Journal of Ethnopharmacology 282 (2022) 114641

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Antimycobacterial activity of acetone extract and isolated metabolites from folklore medicinal lichen *Usnea laevis* Nyl. against drug-sensitive and multidrug-resistant tuberculosis strains

Vinay Bharadwaj Tatipamula [a,b,*], Satya Sowbhagya Priya Annam [c,d]

[a] Center for Molecular Biology, Duy Tan University, Da Nang, 550000, Viet Nam
[b] Institute of Research and Development, Duy Tan University, Da Nang, 550000, Viet Nam
[c] UCB Pharma S.A., Allée de la Recherche 60, 1070, Brussels, Belgium
[d] Pharmaceutical Sciences Department, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, 530003, Andhra Pradesh, India

ARTICLE INFO

Keywords:
Usnea laevis
Tuberculosis
Antimycobacterial activity
Multidrug-resistant strains
Cytotoxicity
Selectivity index

ABSTRACT

Ethnopharmacological relevance: Tuberculosis (Tb) is one of the most infectious diseases caused by *Mycobacterium tuberculosis* (*M.t*) with almost 2 million deaths yearly. Although many Tb control programs have been organised, there is an elevated number of Tb cases due to the appearance of extremely drug-resistant and multidrug-resistant (MDR) Tb strains. In the cultures of Venezuelan Andes, fruticose lichen *Usnea laevis* Nyl. (Usneaceae) with folklore name 'Barba de Piedra, Tusinya' is used as a natural remedy for Tb.

Aim of the study: This study was performed to provide a scientific rationale for the folklore usage of *U. laevis* in treating Tb by validating its antimycobacterial activity against two drug-sensitive and four MDR-Tb strains.

Materials and methods: The mycobacterial inhibitory activities of acetone extract (**UI**), fractions (**F1-10**), and isolated metabolites (**1**–**4**) of *U. laevis* were evaluated against *M.t* H37Ra using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide reduction menadione assay (XRMA). Furthermore, **UI** and **1**–**4** were subjected to antimycobacterial activity against *M.t* H37Ra, *Mycobacterium smegmatis*, and four MDR-Tb (MDR-A8, MDR-V791, MDR-R and MDR-40) strains using resazurin microtitre plate assay (REMA) and cytotoxicity against THP-1 macrophages using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and their selectivity index values were also calculated.

Results: Initially, **UI** has shown prominent inhibitory activity (IC_{50} value: 5.44 ± 0.36 μg/ml) and four of its fractions (**F1, F2, F5** and **F7**) also exhibited the best inhibitory activity (IC_{50} values ranged from 7.46 ± 0.19 to 71.38 ± 2.57 μg/ml) against *M.t* H37Ra using XRMA. Purification of these bioactive fractions identified four metabolites, namely usnic acid (**1**), atranorin (**2**), salazinic acid (**3**), and lobaric acid (**4**). From the MIC values of REMA, it was identified that **UI, 1** and **4** were more effective in inhibiting the growth of all four MDR-Tb strains, compared to first-line drug rifampicin. Interestingly, **UI** has shown better antimycobacterial activity than **1**–**4** and rifampicin against MDR-Tb strains may be due to the synergistic effect of its metabolites. Also, the IC_{50} values of **UI** and **1**–**4** on THP-1 macrophages were found to be far higher than MIC values against tested Tb strains, indicating that THP-1 macrophages were not harmfully affected at concentrations that were effective against Tb strains. Further, the calculated selectivity index values revealed the more active and non-toxicity of **UI, 1** and **4** against MDR-Tb strains than rifampicin.

Conclusions: The current study lends the first evidence for the presence of antimycobacterial metabolites in *U. laevis*. The results exposed the Andean folklore use of *U. laevis* for treating Tb, and the key biomarker metabolites were found to be **1** and **4**. Hence, it can be concluded that *U. laevis* can be used as a potential source for the novel drug development for MDR-Tb.

* Corresponding author. Center for Molecular Biology, Duy Tan University, Da Nang, 550000, Viet Nam
E-mail addresses: vinaybharadwajtatipamula@duytan.edu.vn (V.B. Tatipamula), priya.annam2@gmail.com (S.S.P. Annam).

https://doi.org/10.1016/j.jep.2021.114641
Received 10 August 2021; Received in revised form 3 September 2021; Accepted 11 September 2021
Available online 15 September 2021

1. Introduction

A facultative intracellular bacteria *Mycobacterium tuberculosis* (*M.t*) can survive and propagate within macrophages that cause an infectious disease named tuberculosis (Tb) (Luo et al., 2013). The drug treatment for susceptible *M.t* strains is based on a combination of first-line anti-Tb drugs such as rifampicin, isoniazid, pyrazinamide and ethambutol (World Health Organization, 2013). Although many Tb control programs have been organised, there is an elevated number of Tb cases due to limited drug efficacy, poor patient compliance, and the occurrence of extremely drug-resistant and multidrug-resistant (MDR) Tb strains. The increasing cases of extremely drug-resistant and MDR have created a severe Tb-related health illness globally (Seaworth and Griffith, 2017). As per the World Health Organization, nearly 484,000 novel MDR-Tb cases were reported in 2018, of which 100,000 cases are particularly related to rifampicin-resistant Tb cases (World Health Organization, 2019). Hence, there is an urgent need for more active novel antimycobacterial agents with new chemical moieties that have the capability of penetrating into macrophages and hindering the progress of intracellular pathogens that are required to combat the Tb threat.

