Journal of Molecular Catalysis B: Enzymatic 106 (2014) 46–55

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Novel biotransformation processes of artemisinic acid to their hydroxylated derivatives 3β-hydroxyartemisinic acid and 3β, 15-dihydroxyartemisinic by fungus *Trichothecium roseum* CIMAPN1and their biological evaluation

Rashmi Gaur[a,1], Sudeep Tiwari[b,1], Apurva Jakhmola[d], Jay Prakash Thakur[d], Ram Kishor Verma[c], Rakesh Pandey[b], Rajendra Singh Bhakuni[a,*]

[a] *Medicinal Plant Chemistry Division, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow 226015, India*
[b] *Microbial Technology & Nematology, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow 226015, India*
[c] *Analytical Chemistry Division, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow 226015, India*
[d] *Molecular Bio-prospection Division, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow 226015, India*

ARTICLE INFO

Article history:
Received 7 January 2014
Received in revised form 8 April 2014
Accepted 13 April 2014
Available online 21 April 2014

Keywords:
Trichothecium roseum
Biotransformation
Artemisinic acid
Antimicrobial
Antioxidant

ABSTRACT

The biotransformation of artemisinic acid (**1**) by endophytic fungus *Trichothecium roseum* CIMAPN1 is reported here for the first time. The major biotransformed products appeared as a grayish color spot on thin-layer chromatography (TLC) with transparent crystal-like texture. Based on their infrared (IR) and ^{1}H nuclear magnetic resonance (NMR) spectra, the products were characterized as a 3β-hydroxyartemisinic acid (**2**) and 3β, 15-dihydroxyartemisinic acid (**3**) and were obtained in 51.1% and 37.3% yields, respectively. The highest conversion efficiencies were obtained respectively when 2-day-old cultures of *T. roseum* were fed with 20 mg of compound **1** in 50 ml of medium per culture and the mycelia were harvested after 14 days of incubation. Metabolite **3** is a new compound and metabolite **2** is reported here for the first time from artemisinic acid. Compound **3** was acetylated to the product diacetate derivative **4**. All these compounds were evaluated for their antimicrobial and *in vitro* antioxidant activities. Further, for pharmaceutical utility these compounds were analyzed *in vivo* using well established animal model *Caenorhabditis elegans* for antioxidant/reactive oxygen species scavenging activity. The results clearly showed that the novel derivatives performed well in comparison to the parent compound both *in-vitro* and *in-vivo* studies. The presence of hydroxyl groups in metabolites **2** and **3** could make them interesting synthones for further modification into new clinically potent molecules. Thus, the study suggested a new aisle towards better drug development through the utilization of micro engineers.

1. Introduction

Artemisinin and its derivatives, artemether, arteether, artersunate and artelinate have great importance in malaria chemotherapy [1,2]. Artemisinic acid, an amorphane sesquiterpene and immediate precursor of artemisinin is isolated as major compound from the herb *Artemisia annua* L (Asteraceae). It possesses a variety of pharmacological activities such as antimalarial, antitumor, antipyretic effect, antibacterial activity, allelopathy effect and anti-adipogenesis effect [3]. Chemical synthesis of artemisinin is complex and uneconomical [4,5] and *A. annua* is the only source of it [1,2]. Artemisinic acid has high potential as chemical synthone to produce artemisinin and based antimalarials [6–9]. Microbially produced artemisinic acid by the yeast cells *Saccharomyces cerevisiae* [10,11] could be an economical, high-quality, acceptable from health point and alternate source for new artemisinin analogues which may be difficult or costly to synthesize them by conventional synthetic methods. All these developments, high utility and positive impacts of artemisinic acid dragged attention of scientists and pharmaceuticals to great extinct and thus, the demands have drastically increased worldwide.

