Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *84* (6) 555–562 (2019)
JSCS–5208
UDC 547.582.6+582.29:616.24–002.5:615.28
Original scientific paper

Antimicrobial and anti-tubercular activities of isolates and semi-synthetic derivatives of lichen *Ramalina leiodea* (Nyl.) Nyl.

VINAY BHARADWAJ TATIPAMULA and GIRIJA SASTRY VEDULA*

Pharmaceutical Chemistry Department, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam – 530 003, Andhra Pradesh, India

(Received 24 September, revised 18 December 2018, accepted 19 January 2019)

Abstract: The chemical investigation of lichen *Ramalina leiodea* (Nyl.) Nyl. yielded five known metabolites, *i.e*., usnic acid (**1**), ethyl everninate (**2**), scrobiculin (**3**), methyl 2,6-dihydroxy-4-methylbenzoate (**4**) and 4-[(2-hydroxy-4-methoxy-6-propylbenzoyl)oxy]-2-methoxy-6-propylbenzoic acid (**5**). To develop compound libraries on **4**, a series of semi-synthetic derivatives was prepared (**4a**––**e**). All the metabolites and semi-synthetic analogues were screened for antimicrobial and anti-tubercular activities. The results showed that compounds **3** and **5** were very active against antibacterial and antifungal strains, while the semi-synthetic analogues **4a**–**e** are moderately active on all tested microbial strains. In addition, compounds **4b** and **4d** showed better antimycobacterial activity with *MIC* value of 1.6 μg mL^{-1}, than streptomycin with an *MIC* of 6.25 μg mL^{-1} against *M. tuberculosis*. All the semi-synthetic analogues exhibited better anti-tubercular activity than the isolated metabolites. This is the first report on the synthesis and biological activities of these novel benzohydrazide derivatives.

Keywords: benzohydrazides; derivatization; *Mycobacterium tuberculosis*; well plate method.

INTRODUCTION

Ramalina leiodea (Nyl.) Nyl. is a fruticose lichen belonging to *Ramalinaceae* that is found in regions of eastern India. Generally, *Ramalina* genus has about 246 species distributed around the world, of which only 118 species have been investigated for their chemical and biological properties. A diversity of secondary metabolites has been isolated from the *Ramalina* genus, including dibenzofuran derivatives, depsides, depsidones and orcinol derivatives.[1,2] Moreover, biological screening of this genus resulted in the identification of antioxidant,[3] anti-inflammatory,[4] anticancer,[5] antimicrobial[3] and anti-tubercular[6] activities.

As a part of ongoing research of anti-infective agents from lichens, this study presents the outcomes of chemical and biological investigation of *R. leiodea*.

* Corresponding author. E-mail: vgirijasastry@yahoo.co.in
https://doi.org/10.2298/JSC180924003T

Moreover, previously some novel benzohydrazide derivatives were synthesized that were found to be very active against the *Mycobacterium tuberculosis* H37Rv strain.[7] Now, the acquired synthetic knowledge was applied towards naturally occurring secondary metabolite and some semi-synthetic analogues, *i.e.*, benzohydrazides, were derived and screened along with the isolated metabolites for their antimicrobial and anti-tubercular (anti-TB) activities.

EXPERIMENTAL

General

Commercially available chemicals were used for the extraction, isolation and purification protocols. TLC was performed on silica gel 60 F254 plates (Merck, Germany); elemental analysis was realized on a Carlo Erba 1108 analyzer; nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$, DMSO-d_6 or acetone-d_6 using an Avance (400 MHz) instrument for both ^{1}H-NMR and ^{13}C-NMR; the FT-IR spectra were recorded on a Bruker Alpha-T FT-IR spectrometer (Bruker Optik, Switzerland) and the EI-MS spectra on a JEOL-M-JMS-D-300 instrument at 70eV (Jeol, Tokyo, Japan). The melting points (MP) were recorded on a Boitus melting point apparatus and the UV–Vis spectra on an Electron 420 series spectrophotometer, Elico India Ltd.

