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Short communication

A bioactive labdane diterpenoid from *Curcuma amada* and its semisynthetic analogues as antitubercular agents

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ABSTRACT

A labdane diterpene dialdehyde was first time isolated from the chloroform extract of rhizomes of *Curcuma amada*. This compound exhibited antitubercular activity (MIC = 500 μg/mL) against *Mycobacterium tuberculosis* $H_{37}Rv$ strain in BACTEC-460 assay. Two of its semisynthetic analogues also exhibited antitubercular activity at 250–500 μg/mL. It is the first report on isolation and antimycobacterial activity of this dialdehyde from *C. amada*.

1. Introduction

Tuberculosis is one of the dreadful infectious diseases worldwide causing around 9.27 million incidences in 2007 [1]. Out of these about 86% of total incidences were from Asia and Africa alone. Every year two million people die of this disease. Antibiotic resistance, particularly multidrug resistance (MDR) in *Mycobacterium tuberculosis* is common now in about 27 countries around the globe. New antibiotics and new therapeutic strategies are needed to combat this pathogen. Advances in identifying new antibiotics from plant sources and expanding antibiotic chemical diversity are providing crucial leads for new drugs.

Curcuma amada (family: Zingiberaceae), commonly known as "Mango ginger", is one of the 80 rhizomatous species of the genus *Curcuma*, that manifests most distinct identity of having raw mango flavour combined with that of ginger in the rhizome. *C. amada* is credited with diverse bioactive molecules demonstrating antibacterial, antifungal, insecticidal, aphrodisiac, antipyretic, anti-inflammatory, antihypercholesterolemic and antioxidant properties, which have recently been reviewed [2]. Despite the reported comprehensive phytochemical studies concerning *C. amada*, authentication of the pharmacological actions of the diverse chemical constituents of this species is quite insufficient except a few that involved molecules like demethoxycurcumin, diarylheptanoides, curcumin, bis-demethoxy curcumin [3], difurocumenonol and amadannulen [4,5]. Activity linked characterization of the major chemical components of *C. amada* is the need of the hour in view of the rich, unexplored bioactive constituents.

The present study was aimed at identification of antitubercular principle from *C. amada* extracts. Amongst the different isolated compounds of *C. amada*, we have identified a labdane-type diterpenoid, i.e., labda-8(17), 12-diene-15, 16-dial (**1**) as the major compound accountable for the antitubercular activity and this is its first report from this plant. This diterpenoid was further modified to three analogues (**2**–**4**). One of the analogues exhibited better activity than the parent compound.

2. Chemistry

On phytochemical investigation, a diterpene dialdehyde (**1**) was isolated from the chloroform extract of rhizomes. Its structure was elucidated by spectroscopy, various NMR experiments, i.e., ^1H NMR,

Abbreviations: MIC, minimum inhibitory concentration; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; HRMS, high resolution mass spectrometry; ATCC, American type culture collection.

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^{13}C NMR, DEPT-135, COSY, HSQC, HMBC and Electrospray Mass spectrometry and IR.

Compound **1**, was obtained as thick transparent oil. Its IR spectrum showed absorption bands for two carbonyls as aldehydes (1721, 1734 and 2718 cm^{-1}), olefinic (1654 cm^{-1}), *gem*-dimethyl (1387 cm^{-1}) and cyclohexyl ring (2846 and 2932 cm^{-1}). On the basis of the ESI mass 341 $[M+K]^+$ and ^{13}C NMR the molecular formula was determined as $C_{20}H_{30}O_2$, it was further confirmed by HRMS at 303.2340 for $[M+H]^+$. The ^{1}H NMR spectrum showed three singlets at δ: 0.73 (3H), 0.82 (3H) and 0.90 (3H) for three distinct methyl groups attached to quaternary carbons. A doublet of two protons at δ: 3.42 as allylic methylene, two singlets at δ: 4.38 (1H) and 4.86 (1H), one triplet at δ: 6.76 (1H) for olefinic proton and two singlets at δ: 9.39 and 9.63 (bs) for two aldehydic protons. The ^{13}C NMR and DEPT-135 spectra displayed 3 methyls, 8 methylenes (one $=CH_2$), 5 methines (one =CH and two aldehydes) and 4 quaternary carbons (two double bonded) (Table 1). In HSQC spectrum, two single protons at δ: 4.38 (1H) and 4.86 (1H) were directly connected to methylenic carbon ($=CH_2$, δ: 108.25) which indicated the presence of an exocyclic double bond. In COSY spectrum, one of the aldehydes (δ: 9.63) showed coupling with CH_2 protons at δ: 3.42, indicating a $=C-CH_2-CHO$ type arrangement. The ^{1}H and ^{13}C NMR data of **1** were well in agreement with the earlier published data (Table 1) [6–8]. All these suggested **1** as a labdane-type diterpenoid (Fig. 1).