Artemisinic acid has also been biotransformed to several value added artemisinic acid derivatives which can be used for further modification into clinically important compounds

* Corresponding authors. Tel.: +91 5222718622; fax: +91 5222342666.
E-mail addresses: r.pandey@cimap.res.in (R. Pandey), bhakunirs2000@yahoo.com (R.S. Bhakuni).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.molcatb.2014.04.008
1381-1177/

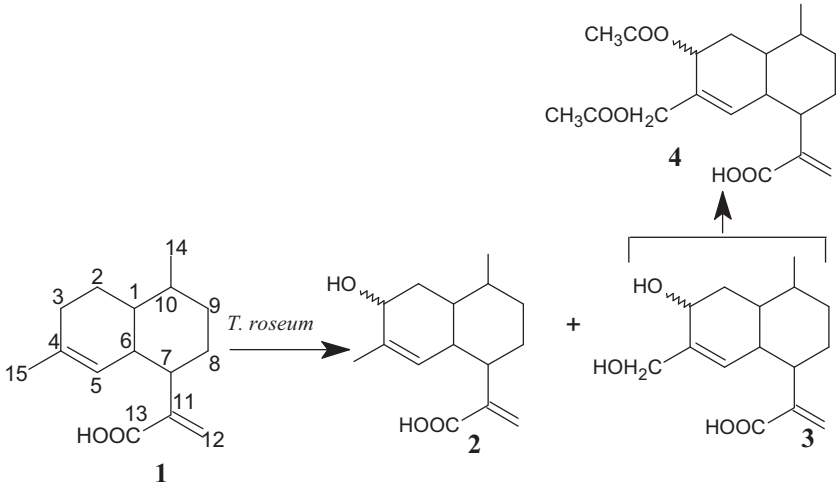

Fig. 1. Structures of artemisinic acid **1**, its metabolites **2**, **3,** diacetate **4**.

[12–17]. Endophytes either bacteria or fungi colonize healthy plant tissue without causing any apparent symptoms of disease [18]. The mutualistic association and metabolism abilities [19,20] of endophytes has opened a new horizon towards synthesis of novel secondary metabolites [21,22] with potential impact on pharmaceutical and food industries [23–25] thus reveling the importance of micro-engineers. In continuation of our earlier biotransformation studies on bioactive compounds [26–28], the endophyte, *Trichothecium roseum* CIMAPN1 isolated from the host plant *A. annua* was chosen as a biocatalyst for the conversion of artemisinic acid into value added products in the present study.

The fungus *T. roseum* is well known for its ability to metabolize a variety of organic compounds. It has earlier been used for O-demethylation, hydroxylation, sulfation, ribosylation of a xanthone, biotransform of tissue-specific hormone tibolone, thymol methyl ether, carvacrol, 3′-fluoroacetophenone, other substituted acetophenones, steroids, oleanolic acid, 18b-glycyrrhetinic acid and related compounds by many investigators [29–33]. In the present study, using artemisinic acid, the fungus has shown 88.4% metabolic potential, producing two major novel metabolites which were identified as 3β-hydroxyartemisinic acid (**2**) and 3β, 15-dihydroxyartemisinic acid (**3**) on the basis of 1D, 2D NMR and ESI-MS techniques (Fig. 1). 3β, 15-Dihydroxyartemisinic acid (**3**) is a new compound. While 3β-hydroxyartemisinic acid (**2**), reported earlier by Zhu et al. [14] and Yang et al. [17] was obtained in higher yield, 51.1% by *T. roseum* for the first time. Further, **1** and its derivatives **2, 3** and **4** were evaluated for their *in vitro* and *in vivo* antioxidant and antimicrobial potentials for revealing their pharmaceutical importance.

2. Materials and methods

Melting points were determined on a Toshniwal melting point apparatus and are uncorrected. IR spectra were recorded using a Perkin Elmer 1719 FT-IR spectrophotometer on KBr plates. ^{1}H NMR (nuclear magnetic resonance) and ^{13}C NMR spectra were obtained in $CDCl_3$ on a Bruker Avance, 300 MHz instrument using TMS as an internal standard. The chemical shift values (δ) are reported in ppm and coupling constants (J) values in Hz. Spectra were interpreted by DEPT 90 and 135 (distortionless enhancement by polarization transfer), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC), ESI-MS spectra were recorded on a Perkin Elmer Turbo Mass/Shimadzu LC–MS. Artemisinic acid, used for the metabolism study was isolated from the herb *A. annua* [8].