Collection

The specimens of lichen, *Ramalina leiodea* (Nyl.) Nyl, were collected from the twigs of mangrove plants, *Ceriops decandra*, from Bhitharkanika Island, Rajnagar, Orissa, India (20°74′ N and 86°87′ E at 0 m elevation) in March 2016. This species was authenticated by Dr. D. K. Upreti, CSIR-National Botanical Research Institute (NBRI), Lucknow, and deposited at the Lichen herbarium, CSIR-NBRI, Lucknow, India, with the accession number 16-027175.

Extraction

The extraction procedure is based on modified procedures of Jug *et al*. 2017.[8] The lichen specimens were collected gently and shade dried. The dried lichen material (150 g) was suspended in ethanol–water (1:1) for a week and evaporated under reduced pressure to obtain the hydroalcoholic extract of *R. leiodea* (**RL-HA,** 40.11 g). The **RL-HA** was re-extracted with acetone solvent, dried over anhydrous sodium sulfate and concentrated to obtain the dry acetone extract of *R. leiodea* (**RL-Ac**, 14.50 g). The **RL-Ac** was subjected to column chromatography using Sephadex LH-20 resulting in four bioactive fractions. Fraction I (612 mg) obtained from 10 % ethyl acetate in hexane, on further purification using column chromatography (hexane in dichloromethane, 9:1) to obtain **1** (236 mg) as yellow needles. Fraction II (1.50 g), obtained from 30 % ethyl acetate in hexane, was subjected to column chromatography (ethyl acetate in dicholormethane, 3:7) to obtain **2** (153 mg) as a greenish solid and **3** (911 mg) as white crystals. Fraction III (4.6 g), obtained in 40 % ethyl acetate in hexane, was recrystallized using acetone and hexane, 9.5:0.5, yielding **4** (4.05 g) as pale-yellow crystals. Fraction IV (20 mg), obtained in 50 % ethyl acetate in hexane, was subjected to column chromatography (ethyl acetate in hexane, 1:1) to obtain **5** (11 mg) as a white solid.

*Procedure for the synthesis of 2,6-dihydroxy-4-methylbenzohydrazide (**4a**)*

4 (2.0 g) was dissolved in ethanol (50 mL) and hydrazine hydrate (3.0 eq., 1.06 mL) was added to the solution. The resultant suspension was refluxed for 6 h and the progress of the reaction was monitored by TLC. After completion of the reaction, ethanol and excess hydra-

zine were removed under vacuum to afford the crude solid product (2.5 g) that was washed and recrystallized from acetone to give **4a** (1.91 g, 95.5 mass %) as a pale yellowish solid.

*General procedure for the synthesis of benzohydrazide derivatives (**4b**–**e**)*

4a (1.0 mmol) was dissolved in ethanol and different aldehydes (**4b**–**e**, 1.1 mmol) was added to the solution and the reaction mixture was heated to reflux for 2–3 h. TLC confirmed the completion of the reaction, the solvent was evaporated under vacuum to obtain a solid that was stirred in *n*-hexane:acetone (1:1) and filtered. The obtained solid was dried under vacuum to afford the solid products **4b**–**e**.

RESULTS AND DISCUSSION

The chemical examination of acetone extract of *R. leiodea* revealed the presence of five known metabolites (**1**–**5**), except **1**, all were reported for the first time from this species. By elemental and spectral analysis, **1**–**5** (Fig. 1) were identified as dibenzofuran derivatives, *i.e.*, usnic acid (**1**); a monocyclic aromatic ester, ethyl everninate (**2**); a depside derivative, scrobiculin (**3**); an orcinol derivative, methyl 2,6-dihydroxy-4-methylbenzoate (**4**) and a depside derivative, 4-[(2-hydroxy-4-methoxy-6-propylbenzoyl)oxy]-2-methoxy-6-propylbenzoic acid (**5**). These metabolites showed moderate to potent antibacterial activity against gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative (*Pseudomonas aeruginosa* and *Salmonella typhi*) strains and antifungal activity against *Candida albicans* and mild anti-tubercular activity against the *Mycobacterium tuberculosis* H37Rv strain.

