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ORIGINAL ARTICLE

Tropidia Curculioides: Secondary Metabolites and Derivatives with Antimycobacterial and Leishmanicidal Activity

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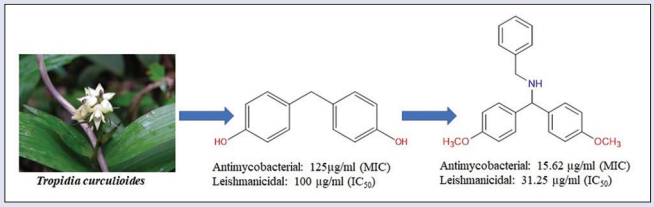
ABSTRACT

Background: Arunachal Pradesh the north-eastern state of India is a natural habitat of 550 species of orchids including 37 species of medicinal importance. Although these plants are regularly used by ethnic population, very few scientific investigations have been conducted to establish their pharmacological potential. In our previous study, we evaluated antimycobacterial and leishmanicidal activity of extracts prepared from three relatively unexplored medicinal orchids. In the present study, we conducted screening of the compounds isolated from *Tropidia curculioides* (Tc) for the above-mentioned activities. Furthermore, we prepared synthetic analogs of the most active compound. **Objective:** Evaluation of antimycobacterial and leishmanicidal activity of the isolated compounds and preparation of synthetic analogs of the most active compound followed by evaluation of biological activities. **Materials and Methods:** Antimycobacterial activity was evaluated by colorimetric redox indicator assay, and the leishmanicidal activity was assessed by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay. The root, stem, and leaves of Tc were extracted with methanol:water (9:1) followed by fractionation with diethyl ether (Et_2O) and *n*-butanol solvent. All these fractions were evaluated for biological activity to identify the most active fraction. Further, chromatography of the most active fraction (Tc root Et_2O fraction) afforded three compounds, namely, 4-hydroxybenzaldehyde (1), 4,4′-dihydroxydiphenylmethane (2), and 3,5-dihydroxy-4-methoxybenzoic acid (3). Standard synthetic procedures were followed to prepare the analogs of the most active compound. **Results:** The screening result identified compound 2 with maximum antimycobacterial activity (minimum inhibitory concentration [MIC] – 125 µg/ml) and leishmanicidal activity (IC_{50} 100 µg/ml). Three synthetic analogs were prepared targeting the methane linkage of compound 2. The N-benzylmethylamine derivative (6) showed the highest activity with MIC 15.62 µg/ml against *Mycobacterium* and IC_{50} 31.25 µg/ml against *Leishmania* spp. **Conclusion:** The promising result of N-benzylmethylamine analog could be explored to obtain new antimycobacterial and leishmanicidal agent.

Key words: 4,4′-dihydroxydiphenylmethane, antimycobacterial, leishmanicidal activity, *Tropidia curculioides*

SUMMARY

- Evaluation of antimycobacterial and leishmanicidal activity of compounds isolated from *Tropidia curculioides*
- Preparation of analogs of the most active compound and evaluation of biological activity.



Abbreviations used: MIC: Minimum inhibitory concentration; NMR: Nuclear magnetic resonance; CRI: Colorimetric redox indicator; OADC: Oleic acid, dextrose, and catalase; MDR: Multidrug Resistant; MTT: 3-(4,5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide; Diethyl ether: Et_2O.

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INTRODUCTION

Orchids are known worldwide for esthetic and economic importance. Many orchids also possess important therapeutic property. Arunachal Pradesh (AP), in India, is a natural habitat of 5000 flowering species of which 550 species belong to *Orchidaceae* family including 37 species of medicinal importance.[1] *Tropidia curculioides* (Tc), a rare epiphytic orchid, found in Meghalaya, Sikkim, and AP possess medicinal property along with its esthetic value.[2] The decoction of roots of Tc is used for cold stage of malaria by the ethnic population. Furthermore, it is used in the symptom of diarrhea and dysentery.[3] Ethnic population of this region depends extensively on folk medicines for a number of common ailments and communicable diseases.[4] Isolated living condition due to poor approach and lack of health-care facility is a threat for survival of this ethnic population. Especially, communicable diseases, for example, tuberculosis (TB), visceral leishmaniasis (VL), and malaria could be a great concern for the locals. TB in humans is caused by *Mycobacterium tuberculosis*, an intracellular microbe belonging to *M. tuberculosis* complex.[5] Recent rise of multidrug-resistant (MDR), extensively drug-resistant, and totally drug-resistant strains of *M. tuberculosis* emphasizes the need of further investigation based on traditional knowledge.[6] Kala azar (VL), as commonly known in India, is a fatal vector-borne disease caused by *Leishmania donovani*. Both these diseases cause considerable morbidity and mortality.[7] Although a number of plant extracts and plant-derived compounds have shown significant leishmanicidal activity,[8] very few have reached to the stage