In Andean folk medicine, a fruticose lichen *Usnea laevis* Nyl. (Usneaceae), popularly known as 'Barba de Piedra, Tusinya' is used for the treatment of tuberculosis, dermatosis, infections, mycosis, and pneumonia (Marcano, 1991; Crawford, 2015). With this background, the Marcano group isolated usnic acid from *U. laevis* and reported its antibiotic activity (Marcano et al., 1999). Therefore, in the current study, we report the bioassay-guided isolation of secondary metabolites from acetone extract of *U. laevis* and evaluated for their *in vitro* antimycobacterial activity using *M.t* H37Ra, *Mycobacterium smegmatis* (*M.s*), and four MDR-Tb strains. Furthermore, acetone extract and all isolated metabolites were subjected to cytotoxicity against human acute monocytic leukaemia cell line (THP-1) macrophages, and the selectivity index (SI) values were also calculated as a degree for the drug lead potential of the extract/metabolites.

2. Materials and Methods

2.1. Lichen material

The entire lichen *Usnea laevis* Nyl. was collected from the tree barks at Western Ghats mountains, Udhagamandalam (Nilgiris District), Tamil Nadu, India, in December 2019, and a voucher specimen (19–035,542) was deposited at the CSIR-National Botanical Research Institute, Lucknow, India.

2.2. Extraction and bioassay-guided isolation

Dried lichen *U. laevis* (~200 g) was blended into powder and extracted with acetone (300 ml × 14 days × 3) by using the maceration method (Nguyen et al., 2021) at room temperature. The combined extracts were evaporated using rotavapor (Shimadzu Rotation evaporator QR, 2005-S, Japan) to obtain acetone extract of *U. laevis* (**UI**, 18.4 g) as a brown solid. Initially, **UI** was screened for antimycobacterial assay against *M.t* H37Ra using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction menadione assay (XRMA) (Singh et al., 2011) and found to be very active (Table S1).

The schematic procedure of bioassay-guided isolation of **UI** was illustrated in Fig. 1. In brief, about 15.0 g of **UI** was exposed to chromatography by sintered disc column (Borosil, India) over 230–400 mesh size silica gel (Merck, India) using dichloromethane-ethyl acetate gradient (0–100%) yielding ten fractions (**F1-10**). All these fractions were also screened against *M.t* H37Ra strain using the XRMA method (Singh et al., 2011). The outcomes of the screening found that only four fractions (**F1, F2, F5** and **F7**) showed >65% inhibition against *M.t* strain (Table S1), signifying additional purification to isolate antimycobacterial metabolites. By repeated column chromatography (sintered disc column, Borosil, India), **F1** (1.2 g) after purification using hexane-dichloromethane gradient (0–100%) yielded **1** (900 mg) as yellow needles. Similarly, **F2** (900 mg) yielded **2** (550 mg) as colourless needles. By using a dichloromethane-ethyl acetate (0–100%, gradient) solvent system, **F5** (840 mg) yielded **3** (650 mg) as colourless needles, and **F7** (800 mg) yielded **4** (350 mg) as pale yellow needles (Fig. 1). For the chemical characterization, all the isolated metabolites were subjected to Mass (LC/MS Triple Quad Portfolio, Agilent, China) and 2D nuclear magnetic resonance (Bruker Avance 400 Spectrometer, Germany) spectral analyses using the Robust mass spectrometry software and Bruker's topspin software, respectively.