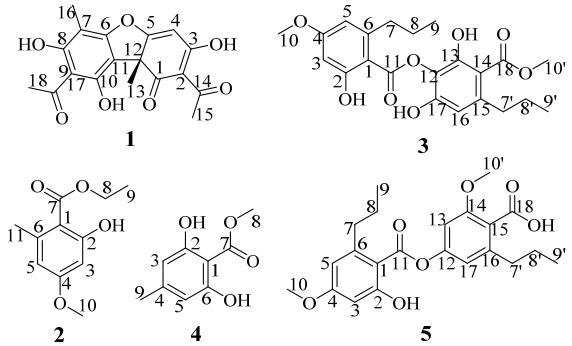
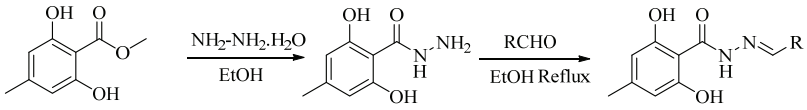


Fig. 1. Chemical constituents isolated from the acetone extract of *R. leiodea*.

It is well known that many today drugs that are commercially available and in the late stage of clinical trials are of natural origin. Additionally, structural modification of natural metabolites is a tool to obtain bioactive molecules with smaller adverse effects than their natural counterparts. Based on the aforementioned observations, an attempt was made to develop new structurally diverse molecular scaffolds from natural metabolites. This paper focuses on the derivatization of 2,6-dihydroxybenzoate into novel benzohydrazides that possess potent anti-tubercular activity. In addition, the potential anti-tubercular activities of ben-

zohydrazide derivatives coupled with functionalities that are present on these natural metabolites make them well suited for semi-synthetic derivatization for the development of more efficacious compounds. Hitherto, there have been no synthetic studies that established or reported on benzohydrazide substituted with 2,6-dihydroxy derivatives. Thus, five analogs were synthesized and evaluated for their antimicrobial and anti-tubercular activities.

As shown in Scheme 1, compounds **4a**–**e** were prepared in good yield from **4**. Initially, the terminal ester group in compound **4** was cleaved by hydrazine monohydrate to yield **4a**, which was further coupled with different aldehydes to yield the corresponding benzohydrazides (**4b**–**e**). The structures of the **4a**–**e** were characterized by elemental analysis, and IR, ESI-MS, ^{1}H- and ^{13}C-NMR spectral data.



4 **4a** **4b-e**

4b: R=p-Ph-OMe; **4c**: R=n-propyl; **4d**: R=Ph; **4e:** R=2,3-di-Chloro-Ph

Scheme 1. Synthesis of benzohydrazide derivatives (**4a**–**e**).

The presence of a carbonyl carbon was confirmed for the benzohydrazides **4a**–**e** by the presence of a carbon signal at δ_C 166–174 ppm in ^{13}C-NMR spectra and an intense FT-IR absorption in the range from 1620–1700 cm^{-1}. Similarly, the amine proton (–NH) of the benzohydrazides was confirmed by a proton signal at δ_H 7.41–8.84 ppm, while the phenolic group was confirmed by a proton signal at δ_H 9.3–10.2 ppm in the ^{1}H-NMR spectra and an intense FT-IR absorption at around 3200 cm^{-1}. The complete NMR and positive ESI-MS analyses confirmed the structures for **4a**–**e**, which are shown in Scheme 1.

Antimicrobial activity

The antimicrobial activity[9] of the isolated metabolites **1**–**5** and their derivatives **4a**–**e** were evaluated against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *S. enterica* and *C. albicans*. Among the isolated metabolites, **3** and **5** having the same skeleton (*i.e.*, depside) depicted better antimicrobial activity against all the tested strains, when compared to the standard drug (streptomycin), which suggested that the presence of depside nucleus plays a vital role in conferring antimicrobial activity. Furthermore, compound **1** that contains a benzofuran moiety showed potent activity only against the tested bacterial strains whereas moderate activity against the fungal strains (Table I). However, compounds **2**, **3** and **4a**–**e** showed only moderate activity against all the tested microbial strains (Table I). Moreover, to explore the importance of the isolates **1**–**5**, their minimum inhibitory concentrations (*MIC*) and minimum bactericidal concentrations

(*MBC*) against selected microbial strains were evaluated, along with streptomycin as the standard using 100 μg/mL each.