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of clinical trials. The advantage with medicinal plants extracts is that they are cost-effective and mostly do not possess any side effect. In our previous study, we investigated three comparatively unexplored orchids of AP, namely, *Satyrium nepalense, Rhynchostylis retusa,* and Tc by bioassay-guided fractionation for antimycobacterial and leishmanicidal activity. In the present study, we concentrated on one plant, namely, Tc for elaborate study from phytochemistry and pharmacognosy viewpoint.

The root, stem, and leaves of Tc were extracted with methanol: water (9:1, rt, 24 h) separately. The concentrated extracts were fractionated with diethyl ether (Et_2O) and *n*-butanol to afford six fractions. All these fractions were evaluated for biological activity which identified Tc root Et_2O, with minimum inhibitory concentration (MIC) of 125 µg/ml against *M. tuberculosis.* Furthermore, the same fraction exhibited most potent leishmanicidal activity with IC_{50} 100 µg/ml against *L. donovani*.[9]

To identify bioactive compounds from the fraction, we conducted column chromatography of Tc root Et_2O fraction which afforded three compounds. The structure of these compounds was established as 4-hydroxybenzaldehyde (1), 4,4'-dihydroxydiphenylmethane (2), and 3,5-dihydroxy-4-methoxybenzoic acid (3) [Figure 1]. As there is no report of the evaluation of antimycobacterial and leishmanicidal activity of the isolated compounds, we conducted screening for the same. Preparation of synthetic analogs of a bioactive compound is a good option to optimize bioactivity. Hence, we followed that approach and prepared three synthetic analogs of the most active compound.

MATERIALS AND METHODS

General

The ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on 500 MHz Bruker spectrometer to characterize synthetic compounds. Silica gel 60–120 and 100–200 (Merck) was used as a stationary phase in chromatography. The TLC plates were examined under UV light at 254 and 366 nm and after staining with iodine.

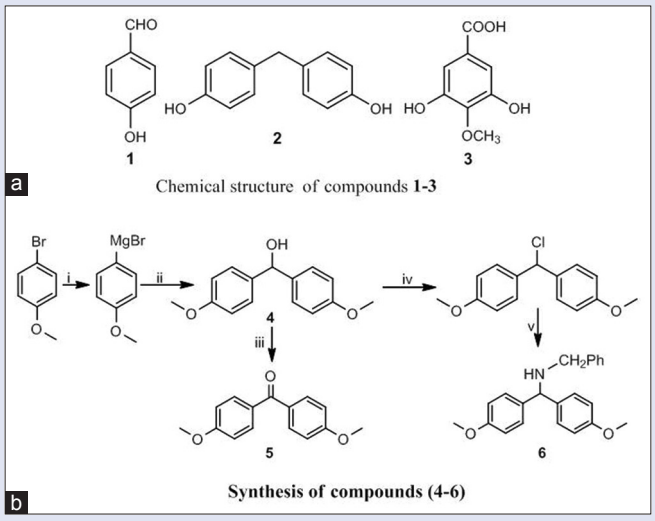

Figure 1: (a) Chemical structure of compounds 1–3 and (b) synthesis of compounds 4–6 (i) Mg metal, tetrahydrofuran, rt to reflux, 4 h, (ii) anisaldehyde, DCM, rt to reflux, 2 h, (iii) $K_2Cr_2O_7$, H_2SO_4, stirring, rt, 2 h, (iv) $SOCl_2$, DCM, stirring overnight, rt, and (v) benzyl amine, DCM, rt, stirring, 24 h

Chemicals

All starting materials, chemicals, reagents, and solvents were procured from Sigma-Aldrich and Merck (India), respectively.