2.3. In vitro antimycobacterial assay

Standard cultures of non-virulent *Mycobacterium tuberculosis* H37Ra

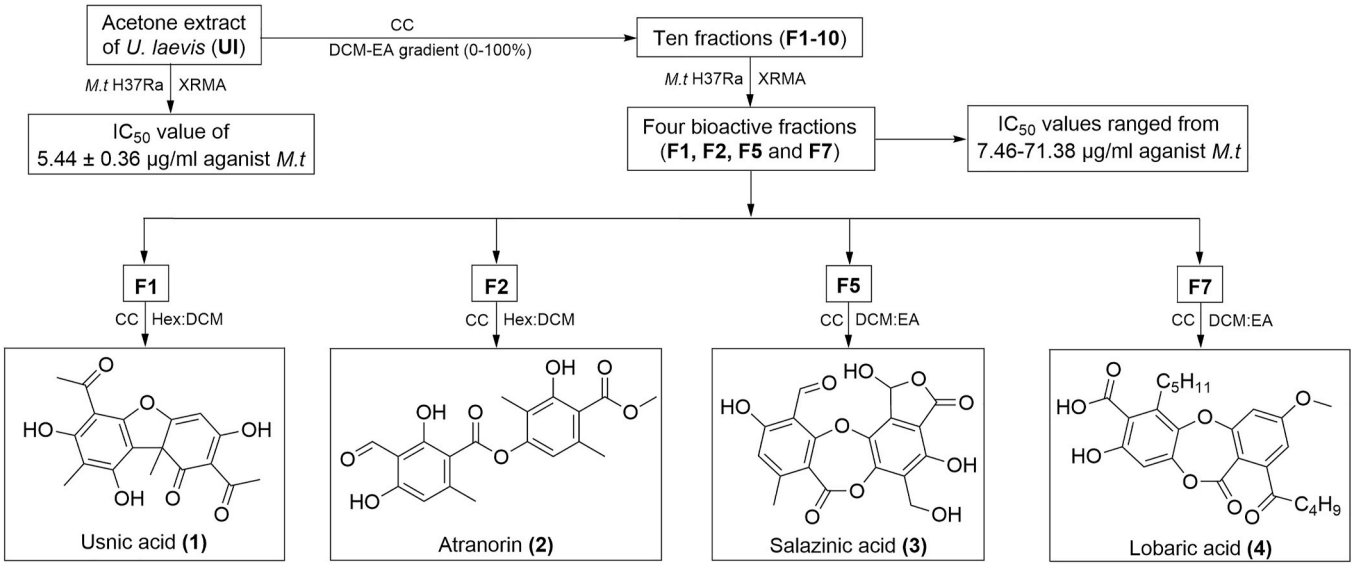

Fig. 1. Bioassay-guided isolation of metabolites **(1–4)** from acetone extract (**UI**) of *Usnea laevis* Nyl. Values of half-maximal inhibitory concentrations (IC_{50}) were expressed as mean ± standard deviation ($n = 3$) against *M.t* H37Ra. *M.t*: *Mycobacterium tuberculosis*; XRMA: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide reduction menadione assay; CC: Column chromatography; DCM: dichloromethane; EA: ethyl acetate; Hex: *n*-hexane.

strain (ATCC 25177), *Mycobacterium smegmatis* (ATCC 1441), and four MDR-Tb strains, namely MDR *Mycobacterium tuberculosis* A8 (MDR-A8), MDR *Mycobacterium tuberculosis* V791 (MDR-V791), *Mycobacterium smegmatis* MDR-R (MDR-R), and *Mycobacterium smegmatis* MDR-40 (MDR-40) were included in this study. The specifications of these MDR strains were listed in Table 1. All strains were grown on Middlebrook 7H9 broth supplemented with albumin dextrose complex (10%) and maintained as glycerol stocks at −70 °C.

For bioassays, 50 μL of the bacterial-glycerol stock was sub-cultured in Middlebrook 7H9 medium supplemented with glycerol (0.05%) and Tween 80 (0.05%) to attain metabolically active mycobacteria and was grown under aerobic conditions with shaking at 190 rpm and 37 °C to log phase (OD595 ~0.5). Later, the clump cultures were sonicated for 5 min and re-suspended by sub-cultured through a 26½ gauge needle (10–15 times) and diluted to 1:20 in Middlebrook 7H9 medium (without Tween 80) and used to carry out the experiments (Singh et al., 2015).

Initial *M.t* inhibitory screening of **Ul, F1-10**, and **1**–**4** were tested at four different concentrations (5–100 μg/ml) against *M.t* H37Ra strain using the XRMA method at 470 nm (Singh et al., 2011) in triplicate. Briefly, to 250 μl of the above-prepared *M.t* H37Ra culture added test sample and 200 μM of XTT in a 96-well plate and incubated at 37 °C for 20 min. Later, optical density (OD) was measured at 470 nm using a Spectramax plate reader. The percentage of inhibition was deliberated with the OD values and half-maximal inhibitory concentration (IC_{50}) values were calculated by logistic regression analysis. Rifampicin (0.1–1.0 μg/ml concentrations) was used as the reference drug, while DMSO (up to 2%) was used as a negative control.

Further evaluation of the antimycobacterial activity of **Ul** and **1**–**4** (200–0.10 μg/ml concentrations) was carried out against *M.t,* MDR-A8, MDR-V791, *M.s,* MDR-R, and MDR-40 strains using resazurin microtitre plate assay (REMA) (Lakshmanan et al., 2011) in triplicate. The two-fold serial dilutions of Ul and 1–4 from 200 to 0.10 μg/ml were carried out in a 7H9 medium. In a 96-well plate, the test sample and 100 μl of the above prepared MDR culture were added and incubated at 37 °C for 7 days. Later, each well was treated with 30 μl of resazurin solution (0.02%) and further incubated for 2 days. The minimum inhibitory concentration (MIC) values were deliberated by a colour change from blue to pink. Rifampicin (200–0.10 μg/ml concentrations) was used as the reference drug, while DMSO (up to 2%) was used as a negative control.