TABLE I. Diameters of the zones of inhibition for all the isolates and benzohydrazide derivatives of *R. leiodea* against bacterial and fungal stains; loading concentration: 100 μL per well

Sample (1 μg mL^{-1})	Average zone of inhibition[a], mm				
	B. subtilis	*S. aureus*	*S. enterica*	*P. aeruginosa*	*C. albicans*
1	23±0.1	25±0.1	20±0.1	28±0.2	18±0.1
2	18±0.1	18±0.1	16±0.1	20±0.1	16±0.1
3	25±0.2	26±0.2	24±0.1	26±0.2	20±0.1
4	20±0.1	21±0.1	20±0.1	20±0.1	18±0.1
5	25±0.1	25±0.2	24±0.2	25±0.1	20±0.1
4a	17±0.1	18±0.1	18±0.1	18±0.1	15±0.1
4b	16±0.1	15±0.1	15±0.1	19±0.1	15±0.1
4c	12±0.1	21±0.1	20±0.1	20±0.1	18±0.1
4d	18±0.1	16±0.1	18±0.1	20±0.1	16±0.1
4e	16±0.1	17±0.1	22±0.1	19±0.1	15±0.1
Streptomycin	22±0.1	22±0.1	23±0.1	24±0.2	20±0.1

[a]$n = 3$; the diameter of the inhibition zone includes the diameter of the well

The *MIC* data suggested that all the selected strains were susceptible to **1**, **3** and **5** and were more potent than streptomycin. However, most potent activity was found against *P. aeruginosa* (nosocomial infections), with an *MIC* value for **1** and **5** of 0.007 μg mL^{-1}, whereas the value for streptomycin was 0.03 μg mL^{-1}; against *S. aureus* (causes disease due to direct infection/production of toxins) with an *MIC* value of 0.007 μg mL^{-1} for **3**, while the value for streptomycin was 0.03 μg mL^{-1}; **5** showed potent activity against *S. typhi* (typhoid fever) with an *MIC* of 0.007 μg mL^{-1}, whereas, it was 0.01 μg mL^{-1} for streptomycin (Table II). The calculated *MBC* values against *P. aeruginosa* were found to be 0.03 μg mL^{-1} for **1**, **3** and **5**; against *S. aureus*, it was found to be 0.01 μg mL^{-1} for **3** and against *S. enterica*, it was found to be 0.01 μg mL^{-1} for **5** (Table II).

TABLE II. *MIC* values for all the isolates and benzohydrazide derivatives of *R. leiodea* against bacterial and fungal stains

Sample	*MIC*[a] / μg mL^{-1}					*MBC*[b] / μg mL^{-1}				
	B. subtilis	*S. aureus*	*S. enterica*	*P. aeruginosa*	*C. albicans*	*B. subtilis*	*S. aureus*	*S. enterica*	*P. aeruginosa*	*C. albicans*
1	0.03	0.01	0.01	0.007	0.06	0.06	0.03	0.03	0.01	0.12
2	0.06	0.03	0.03	0.01	0.12	0.12	0.06	0.06	0.03	0.25
3	0.01	0.007	0.01	0.01	0.01	0.03	0.01	0.03	0.03	0.03
4	0.06	0.03	0.03	0.01	0.12	0.12	0.06	0.06	0.03	0.25
5	0.01	0.01	0.007	0.007	0.03	0.03	0.03	0.01	0.01	0.06
Streptomycin	0.03	0.03	0.01	0.03	0.12	0.06	0.06	0.03	0.06	0.25

[a]$n = 3$; *MIC*: minimum inhibitory concentration; [b]*MBC*: minimum bacterial concentration

Anti-tubercular activity

The anti-TB activity[7] of the isolated compounds **1**–**5** and benzohydrazide derivatives **4a**–**e** was investigated against the *M. tuberculosis* H37Rv strain since the benzohydrazides are also known for their anti-TB properties.[7] The results are summarized in Table III. They confirmed that the benzohydrazides **4a**–**e** showed better inhibitory profiles against the *M. tuberculosis* H37Rv strain than the isolated compounds **1**–**5** (Table III). Among the semi-synthetic derivatives, compounds **4b** and **4d** depicted a better *MIC* value of about 1.6 μg mL^{-1}, which suggested that the presence of benzyl moiety played a major role in conferring anti-TB activity. In addition, **4c** exhibited an equivalent *MIC* to that of streptomycin with an *MIC* of 6.25 μg mL^{-1} (Table III). The interesting mechanism of action attributed to **4a**–**e** derivatives, especially inhibition of synthesis of mycolic acids, arises due to their structural similarity with the first line anti-TB drug, isoniazid.[10]