Plant material

The whole plant material was collected from Tipi district of AP in April 2014 and was authenticated by Dr. Ona Apang, Scientist, State Forest Research Institute, Itanagar. A voucher specimen (AUUP/AIB/2014/02) has been maintained in the herbarium of Amity Institute of Biotechnology, Amity University, Noida.

Extraction, Isolation, and characterization

Briefly, 1.6 kg of Tc was divided into root 0.922 g; stem 0.302 g; and leaves and flowers 0.310 g. Each part was macerated separately and was extracted with methanol:water (9:1, 1 l × 24 h) for 3 consecutive days. The concentrated aqueous extract was suspended in water and was partitioned with Et_2O and *n*-BuOH successively. Column chromatography of 5.18 g of Et_2O fraction on silica gel (100–200 mesh, 0.8 kg) with *n*-hexane and EtOAc (0-100%) as eluent afforded nine fractions (F-1 to F-9). Repeated chromatography of F-4 (540 mg) with *n*-hexane and EtOAc (0-30%) afforded eight subfractions (SF4.1 to SF4.8). Further separation of SF4.3 by high performance liquid chromatography (HPLC) on C-18 column (10 mm × 250 mm) using MeCN-H_2O (45:55, v/v, 20 min, flow rate of 5 ml/min) as mobile phase afforded compound 1 (40 mg, 0.0044%) at 12.5 min of the run. Similarly, repeated column chromatography followed by HPLC afforded compound 2 (35 mg, 0.0038%) and 3 (20 mg, 0.0022%) from F-5 and F-6, respectively. The structure of the compounds was characterized by ^1H and ^{13}C NMR and mass spectral analysis.[10]

Preparation of synthetic derivatives

Bis (4-methoxyphenyl) methanol (4)

First, Grignard reagent of *p*-methoxybromobenzene (2 g, 10.69 mmol) was prepared by refluxing eight equivalents of magnesium (2.05 g, 85.52 mmol) in dry tetrahydrofuran. The product was treated with 1.1 equivalent of 4-methoxybenzaldehyde (1.6 g, 11.75 mmol) at 0°C, and stirring was continued for 2 h at room temperature. Thereafter, compound 4 was (1.5 g, 75%) extracted in organic layer by partition and was purified further by chromatography using *n*-hexane and ethyl acetate (3:1) as mobile phase.

Bis (4-methoxyphenyl) methanone (5)

The solution of 4 (1.1 g, 4.13 mmol) in CH_2Cl_2 was treated with potassium dichromate (1.2 g, 1.09 mmol) and sulfuric acid (4 g, 10 mmol) dissolved in DCM (30 ml) at 0°C. Saturated aqueous $Na_2S_2O_3$ solution (5 ml) was used for quenching of the reaction mixture and filtered. The solvent was removed by following standard procedures and was purified by chromatography to obtain compound 5 (0.8 g, 80% yield) as a white crystalline solid.

N-benzyl-1,1-bis (4-methoxyphenyl) methanamine (6)

The solution of compound 4 (0.2 g, 0.98 mmol) in DCM was treated with 1.2 equivalent of $SOCl_2$ at room temperature for overnight to obtain the corresponding chloromethane derivative. The solvent was removed under vacuum, and the product was refluxed with benzylamine (1.2 g, 10 mmol) at 90°C for 18 h. The desired compound was obtained in ethyl acetate (250 ml) by solvent extraction. The residue after removal of solvent was purified by chromatography using *n*-hexane and ethyl acetate (5:1) to afford compound 6 (0.15 g, 75% yield).

Biological Activity

Cell culture

The H37Rv strain of *M. tuberculosis* (TMC-102; vulnerable to isoniazid, streptomycin, rifampicin, pyrazinamide, and ethambutol) and MDR clinical isolate (Tb-14, 348/16, resistant to isoniazid and rifampicin) was acquired from JALMA Institute of Leprosy and other mycobacterial diseases, India, through Dr. V.M. Katoch. The DD8 strain of *L. donovani* promastigotes was acquired from "Clinical Microbiology and Laboratory Medicine" Department of AIIMS, New Delhi. Murine macrophage J774.G8 and fibroblast cells were collected from the 'Cell Death and Differentiation Research Laboratory' of National Institute of Immunology, New Delhi.