2.4. Cytotoxicity assay

Ul and **1**–**4** were subjected for cytotoxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Tatipamula and Vedula, 2020) against THP-1 macrophages in triplicate. From the National Centre for Cell Science (Pune, India), the human acute monocytic leukaemia cell line (THP-1) was procured and maintained in RPMI-1640 medium (Merck, India) in a 5% CO_2 atmosphere at 37 °C. A day before infection, differentiation of THP-1 monocytes into macrophages was induced by seeding 1×10^5 cells in a 96-well plate and added with 100 nM phorbol-12-myristate-13-acetate (Sigma-Aldrich, India) and incubated at 37 °C overnight in a cell culture medium. On the experiment day, the medium was replaced with a fresh cell culture medium and incubated with test samples (50–300 μg/ml concentration dissolved in DMSO) in a 5% CO_2 atmosphere at 37 °C for 48 h. Later, 20 μL of 5.0 mg/ml of MTT dye (Sigma-Aldrich) solubilized in DMSO was added to each well and incubated in a 5% CO_2 atmosphere at 37 °C for 4 h. The absorbance was recorded at 570 nm using a microplate reader (Molecular Devices, USA). From the dose-response curves, IC_{50} values were deliberated against THP-1 macrophages. Doxorubicin (2.5–10 μg/ml concentration dissolved in DMSO) was used as the reference drug, while DMSO was used as a negative control.

Table 1
List of mycobacterial multidrug-resistant (MDR) strains.

Strain	Code	Specification	Reference
MDR *Mycobacterium tuberculosis* A8	MDR-A8	Resistant to rifampicin and isoniazid	Horváti et al. (2015)
MDR *Mycobacterium tuberculosis* V791	MDR-V791	Resistant to rifampicin, isoniazid, streptomycin, and ofloxacin	Yang et al. (2018)
Mycobacterium smegmatis MDR-R	MDR-R	Resistant to rifampicin, tetracycline, and chloramphenicol	Gupta et al. (2011)
Mycobacterium smegmatis MDR-40	MDR-40	Resistant to rifampicin, ampicillin, and kanamycin	Gupta et al. (2011)

2.5. Statistical analysis

The statistical significance for IC_{50} values was determined using one-way analysis of variance followed by Dunnett's multiple comparison test, where $^*P < 0.05$ and $^{***}P < 0.0001$ were considered statistically significant compared to positive control.

3. Results and discussion

3.1. Bioassay-guided isolation

To isolate the biological active antimycobacterial substances from **Ul**, it was initially fractionated to afford ten fractions (**F1-10**) using column chromatography. These fractions were then screened for antimycobacterial activity against *M.t* H37Ra using the XRMA method. The outcomes of the preliminary screening revealed that only **Ul, F1, F2, F5** and **F7** showed above 65% inhibition against *M.t* strain, while other fractions revealed *M.t* inhibition in the range of 5–18% (Table S1). Among these, **Ul** and **F1** presented to be the most potent antimycobacterial agents against *M.t* H37Ra strain with IC_{50} values of 5.44 ± 0.36 and 7.46 ± 0.19 μg/ml, respectively. Whereas **F2, F5** and **F7** showed significant ($P < 0.0001$) antimycobacterial activity with IC_{50} values of 71.38 ± 2.57, 48.02 ± 2.91, and 12.71 ± 2.47 μg/ml, respectively, against *M.t* H37Ra, compared to rifampicin (IC_{50} value: 0.14 ± 0.00 μg/ml) (Fig. 2). On the other hand, the negative control (DMSO, up to 100 μg/ml) used for dilution did not display any inhibition of *M.t* H37Ra growth.

Later, the bioactive fractions (**F1, F2, F5** and **F7**) were purified using column chromatography, which yielded four metabolites (**1**–**4**). Upon characterization using spectral and elemental analysis, these

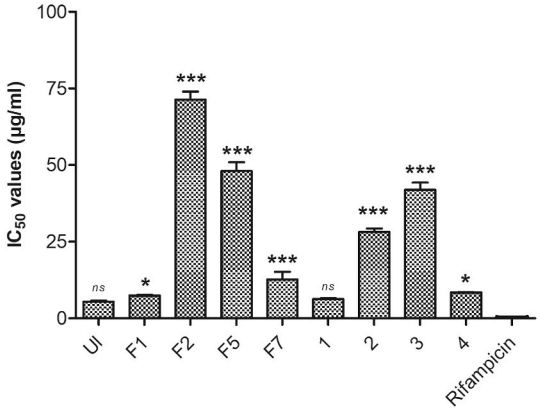

Fig. 2. Half-maximal inhibitory concentrations (IC_{50}) of acetone extract (**Ul**), bioactive fractions (**F1, F2, F5** and **F7**), and isolated metabolites (**1**–**4**) from *Usnea laevis* Nyl. against *Mycobacterium tuberculosis* H37Ra using the XRMA method. Statistical significance was determined using one-way analysis of variance followed by Dunnett's multiple comparison test, where $^*P < 0.05$ and $^{***}P < 0.0001$ indicated a significantly higher compared to rifampicin. XRMA: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide reduction menadione assay; ns: non-significant compared to rifampicin.