TABLE III. *In vitro* anti-tubercular activity of all the isolates and benzohydrazide derivatives of *Ramalina leiodea* against the *M. tuberculosis* H37Rv strain; $n = 3$; *MIC*: minimum inhibitory concentration

Sample	*MIC* / μg ml^{-1}
1	50
2	50
3	50
4	50
5	25
4a	12.5
4b	1.6
4c	6.25
4d	1.6
4e	12.5
Ciprofloxacin	3.12
Pyrazinamide	3.12
Streptomycin	6.25

Structure–activity relationship of benzohydrazides as anti-tubercular agents

The *in vitro* anti-TB activity assisted in the identification of the potency of benzohydrazides against *M. tuberculosis*, thereby in elucidating the structure––activity relationship. The syntheses of phenyl containing analogues (**4b** and **d**) were found to increase the anti-TB activity. Additionally, the replacement of electron donating groups such as methoxy (**4b**) with a phenyl moiety led to better anti-TB activity than substitution with an electron withdrawing group such as chlorine (**4e**). In addition, the electron releasing (such as alkyl, **4c**) derivatives improved the anti-TB activity.

CONCLUSIONS

To conclude, the present study involved the isolation, semi-synthesis and biological profile of metabolites from the lichen *R. leiodea*. Among all the compounds, **3** and **5** revealed good antibacterial as well as antifungal activity. Additionally, compound **1** showed significant antibacterial activity against the tested strains. Moreover, the semi-synthetic analogs **4a**–**e** exhibited better anti-TB activity with moderate antimicrobial activity. Hence, this research suggested a solid foundation for further lead optimization of this class of benzohydrazides by a systematic refinement, including the synthesis of compounds to improve their overall biological properties.

SUPPLEMENTARY MATERIAL

The structural elucidation and spectral data of all the isolated metabolites and semi-synthetic derivatives were provided as Supplementary Material, available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

Acknowledgements. We thank the Ministry of Earth Sciences for the financial support and the Directors of Maratha Mandal Dental College (Belgaum, India) for providing the necessary facilities.

ИЗВОД

АТИМИКРОБНА И АНТИТУБЕРКУЛОЗНА АКТИВНОСТ ИЗОЛАТА И ПОЛУСИНТЕТИЧКИХ ДЕРИВАТА ЛИШАЈА *Ramalina leiodea* (Nyl.) Nyl.

VINAY BHARADWAJ TATIPAMULA и GIRIJA SASTRY VEDULA

Pharmaceutical Chemistry Department, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, Andhra Pradesh, India

Хемијском анализом лишаја *Ramalina leiodea* (Nyl.) Nyl. идентификовано је пет познатих метаболита: уснинска киселина (**1**), етил-евернинат (**2**), скробикулин (**3**), метил-2,6-дихидрокси-4-метилбензоат (**4**) и 4-[(2-хидрокси-4-метокси-6-пропилбензоил)окси]-2-метокси-6-пропилбензоева киселина (**5**). Даље је направљена серија полусинтетичких једињења од метаболита **4** (**4a**–**e**). Сви метаболити и полусинтетички аналози су тестирани на антимикробну и антитуберкулозну активност. Резултати су показали да су једињења **3** и **5** веома активна спрам бактерија и гљива, док полусинтетички аналози **4a**–**e** испољавају умерену активност спрам тесираних врста микроорганизама. Најјачу активност су испољила једињења **4b** и **d** спрам *M. tuberculosis*; *MIC* вредност је била 1,6 μg mL^{-1}, док је *MIC* вредност за стрептомицин 6,25 μg mL^{-1}. Сви полусинтетички аналози су испољили већу антитуберкулозну активност од изолованих метаболита. Ово је први рад у коме су описане синтеза и биолошка активност наведених деривата бензохидразида.

(Примљено 24. септембра, ревидирано 18. децембра 2018, прихваћено 19. јануара 2019)

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