Antimycobacterial activity

Colorimetric redox indicator Assay

Briefly, compounds dissolved in 2% dimethyl sulfoxide (DMSO) were diluted in Middlebrook 7H9 broth supplemented with oleic acid, dextrose, and catalase to attain concentrations in the range of 10–250 µg/ml. Thereafter, the bacterial suspension at 1.5×10^8 cells/ml in Lowenstein-Jensen medium was added to each well and two wells at the edge were considered as control. Further, incubation was continued for 7 days at 37°C, subsequently, 25 µl of resazurin (0.02% w/v) was added to each well followed by reincubation at 37°C for 24 h to develop the color. The result was expressed in MIC values. Bacterial suspension in media and bacterial suspension in 2% DMSO were used as positive and DMSO control, respectively. All the experiments were performed thrice, and the mean values were considered.

Leishmanicidal activity

Parasite culture

The M-199 medium-containing 10% fetal calf serum (FCS) supplemented with streptomycin (100 µg/ml) and penicillin (100 U/ml) was used for the maintenance of DD8 strain of *L. donovani* promastigotes. Parasites after sixth passage were not used to carry out *in vitro* experiments. Murine macrophage J774.G8 cells were preserved in RPMI 1640 medium supplemented with 10% FCS in a carbon dioxide incubator (5% CO_2) at 37°C for 12 h. The chicken fibroblast cells were cultured in Dulbecco's modified Eagle's medium supplemented with tetracycline and fetal bovine serum.

Leishmanicidal assay (promastigotes)

The promastigotes of *L. donovani* (DD8) were cultured in M-199 medium as described previously, and the cell count was monitored by direct counting with Neubauer hemocytometer to attain the logarithmic phase (a cell density of 5×10^6 cells/ml). The log culture was appropriately diluted with media to adjust the cell count to 1×10^6 cells/ml.[11] Briefly, promastigotes at logarithmic phase at a cell count of 1×10^6 cells/ml were seeded with compounds at different dilutions and incubated at 25°C for 2 days. Thereafter, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline (PBS) at 5 mg/ml concentration was added for the development of color. Then, solvent DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 570 nm. The cell viability was determined in terms of IC_{50} values, where, miltefosine and amphotericin B were considered as reference. The experiment was repeated thrice.

Leishmanicidal assay (amastigotes)

Intracellular amastigotes were generated by infecting J774.G8 macrophage cells with stationary phase promastigotes at a density of 5×10^5 cells/ml. The parasite:macrophage ratio was maintained at 10:1, and the plates were incubated at 37°C for 4 h. After incubation, the unbound promastigotes were excluded by washing with PBS buffer and different concentrations of the compound were added, further incubated at 25°C for 2 days. The number of viable amastigotes relative to control was expressed in IC_{50} values.[12]

Cell cytotoxicity assay

Cell cytotoxicity[13] was also measured by MTT assay. Briefly, the fibroblast cell suspension was encrusted (2 ml) on microtiter plate and was treated with sample solution at different dilutions. Further incubated at 37°C for 1 day in a CO_2 incubator. Thereafter, the cells were disaggregated using 0.25% trypsin, and viability of the cells was evaluated by MTT assay.

Statistical analysis

All experiments were carried out in triplicates, and the results were expressed as mean ± standard deviation values wherever applicable.

RESULTS

Previous study conducted by our group afforded three compounds from the bioactive diethyl ether fraction of root parts of Tc. The compounds were characterized as 4-hydroxy benzaldehyde (1), 4,4'-dihydroxydiphenylmethane (2), and 3,5-dihydroxy-4-methoxybenzoic acid (3) [Figure 1]. The compounds were evaluated for antimycobacterial and leishmanicidal activity, and the result has been presented in Table 1. Among the plant-derived compounds, compound 2 characterized as 4,4'-dihydroxydiphenylmethane exhibited maximum potency of 125 µg/ml (MIC) against both H37Rv and MDR strains of *M. tuberculosis*. The same compound also exhibited good leishmanicidal activity with IC_{50} of 100 µg/ml against both promastigotes and intracellular amastigotes of *L. donovani*. While rest two compounds, namely, 1 and 3 showed bioactivity ≥500 µg/ml [Table 1] hence, were considered as inactive. Inspired by the result, we