metabolites were identified as usnic acid (**1**), atranorin (**2**), salazinic acid (**3**), and lobaric acid (**4**) (Fig. 1). Except **1**, all other metabolites were probably reported for the first time from *U. laevis*. The preliminary screening of these metabolites revealed that only metabolite **1** (5–100 μg/ml) had a higher percentage of *M.t* H37Ra inhibition as compared to all fractions (**F1-10**), which was also noted to be almost equivalent to that of the **Ul** (Table S1). Metabolites **1** (IC_{50} value: 6.32 ± 0.20 μg/ml) and **4** (IC_{50} value: 8.49 ± 0.11 μg/ml; $P < 0.05$) obtained from bioactive fractions **F1** and **F7**, respectively, exhibited profound inhibitory action against *M.t* H37Ra, while metabolites **2** and **3** obtained from **F2** and **F5** showed significantly ($P < 0.0001$) higher IC_{50} values of 28.40 ± 1.13 and 41.96 ± 2.32 μg/ml, respectively, against *M.t* H37Ra, compared to rifampicin (Fig. 2). This emphasized that the isolated metabolites (**1**–**4**) might be responsible for the antimycobacterial activity of bioactive fractions (**F1, F2, F5** and **F7**), as well as **Ul**.

3.2. In vitro antimycobacterial assay

Depending upon the outcomes of the initial screening, **Ul** and **1**–**4** at a concentration ranged from 0.10 to 200 μg/ml were further evaluated to determine MIC values against two drug-sensitive (*M.t* H37Ra and *M.s*), four MDR-Tb (two MDR *M.t*: MDR-A8 and MDR-V791; two MDR *M.s*: MDR-R and MDR-40) strains using the REMA method. The outcomes of REMA were presented in the form of MIC values in Table 2. Among all tested samples, **Ul, 1** and **4** revealed significant antimycobacterial activity with MIC values of 25, 50 and 100 μg/ml, respectively, against *M.t* H37Ra stain, compared to rifampicin (MIC value of 0.2 μg/ml) (Table 2). While metabolites **2** and **3** didn't show any inhibition against *M.t* H37Ra at all concentrations tested and their MIC value was considered as >200 μg/ml (Table 2).

Few studies have elucidated the antimycobacterial properties of metabolites **1**–**4** against various Tb strains, namely *M.t* H37Rv, *M. kansasii*, *M. avium,* and *M. aurum* (Honda et al., 2010; Ingólfsdóttir et al., 1998; Lira et al., 2009; Lucarini et al., 2014). Their inhibitory results of metabolites **1**–**4** against *M.t* strain were found similar to our present research findings. Besides, literature search of these metabolites did not fetch any reports of their mechanism of action against *M.t*. However, Gupta et al. (2012) postulated that usnic acid exerts its antibacterial activity against methicillin-resistant *Staphylococcus aureus* by disruption of the cell membrane.

In the present study, metabolites **1**–**4** have been assessed for the first time against *M.s* and four MDR-Tb (MDR-A8, MDR-V791, MDR-R and MDR-40) strains in this paper. Similar to *M.t* strain, **Ul, 1, 3** and **4** revealed significant antimycobacterial activity with MIC values of 6.25, 12.50, 50.0 and 50.0 μg/ml, respectively, against *M.s* stain, compared to rifampicin (MIC value of 0.2 μg/ml) (Table 2). While metabolite **2** was found to be inactive against *M.s* up to 200 μg/ml concentration (Table 2).

On the other hand, all four MDR-Tb strains were found to be more susceptible to **Ul**, compared to *M.t* H37Ra and *M.s*. The MIC values of **Ul** against MDR-Tb strains were in the range of 0.41–6.25 μg/ml (Table 2). From these results, it was noticed that **Ul** was more potent in inhibiting the growth of MDR-Tb strains in comparison to drug-sensitive strains (*M.t* H37Ra and *M.s*).

Among isolated metabolites, the two MDR *M.t* strains (MDR-A8 and MDR-V791) were found to be more susceptible to **1** and **4**, when compared to *M.t* H37Ra. Metabolites **1** (MIC values: 25.0 and 12.5 μg/ml) and **4** (MIC values: 50 and 50 μg/ml) exhibited the highest mycobactericidal effect against MDR-A8 (resistant to rifampicin and isoniazid) and MDR-V791 (resistant to rifampicin, isoniazid, streptomycin, and ofloxacin), respectively, compared to rifampicin (MIC values: 100 and > 200 μg/ml, respectively). Instead, metabolites **2** and **3** were found to be inactive against both MDR-A8 and MDR-V791 up to 200 μg/ml concentration (Table 2).