The parent compound **1** was modified to three different derivatives as shown in Scheme 1. Both aldehyde groups of **1** were reduced to alcohols using sodium borohydride to produce a diol analogue (**2**). The diol was further acetylated with acetic anhydride to afford a diacetyl analogue (**3**). Parent dialdehyde (**1**) was refluxed with hydroxylaminehydrochloride to produce a bis-oxime analogue **4**.

Labda-8(17), 12-diene-15, 16-dial (**1**) was previously isolated from several species of Zingiberaceae family [6–12], but there is no report so far about its isolation from *C. amada*.

3. Biological results and discussion

Antitubercular activities of compound **1** and its semisynthetic analogues (**2**–**4**) were evaluated against *M. tuberculosis* H_{37}Rv strain in BACTEC-460 assay. The results were calculated and expressed as minimum inhibitory concentrations (MIC) in μg/mL

Table 1
^{1}H NMR and ^{13}C NMR assignments of compound **1**.

Carbon No.	Chemical shift ^{13}C NMR (δ ppm)	Type of carbon	Chemical shift ^{1}H NMR (δ ppm)
1	39.57	CH_2	1.077, 1.769
2	19.64	CH_2	1.525, 1.575
3	42.33	CH_2	1.187, 1.421
4	33.93	QC	–
5	55.72	CH	1.159
6	24.47	CH_2	1.336, 1.724
7	38.21	CH_2	2.04, 2.431
8	148.36	QC	–
9	56.80	CH	1.92
10	39.96	QC	–
11	25.02	CH_2	2.336, 2.495
12	160.25	CH	6.743–6.786
13	135.26	QC	–
14	39.70	CH_2	3.415, 3.435
15	197.65	CHO	9.63
16	193.87	CHO	9.396
17	108.25	CH_2	4.38, 4.86
18	33.95	CH_3	0.90
19	22.11	CH_3	0.824
20	14.76	CH_3	0.732

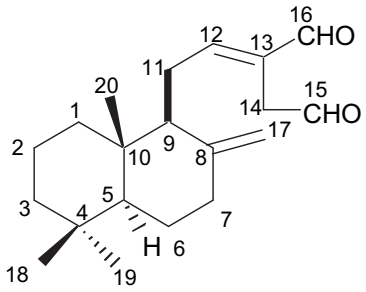


Fig. 1. Structure of compound **1**.

(Table 2). Compound **1** exhibited antitubercular activity at MIC 500 μg/mL. Its semisynthetic analogues **2** and **4** possessed antitubercular activity at MIC 250 μg/mL and 500 μg/mL, respectively, (Fig. 2). Analogue **3** was inactive at 1000 μg/mL concentration. There was twofold increment in the activity of analogue **2** over **1**.

Compound **1** was previously reported as cytotoxic [13,14] and antifungal agent. We found it as antitubercular agent against *M. tuberculosis* H_{37}Rv strain in BACTEC-460 assay at MIC 500 μg/mL. Demethoxycurcumin is another antitubercular principle reported earlier from *Curcuma sp.* by Agrawal et al [15], which is also present in *C. amada*. However, this is the first report of isolation of **1** from *C. amada* exhibiting antitubercular activity.

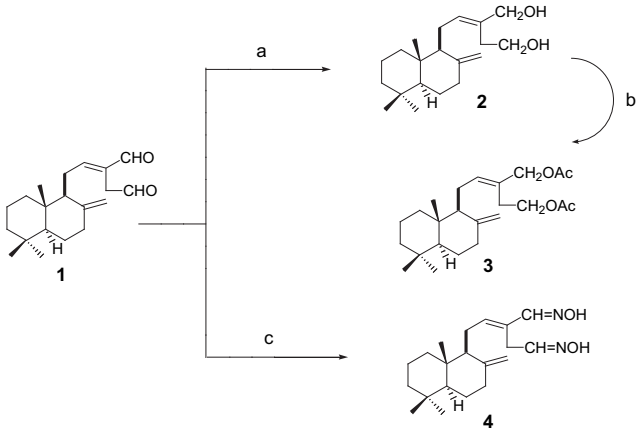
Plants are excellent sources of diverse molecules and further modifications of the active ones might usher us to the discovery of some leads. The present study has brought out a labdane-type diterpenoid molecule for the first time from *C. amada* endowed with antitubercular activity and a semisynthetic better analogue was achieved. Further modifications of this parent compound may lead to better antitubercular principle.

