupt the mitochondrial membrane potential. In addition, the compounds showed the ability to block the cell cycle of the *Leishmania* promastigotes in the G2-M phase. This study has shown that some of the stilbene derivatives have high-level antileishmanial activity and that as a pro-apoptotic agent at an appropriate time to in vivo studies of TTAS (*Trans*-3,40,5-trimethoxy-30-amino-stilbene) for

promastigotes of *Leishmania* is appropriate. In our study, a new mechanism has been established in which usnic acid inhibits proliferation of *Leishmania* promastigotes and downregulates the level of *Bcl-2* gene expression, which is caused by apoptosis, and upregulates *p53*, *Bax*, *Casp-3*, and *Casp-9* gene expression. This study clearly demonstrates that usnic acid plays a critical role in the apoptotic process induced by usnic acid in the promastigotes of *Leishmania*. In parallel, *L. infantum* and *Leishmania panamensis* promastigotes, the cell cycle with the HSP90 inhibitor 17-AAG (≥ 100 nM 17-AAG) was stopped at G0/G1 phase, and that cell death is highly upregulated by an apoptotic-like process mediated by the ether phospholipid edelfosine (5–20 μ M) (Varele-M et al. 2014).

Zu et al. determined that the effects of emodin on the proliferation of human breast cancer cells by qRT-PCR and western blot analysis. In breast cancer cells, emodin treatment downregulated Bcl-2 expression and upregulated p53 and Bax levels in protein level. It has been determined that the gene expression results in our study are consistent with the protein results of Zu et al. (2015).

It has been shown that the antileishmanial activity of spinigerin is originally derived from *Pseudacanthotermes spiniger* and *L. donovani* promastigotes, and provides apoptosis-like cell death when exposed to spinigerin (IC₅₀, 150 μ M) (Sardar et al. 2013). Approximately 97% of the cell populations were found to be annexin-V positive and 44% higher annexin-V positive. Furthermore, apoptosis is a similar death (Sardar et al. 2013), as cell rounding, nuclear condensation, oligonucleosomal DNA degradation, and TUNEL-positive cells show morphological changes without loss of membrane integrity in spinigerin exposure. In summary, it has been shown that spinigerin causes caspase-independent apoptosis-like cell death in *L. donovani*. In our study, it was determined that usnic acid, a naturally occurring lichen secondary metabolite compound, showed strong antiproliferative effects in vitro on three *Leishmania* species like spinigerins. We have suggested that usnic acid-induced cell survival inhibition is caused by cell apoptosis, and so, we have applied qRT-PCR to observe molecular changes in apoptosis as described by Livak and Schmittgen (2001). Usnic acid mostly induced apoptosis in *L. major* treated with 10.76 μ M of usnic acid for 48 h and the most gene expression value obtained from *P53* gene.

Berberine chloride, a quaternary isoquinoline alkaloid, emerges as a promising antileishmanial compound. The IC₅₀ value of this compound is 7.1 mM in the *L. donovani* promastigotes; The G0/G1 population was accompanied by DNA laddering, hallmarks of apoptosis, and mitochondrial membrane potential (Saha et al. 2009). Berberine did not stimulate chloride caspase activity and antileishmanial activity in the presence of a caspase inhibitor. Z-Val-Ala-DL-Asp (methoxy)-fluoromethyl ketone remained

unchanged, indicating that apoptosis was caspase-independent and an apoptosis-like death following improved reactive oxygen species formation (Saha et al. 2009). Our study has also shown that usnic acid causes downregulation *Bcl-2* gene expression. After administration of usnic acid at IC_{50} values determined for each *Leishmania* species, qRT-PCR showed a statistically significant increase in *P53* and *Bax* genes as well as *Casp-3* and *Casp-9*, members of the caspase family, relative to the control. These genes, which are used as apoptosis indicators, show that the parasitic killer effect is over apoptosis pathways. qRT-PCR assay provides an opportunity to the level of gene expression changes apoptosis induced by usnic acid as treatment of leishmaniasis.

Conclusion

Chemotherapeutic agents currently available in the treatment of visceral leishmaniasis have difficulties in being routinely used due to acquired drug resistance and toxicity. In this sense, our study suggests that usnic acid may be an attractive alternative to the conventional pharmaceutical therapies. We first reported the effectiveness of usnic acid as an antiproliferative natural compound to inhibit *Leishmania* species. This valuable naturally occurring growth inhibitory mechanism of the lichen compound is associated with the downregulation of the gene expression resulting in apoptosis.

In conclusion, the higher efficacy of usnic acid on the cutaneous forms of leishmaniasis has been evaluated as highly promising for local (topical) or intralesional treatment. Our study provides a basis for the treatment of usnic acid lichen secondary metabolite which is more effective, easily accessible, and inexpensive agent against the more common *Leishmania* spp. in our region. Thus, the molecular pathways responsible for the efficacy of usnic acid, which can be used as a drug candidate, are more clearly defined. Our findings support that usnic acid may be a powerful new natural compound for the clinical treatment of leishmaniasis in the future, although in vivo studies are still needed in animal models.

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Compliance with ethical standards

Conflict of interest No competing financial interest exists.

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