Interestingly, metabolite **1** was found to be extremely effective in inhibiting both MDR *M.s* strains, namely MDR-R (resistant to rifampicin, tetracycline, and chloramphenicol) and MDR-40 (resistant to rifampicin, ampicillin, and kanamycin), with a MIC value of 12.50 μg/ml. Similarly, metabolites **3** and **4** showed better mycobactericidal activity against MDR-R and MDR-40 with a MIC value of 50 μg/ml than rifampicin (MIC value: >200 and 100 μg/ml, respectively). While, metabolite **2** didn't show any inhibition against all tested MDR *M.s* stains at all concentrations tested from 200 to 0.10 μg/ml, hence, MIC was considered as >200 μg/ml (Table 2). In contrast, the negative control (DMSO, up to 2%) used for two-fold serial dilution did not display any growth inhibition of all tested mycobacterial strains.

Generally, synergistic effects can be created if the chemical constituents of an extract affect multiple targets or interact with one another in order to improve the bioavailability of one or more substances in the mixture (Wagner and Ulrich-Merzenich, 2009). For instance, the combination of rifampicin with 7-methyljuglone (a natural secondary metabolite) reduced (four-fold) the MIC value, and also showed both intracellular and extracellular synergistic activity against tested *M.t* strains (Bapela et al., 2006). Similarly, in the present study, **Ul** has shown better antimycobacterial activity than **1**–**4** and rifampicin against MDR-Tb strains may be due to the synergistic effect of its metabolites. Concurrently, MDR-Tb strains were found to be more susceptible to metabolites **1, 3** and **4** with significantly lower MIC values than rifampicin, indicating these metabolites were identified as the chief biomarker substances present in traditional medicinal lichen *U. laevis*.

Table 2
Antimycobacterial activity, cytotoxicity, and selectivity index of isolated metabolites (**1**–**4**) and acetone extract (**Ul**) of *Usnea laevis* Nyl.

Sample	MIC values[a] (Selectivity index value[b])						Cytotoxicity[c] (IC_{50} values on THP-1 macrophages)
	M.t H37Ra	Multidrug-resistant *M.t* strains		*M.s*	Multidrug-resistant *M.s* strains		
		MDR-A8	MDR-V791		MDR-R	MDR-40	
Ul	25.00 (6)	6.25 (22)	1.63 (85)	6.25 (22)	0.81 (172)	0.41 (340)	139.36 ± 6.15***
1	50.00 (3)	25.00 (5)	12.50 (10)	12.50 (10)	12.50 (10)	12.50 (10)	130.60 ± 2.83***
2	>200 (ND)	>200 (ND)	>200 (ND)	>200 (ND)	>200 (ND)	>200 (ND)	286.13 ± 10.09***
3	>200 (ND)	>200 (ND)	>200 (ND)	100.00 (3)	50.00 (6)	50.00 (6)	243.85 ± 9.26***
4	100.00 (2)	50.00 (4)	50.00 (4)	50.00 (4)	50.00 (4)	50.00 (4)	190.56 ± 7.16***
Rifampicin	0.20 (467)	100.00 (1)	>200 (ND)	0.20 (467)	>200 (ND)	100.00 (1)	93.35 ± 3.81***
Doxorubicin	NT	NT	NT	NT	NT	NT	6.46 ± 0.18

[a] Values are expressed as μg/ml ($n = 3$); MIC: Minimum inhibitory concentration is the lowest concentration of the sample exhibiting percentage growth inhibition of ≥90%, relative to the growth control; *M.t: Mycobacterium tuberculosis; M.s: Mycobacterium smegmatis*; NT: Not tested; ND: Not determined.

[b] Selectivity index: Cytotoxicity (IC_{50} value)/Antimycobacterial activity (MIC value). The higher selectivity index values indicate the more active and non-toxicity of a metabolite/extract.

[c] Values are expressed as μg/ml (mean ± standard deviation, $n = 3$), where statistical analysis was determined using one-way analysis of variance followed by Dunnett's multiple comparison test, where ***$P < 0.0001$ indicated a significantly higher as compared to doxorubicin; IC_{50}: Half-maximal inhibitory concentration is the lowest concentration of the sample exhibiting percentage growth inhibition of 50%, relative to the growth control.

3.3. Cytotoxicity assay

As mentioned earlier, *M.t* is an intracellular pathogen that initiates pathogenesis in alveolar macrophages (Luo et al., 2013). THP-1 cells were commonly used to differentiate *in vitro* from macrophages (Pick et al., 2004). Therefore, **Ul** and **1**–**4** were tested on THP-1 macrophages for their safety assessment using MTT assay. From the results, it was noticed that the IC_{50} values of **Ul** (139.36 ± 6.15 μg/ml) and **1**–**4** (ranged from 130 to 286 μg/ml) on THP-1 macrophages were found to be much higher than MIC values against tested mycobacterial strains (Table 2). All these cytotoxicity IC_{50} values were found to be significantly ($P < 0.0001$) higher than that of the standard drug, doxorubicin (IC_{50} value: 6.46 ± 0.18 μg/ml) (Table 2). This indicates that THP-1 macrophages were not harmfully affected at concentrations that were effective against *M.t,* MDR-A8, MDR-V791, *M.s,* MDR-R and MDR-40 strains. Hence, the cytotoxicity results propose the biocompatible nature of all isolated metabolites.