Table 1: *In vitro* antimycobacterial and leishmanicidal activity of compounds 1-6 against H37Rv and multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* and promastigotes and intracellular amastigotes of *Leishmania donovani*

Compound	Antimycobacterial activity (expressed as MIC in µg/ml)		Leishmanicidal activity (expressed as IC_{50} in µg/ml)	
	H37Rv	MDR clinical isolate	Promastigotes	Amastigotes
1	500	500	500	500
2	125	125	100	100
3	1000	1000	1000	1000
4	31.25	31.25	62.5	62.5
5	62.5	62.5	62.5	62.5
6	15.62	15.62	31.25	31.25
Rifampicin	0.8	1	-	-
Amphotericin B	-	-	0.55	0.65
Miltefosine	-	-	8.11	4.37

MIC: Minimum Inhibitory Concentration; MDR: Multidrug Resistant

prepared three analogs of compound 2, namely, bis (4-methoxyphenyl) methanol (4), bis (4-methoxyphenyl) methanone (5), and N-benzyl-1,1-bis (4-methoxyphenyl) methenamine (6) by standard methods as described in material and method section. The synthetic compounds were also evaluated for biological activity which showed that the most significant antimycobacterial activity (MIC 15.62 μg/ml) was observed with compound 6 against both H37Rv strain and MDR clinical isolates. Moreover, the highest leishmanicidal activity was also observed with compound 6 exhibiting IC_{50} 31.25 μg/ml against both promastigotes and amastigotes of *L. donovani*. The second highest activity was observed with compound 4**,** which exhibited good antimycobacterial activity, with MIC 31.25 μg/ml against the experimental strains. The same compound exhibited substantial leishmanicidal activity with IC_{50} of 62.5 μg/ml against both promastigotes and amastigotes of *L. donovani*. Compound 5 also exhibited moderately good antimycobacterial and leishmanicidal activity.

DISCUSSION

As any previous report of antimycobacterial and leishmanicidal activity of the isolated compounds was not available, we evaluated the compounds against H37Rv and MDR strains of *M. tuberculosis* and promastigotes and intracellular amastigotes of *L. donovani*. The result showed that only compound 2 exhibited maximum potency against all of the strains whereas compounds 1 and 3 remained inactive in biological viewpoint. Analysis of structures of these compounds revealed that only compound 2 constituted a biphenyl skeleton while rest two compounds were benzenoid in character. A literature search indicated that most of the diphenylamine derivatives exhibit significant bactericidal property.[14] Besides, many diphenylmethane derivatives also exhibited prominent biological activity against arteriosclerosis and hypercholesterolaemia.[15] One of the studies indicated that bioactivity and toxicity of the analogs are directly proportional to the number of halogen substituents present in a phenyl ring.[16] By following standard procedures of condensing a phenol or substituted phenol with a reactive benzyl species bearing suitable substitution, a number of analogs have been prepared. However, any pharmacological study by incorporating modifications at the methane linkage of a diphenylmethane has not been conducted so far. Furthermore, any systematic investigation for leishmanicidal or antimycobacterial activity with diphenylmethane analogs has not been reported; hence, we prepared three synthetic analogs where we introduced a hydroxyl group (4), a ketone group (5), and a benzylamine linkage (6) at methane linkage of compound 2. It is interesting to note that all the analogs exhibited substantially better activity than compound 2. The compound 6 exhibited eight times higher activity than the parent compound. It is worthy to mention that currently used antitubercular drugs such as pyrazinamide, isoniazid, and ethionamide also possess nitrogenous pharmacophore as an integral part in the skeleton.[17] This suggests that electron withdrawing groups and bulkier groups may be responsible for enhancing the activity. Since amines are far more basic than any oxygenated functionality such as an alcohol, ether, or ketone, they are much more nucleophilic. Thus, compound 6, being a secondary arylalkylamine, the lone pair of nitrogen does not take part in resonance with the benzene ring, and hence, the lone pair was freely available, which could be the reason for effective binding of the ligand at certain receptors of *Mycobacterium* or *Leishmania* parasites. In addition, the presence of phenyl ring might support favorable binding of the ligand at a hydrophobic pocket. These encouraging results could further be investigated to develop new antimycobacterial agents in the future.

CONCLUSION

The encouraging results of naturally occurring and synthetic analogs could further be explored to develop new antimycobacterial agents in the future.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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