2.1. Isolation of endophytes

For the isolation of endophytic fungus, leaves of the herb *A. annua* were collected from the experimental field at CSIR-CIMAP, Lucknow, India. The leaves were thoroughly washed in running tap water for removing the adhering dirt particles. The washed leaves were surface sterilized with 70% ethanol and 0.1% $HgCl_2$ for 5 min followed by 3–4 subsequent washing through sterilized distilled water. The sterilized leaves were aseptically dried on filter paper for removing the excess water. The partially dried sterilized leaves were placed on potato dextrose agar (PDA) plates, supplemented with streptomycin (100 μg/ml) and incubated at 25 °C for 3–10 days [22]. After 10 days the pure looking isolates were transferred to fresh PDA plates, supplemented with the same streptomycin concentrations.

2.2. Screening of fungal isolates for biotransformation potentials

For biotransformation experiments the fungal isolates were cultured in minimal media as described by [34] with slight alterations. Briefly, the fungal isolates were grown in media containing the following chemical constituents (g/l): sucrose (30), peptone (5.0), NaCl (3.0), NaH_2PO_4 (3.0), $NaNO_3$ (3.0), $MgSO_4{\cdot}3H_2O$ (5.0), KCl (4.15), $FeSO_4$ (1.0), $MnSO_4$ (1.0) at pH 6.0 for 48 h followed by addition of artemisinic acid/acetone (40 mg/ml) solution. The fermentation medium thus, obtained was distributed among 20 flasks of 200 ml capacity (50 ml in each) and autoclaved. Compound **1** was dissolved in 10 ml acetone and the resulting clear solution was evenly distributed among 20 flasks (20 mg/0.5 ml in each flask), containing 48-h-old cultures. The fermentation was carried out for further 14-days. The inoculated flasks were placed on the rotary shaker at 28 °C with 200 rpm orbital shaking. During the fermentation, aliquots from one culture flask were taken daily, and analyzed by HPLC in order to determine the degree of transformation of substrate. In all experiments, medium without fungal culture was taken as control. No metabolite was detected in the control.

2.3. Purification and identification of novel compounds

The fungus CIMAPN1 was inoculated in 1L Erlenmeyer flasks containing 500 ml minimal media and after 48 h of growth, artemisinic acid was added at a concentration of 400 mg/l. The flasks were incubated at 28 ± 2 °C with 200 rpm for 14 days. On day 14th, the entire culture broth and mycelia were extracted three times with equal volumes of ethyl acetate, the combined

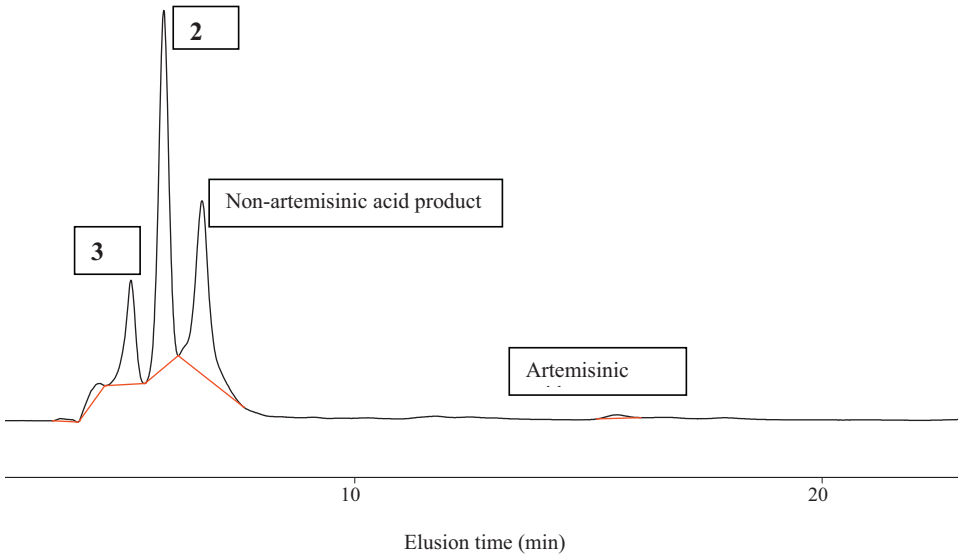

Fig. 2. HPLC profile of ethyl acetate extract of culture broth showing conversion of artemisinic acid to metabolites 3β-hydroxyartemisinic acid (**2**) and 3β, 15-dihydroxyartemisinic acid (**3**) by fungi *T. Roseum*.