3.4. Selectivity index

Theoretically, SI values for **Ul** and **1**–**4** were calculated (IC_{50}/MIC) to estimate the efficiency and safety of any drug during *in vivo* treatment for any bacterial infection (Dzoyem et al., 2016). The higher SI values indicate the more active and non-toxicity of a compound. In the present study, the SI ratio values of **Ul** (SI: 22–340), **1** (SI: 5–10) and **4** (SI: 4) towards all tested MDR-Tb isolated strains (MDR-A8, MDR-V791, MDR-R, and MDR-40) were far higher than rifampicin (SI: 1) (Table 2). Particularly, metabolite **3** (SI: 6) showed a significantly higher SI value towards two MDR *M.s* strains (MDR-R and MDR-40) than rifampicin (SI: 1). This theoretical analysis indicates that **Ul**, **1** and **4** displayed strong antimycobacterial activity against four MDR-Tb strains, compared to drug-sensitive strains (*M.t* H37Ra and *M.s*). These outcomes were considered very promising since **Ul**, **1** and **4** exhibited better selectivity against four MDR-Tb strains than toxicity to THP-1 macrophages.

4. Conclusions

To conclude, this is the first study investigation that provides support to the traditional usage of lichen *U. laevis* in Tb *via in vitro* antimycobacterial activity. The bioassay-guided isolation of *U. laevis* yielded four metabolites, of these, **1** and **4** possess significant antimycobacterial actions against *M.t* H37Ra, *M.s*, as well as four MDR-Tb strains, reveal its potential usage to combat drug resistance problem in Tb. Also, the cytotoxicity and SI index values found to be far higher than rifampicin indicate **1** and **4** would be non-toxic and more effective during *in vivo* studies. On a whole, **Ul** exhibited better antimycobacterial activity than the isolated metabolites against the MDR-Tb strains may be due to the synergistic effect of its metabolites. However, further studies with these isolated metabolites in combination with one another and/or with first-line anti-Tb drugs will be conducted to enhance the biological activity through the synergistic effects. Additionally, a systematic investigation is further required to explore the antimycobacterial activities of **1** and **4** in animals and humans, after which they may be used as a first-line treatment for Tb or as an adjunctive treatment with standard antimycobacterial drugs.

CRediT authorship contribution statement

Vinay Bharadwaj Tatipamula: conceived the study, analysed the data, and wrote the manuscript. **Satya Sowbhagya Priya Annam:** helped with investigations, Formal analysis, Software, Both the authors have read and approved the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114641.

References

Bapela, N.B., Lall, N., Fourie, P.B., Franzblau, S.G., Van Rensburg, C.E.J., 2006. Activity of 7-methyljuglone in combination with antituberculous drugs against Mycobacterium tuberculosis. Phytomedicine 13, 630–635.

Crawford, S.D., 2015. Lichens used in traditional medicine. In: Lichen Secondary Metabolites. Springer International Publishing, Cham, pp. 27–80. https://doi.org/10.1007/978-3-319-13374-4_2.

Gupta, S., Dwivedi, G.R., Darokar, M.P., Srivastava, S.K., 2011. Antimycobacterial activity of fractions and isolated compounds from Vetiveria zizanioides. Med. Chem. Res. 21, 1283–1289. https://doi.org/10.1007/S00044-011-9639-8.

Gupta, V.K., Verma, S., Gupta, S., Singh, A., Pal, A., Srivastava, S.K., Srivastava, P.K., Singh, S.C., Darokar, M.P., 2012. Membrane-damaging potential of natural L-(−)-usnic acid in Staphylococcus aureus. Eur. J. Clin. Microbiol. Infect. Dis. 31, 3375–3383. https://doi.org/10.1007/s10096-012-1706-7.

Honda, N.K., Pavan, F.R., Coelho, R.G., de Andrade Leite, S.R., Micheletti, A.C., Lopes, T. I.B., Misutsu, M.Y., Beatriz, A., Brum, R.L., Leite, C.Q.F., 2010. Antimycobacterial activity of lichen substances. Phytomedicine 17, 328–332. https://doi.org/10.1016/J.PHYMED.2009.07.018.

Horváti, K., Bacsa, B., Szabó, N., Fodor, K., Balka, G., Rusvai, M., Kiss, É., Mező, G., Grolmusz, V., Vértessy, B., Hudecz, F., 2015. Antimycobacterial activity of peptide conjugate of pyridopyrimidine derivative against Mycobacterium tuberculosis in a series of in vitro and in vivo models. Tuberculosis 95 (Suppl. 1). https://doi.org/10.1016/J.TUBE.2015.02.026. S207–S211.

Ingólfsdóttir, K., Chung, G.A.C., Skúlason, V.G., Gissurarson, S.R., Vilhelmsdóttir, M., 1998. Antimycobacterial activity of lichen metabolites in vitro. Eur. J. Pharmaceut. Sci. 6, 141–144. https://doi.org/10.1016/S0928-0987(97)00078-X.