4. Experimental protocols

4.1. Chemistry

4.1.1. Plant material

Rhizomes of *C. amada* (family: Zingiberaceae) were collected from cultivated fields of Jadopur village, Chappra, Bihar, India in March, 2009. The plant material was authenticated (Herbarium no. 9419) by a taxonomist of Botany and Pharmacognosy division, CIMAP Lucknow.



Scheme 1. Synthesis of analogues **2**–**4**, reagents and conditions: (a) $NaBH_4$-MeOH, RT, 1 h, 82%; (b) acetic anhydride, dry pyridine, RT, overnight 16–18 h, 91%; and (c) ethanol, pyridine, hydroxylamine hydrochloride, reflux, 3 h, 86%.

Table 2
Antimycobacterial activity of compound **1** and three of its analogues against *M. tuberculosis* $H_{37}Rv$ strain by BACTEC assay.

S. no.	Compound no.	MIC (μg/mL)
1.	**1**	500
2.	**2**	250
3.	**3**	>1000
4.	**4**	500
5.	Rifampicin	2.0
6.	Stretomycin	2.0

4.1.2. Extraction and isolation

Fresh rhizomes were used after crushing with mortar & pestle. The crushed rhizomes (650 g, dry matter = 18.98%) were first extracted with methanol (1 L × 5) at room temperature for five times each for 24 h to get 10.55 g (1.62% fresh weight basis) of a residue. The leftover mass was then soaked in chloroform (1 L × 3) for three extraction of each for 24 h to get 2.2 g (0.34% fresh weight basis) of crude extract. The chloroform residue (2.1 g) was chromatographed on silica gel (80 g, 60–120 mesh, 2.5 × 75 cm glass) and was eluted with increasing polarity mixture of ethyl acetate–hexane, to get a pure compound (**1**) at 6% ethyl acetate–hexane in 418 mg (0.064% of fresh weight basis) yield. The purity of the isolated compound was found to be about 94% based on area normalization method (chromatographic conditions as below). The structure of compound **1** was elucidated by spectroscopy.

4.1.3. HPLC analysis

The quantification of compound **1** was performed by reverse phase HPLC using C-18 (Waters® ODS2, 250 × 4.6 mm, 10 μm) column and water (0.5%AcOH): acetonitrile 25:75 as mobile phase with flow rate of 1.0 mL/min. Data acquisition was done in the range of 200–600 nm while for quantification, 235 nm was selected on the basis of UV maxima of the compound **1** (Retention time = 10.6 min). Concentration of compound **1** was also determined in the rhizomes of *C. amada* as well as in active fraction separately. The concentration of compound **1** in rhizome and active fraction were 0.096% and 28.25%, respectively, on fresh weight basis. The method for quantitation was validated following the ICH guidelines. The purity of the semisynthetic analogues (**2**–**4**) was found 91–94% by RP-HPLC-PDA method.

*4.1.4. Derivatisation of compound **1***

The isolated labdane diterpenoid was further modified to three simple derivatives diol (**2**), diacetate (**3**) and dioxime (**4**).

*4.1.4.1. Synthesis of labda-8(17), 12-diene-15, 16-diol (**2**).* Labda-8 (17), 12-diene-15, 16-dial (**1**, 100 mg, 0.32 mmol) was taken in methanol (10 mL). The reaction mixture was stirred at room temperature and to this sodium borohydride (20 mg, 0.52 mmol) was added and further stirred for 30 min. On completion, methanol was evaporated and it was acidified with dil. HCl (10%, 2 mL). It was extracted with chloroform, washed with water and dried over anhydrous sodium sulphate. The organic layer was evaporated *in vacuo* to get **2** as oil in 82% yield.

^{1}H NMR ($CDCl_3$, 300 MHz): δ: 0.63 (*s*, 3H, CH_3), 0.74 (*s*, 3H, CH_3), 0.81 (*s*, 3H, CH_3), 0.99–2.38 (*m*, 16H, Rest of 7 × CH_2 and 2 × CH of diterpenoid ring), 3.67–3.69 (bd, 2H, 15CH_2OH), 3.94 (*s*, 2H, 16CH_2OH), 4.37 (*s*, 1H, 17=CH), 4.76 (*s*, 1H, 17=CH), 5.43–5.45 (distorted triplet, 1H, 12CH=); ESI mass (MeOH): 329 $[M + Na]^+$, 345 $[M + K]^+$.

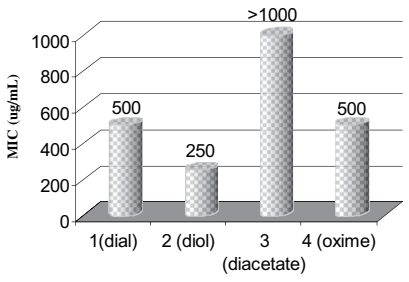


Fig. 2. Antimycobacterial activity of compound **1** and its three derivatives, i.e., diol, diacetate and oxime.