Lakshmanan, D., Werngren, J., Jose, L., Suja, K.P., Nair, M.S., Varma, R.L., Mundayoor, S., Hoffner, S., Kumar, R.A., 2011. Ethyl p-methoxycinnamate isolated from a traditional anti-tuberculosis medicinal herb inhibits drug resistant strains of Mycobacterium tuberculosis in vitro. Fitoterapia 82, 757–761. https://doi.org/10.1016/J.FITOTE.2011.03.006.

Lira, M.C.B., Siqueira-Moura, M.P., Rolim-Santos, H.M.L., Galetti, F.C.S., Simioni, A.R., Santos, N.P., Egito, E.S.T. Do, Silva, C.L., Tedesco, A.C., Santos-Magalhães, N.S., 2009. In vitro uptake and antimycobacterial activity of liposomal usnic acid formulation. J. Liposome Res. 19, 49–58. https://doi.org/10.1080/08982100802564628.

Lucarini, R., Tozatti, M., Salloum, A. de O., Crotti, A., Silva, M., Gimenez, V., Groppo, M., Januário, A., Martins, C., Cunha, W., 2014. Antimycobacterial activity of *Usnea steineri* and its major constituent (+)-usnic acid. Afr. J. Biotechnol. 11, 4636–4639. https://doi.org/10.4314/ajb.v11i20.

Luo, X., Pires, D., Aínsa, J.A., Gracia, B., Duarte, N., Mulhovo, S., Anes, E., Ferreira, M.J. U., 2013. Zanthoxylum capense constituents with antimycobacterial activity against Mycobacterium tuberculosis in vitro and ex vivo within human macrophages. J. Ethnopharmacol. 146, 417–422. https://doi.org/10.1016/J.JEP.2013.01.013.

Marcano, V., 1991. Propriedades biomédicas de las embriofitas asifonogamas andinas: II líquenes. Rev. Fac. Farm. 28, 1–13.

Marcano, V., Rodriguez-Alcocer, V., Morales Méndez, A., 1999. Occurrence of usnic acid in Usnea laevis Nylander (lichenized ascomycetes) from the Venezuelan Andes. J. Ethnopharmacol. 66, 343–346. https://doi.org/10.1016/S0378-8741(98)00181-0.

Nguyen, T.-T., Ketha, A., Hieu, H.V., Tatipamula, V.B., 2021. In vitro antimycobacterial studies of flavonols from Bauhinia vahlii Wight and Arn. 3 Biotech 11, 128. https://doi.org/10.1007/s13205-021-02672-4.

Pick, N., Cameron, S., Arad, D., Av-Gay, Y., 2004. Screening of compounds toxicity against human monocytic cell line-THP-1 by flow cytometry. Biol. Proced. Online 6, 220–225. https://doi.org/10.1251/bpo92.

Seaworth, B., Griffith, D., 2017. Therapy of multidrug-resistant and extensively drug-resistant tuberculosis. Microbiol. Spectr. 5 https://doi.org/10.1128/MICROBIOLSPEC.TNMI7-0042-2017. TNMI7-0042–2017.

Singh, R., Nawale, L.U., Arkile, M., Shedbalkar, U.U., Wadhwani, S.A., Sarkar, D., Chopade, B.A., 2015. Chemical and biological metal nanoparticles as antimycobacterial agents: a comparative study. Int. J. Antimicrob. Agents 46, 183–188. https://doi.org/10.1016/j.ijantimicag.2015.03.014.

Singh, U., Akhtar, S., Mishra, A., Sarkar, D., 2011. A novel screening method based on menadione mediated rapid reduction of tetrazolium salt for testing of anti-mycobacterial agents. J. Microbiol. Methods 84, 202–207. https://doi.org/10.1016/j.mimet.2010.11.013.

Tatipamula, V.B., Vedula, G.S., 2020. Fibrinolytic, anti-inflammatory and cytotoxic potentialities of extracts and chemical constituents of manglicolous lichen. Graphis ajarekarii Patw. & C. R. Kulk. Nat. Prod. J. 10, 87–93. https://doi.org/10.2174/2210315508666180604101813.

World Health Organization, 2013. Global tuberculosis report 2013. Geneva, Switzerland. https://apps.who.int/iris/handle/10665/91355.

World Health Organization, 2019. Global Tuberculosis Report 2019. Geneva, Switzerland.

Wagner, H., Ulrich-Merzenich, G., 2009. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16, 97–110. https://doi.org/10.1016/j.phymed.2008.12.018.

Yang, E., Yang, R., Guo, M., Huang, D., Wang, W., Zhang, Z., Chen, C., Wang, F., Ho, W., Shen, L., Xiao, H., Chen, Z.W., Shen, H., 2018. Multidrug-resistant tuberculosis (MDR-TB) strain infection in macaques results in high bacilli burdens in airways, driving broad innate/adaptive immune responses. Emerg. Microb. Infect. 7, 207. https://doi.org/10.1038/S41426-018-0213-Z.