*4.1.4.2. Synthesis of labda-8(17), 12-diene-15, 16-diacetate (**3**).* Labda-8(17), 12-diene-15, 16-diol (**2**, 50 mg, 0.16 mmol) was taken in dry chloroform (10 mL). To this stirred solution, dry pyridine (0.2 mL) and acetic anhydride (0.1 mL) were added and further stirred at room temperature. On completion, the reaction mixture was acidified with dil. HCl (10%, 4 mL) and extracted with chloroform. Organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated *in vacuo* to get **3** as oil in 91% yield.

^{1}H NMR ($CDCl_3$, 300 MHz): δ: 0.65 (*s*, 3H, CH_3), 0.74 (*s*, 3H, CH_3), 0.80 (*s*, 3H, CH_3), 1.01–2.40 (*m*, 16H, Rest of 7 × CH_2 and 2 × CH of diterpenoid ring), 2.02 (*s*, 3H, $OCOCH_3$), 2.07 (*s*, 3H, $OCOCH_3$), 4.05–4.07 (*t*, 2H, 15CH_2O–), 4.36 (*s*, 1H, 17=CH), 4.41 (*s*, 2H, 16CH_2O–), 5.49 (*t*, 1H, 12CH=); ESI mass (MeOH): 391 $[M + H]^+$, 413 $[M + Na]^+$.

*4.1.4.3. Synthesis of labda-8(17),12-diene-15, 16-dioxime (**4**).* Labda-8(17), 12-diene-15, 16-dial (**1**, 50 mg, 0.16 mmol) was taken in ethanol (20 mL) and pyridine (0.1 mL). To this stirred solution, hydroxylaminehydrochloride (20 mg, 0.29 mmol) was added and further refluxed for 2 h. On completion, the solvent was evaporated, residue thus obtained was dissolved in chloroform, acidified with dilute HCl. Organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated *in vacuo* to get **3** as oil in 86% yield.

^{1}H NMR ($CDCl_3$, 300 MHz): δ: 0.64 (s, 3H, CH_3), 0.74 (*s*, 3H, CH_3), 0.80 (*s*, 3H, CH_3), 1.02–2.34 (*m*, Rest of *x*CH_2 and *x*CH of diterpenoid ring), 4.32 (*s*, 1H, 17=CH), 4.75 (*s*, 1H, 17=CH), 5.72 (distorted *s*, 1H, 12 CH=), 7.29 (bs, 1H, CH=N–O–), 7.44 (bs, 1H, CH=N–O–); ESI mass (MeOH): 333 $[M + H]^+$.

4.2. Bioevaluation

4.2.1. BACTEC radiometric susceptibility assay

M. tuberculosis $H_{37}Rv$ (ATCC 27294) was maintained on Löwenstein-Jansen media slant at 37° C and after 21 days of incubation bacterial cells were scraped from slants and transferred in 1.0 mL of BACTEC diluting fluid and made complete homogenized suspension by vortexing with glass beads (2 mm diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps if any. The turbidity of the homogenous suspension was adjusted to McFarland standard 1.0 with diluting fluid. A BACTEC 12B vial (Becton–Dickinson) was injected with 0.1 mL of this suspension. This vial was used as primary inoculum after the growth index (GI) reached a value of about 500 (approximately 1×10^6 cfu/mL).

Briefly, 0.1 mL of bacterial suspension from the primary inoculum culture vial (GI 500) was injected into test compound containing vials using 1.0 mL insulin syringe. To comply with 1% proportion method, 0.1 mL of primary inoculum was added to 9.9 mL BACTEC diluting fluid to obtain 1:100 dilutions. From this 0.1 mL was injected into two 12B vials containing 4.0 mL medium along with 40 μL of DMSO. Vials were incubated at 37 °C, and the GI

was recorded every 24 h in a BACTEC 460TB instrument (Becton–Dickinson). Once the GI of the control vial (1:100) reached 30 then the GI values of the test (compound containing) vials were compared with that of control vials based on difference in growth (ΔGI). The result was interpreted as follows: If the difference (called as ΔGI) of current GI from previous day GI in the case of test compound containing vials is lower than the ΔGI of 1:100 control vial for the same period then the test compound is termed as active against MTB or otherwise inactive.

Twofold serial dilution technique was used to assess the minimum inhibitory concentration (MIC) of a test compounds. Only broth culture was used as a positive control and media as a negative control.

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