extract was dried over anhydrous Na_2SO_4 and evaporated to dryness at 40 °C under reduced pressure to afford an orange-brown residue. The residue was analyzed by TLC, showed two fast moving metabolites along with traces of unreacted artemisinic acid. The metabolites were purified by flash chromatography over a silica gel (230–400 mesh, Avra Synthesis, India) column, eluting with gradient mode solvent, hexane–ethyl acetate (75:25, 1:1) at 10 ml/min flow rate. Fractions of 25 ml each were collected. Early hexane–ethyl acetate (1:1) fractions upon crystallization provided colourless crystals of metabolites **2** with 51.1% (204.6 mg) yield and 97.2% HPLC purity, R_f = 0.55 (hexane–acetone 1:1). Crystallization of hexane–ethyl acetate (1:1) mid fractions yielded metabolite **3** with 37.3% (149.2 mg) yield and 98.7% HPLC purity, R_f = 0.36 (hexane–acetone 1:1), mp 91–92 °C.

2.4. HPLC analysis

HPLC analysis of the extracts and pure compounds were performed on a Shimadzu LC-10AD liquid chromatography equipped with an SPD-M10A VP diode array detector, an SIL-10ADVP auto injector and a CBM-10 interface Module. Data were collected and analyzed using a class LC-10 work station. The samples were analyzed by using reverse phase chromatography on waters spherisorb ODS2 (250 × 4.6 mm i.d., 10 mm) column using binary gradient elution with methanol and water containing 0.1% TFA mobile phase (70:30) at a flow rate of 0.6 ml/min, a column temperature of 25 °C and UV detection at λ 220 nm.

2.5. 3-β-Hydroxyartemisinic acid (*2*)

Colorless needles (hexane–ethyl acetate 1:1); IR ν^{max} (KBr): 3445 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.48 (1H, brs, Ha-13), 5.59 (1H, brs, Hb-13), 5.15 (1H, brs, H-5), 3.94 (1H, d, J = 5.4 Hz, H-3), 2.73 (1H, m, H-7), 2.52 (1H, brs, H-6), 2.20 (1H, dd, J = 2.6,15.1 Hz, Ha-2), 1.92 (1H, m, H-10), 1.74 (3H, s, H-15), 1.72–1.81 (2H, m, Hb-2 and Ha-9), 1.36–1.44 (2H, m, H-1 and H-8), 1.06 (1H, m, Hb-9), 0.97 (3H, d, J = 6.0 Hz, H-14). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 40.81 (C-1), 34.73 (C-2), 68.44 (C-3),135.62 (C-4), 124.65 (C-5), 38.47 (C-6), 42.19 (C-7), 26.48 (C-8), 36.04 (C-9), 29.71 (C-10), 142.71 (C-11), 172.63 (C-12), 127.47 (C-13), 20.37 (C-14), 21.10 (C-15). ES-MS (positive): m/z 273[M + Na]$^+$, 251[M + H]$^+$ ($C_{15}H_{22}O_3$ for **2**).

2.6. 3β, 15-dihydroxyartemisinic acid (*3*)

Colorless needles (hexane–ethyl acetate 1:1); IR ν^{max} (KBr): 3430 (OH), 2925, 2856, 1686 (α β-unsaturated CO group) and 1623 cm^{-1} (double bond); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.34 (1H, brs, Ha-13), 5.54 (1H, brs, Hb-13), 5.44 (1H, brs, H-5), 4.21 (1H, d, J = 5.1 Hz, H-3), 4.10 (2H, brs, H_2-15), 2.77 (1H, m, H-7), 2.54 (1H, brs, H-6), 2.22 (1H, dd, J = 2.3,14.1 Hz, Ha-2), 1.92 (1H, m, H-10), 1.63–1.72 (1H, m, Hb-2 and Ha-9), 1.28–1.43 (2H, m, H-1 and H-8), 1.09 (1H, m, Hb-9), 0.95 (3H, d, J = 6.3 Hz, H-14). ^{13}C NMR (CDCl3, 75 MHz): δ 41.47 (C-1), 34.85 (C-2), 64.95 (C-3), 140.40 (C-4), 124.37 (C-5), 39.17 (C-6), 42.71 (C-7), 26.66 (C-8), 36.24 (C-9), 29.17 (C-10), 144.00 (C-11), 172.63 (C-12), 124.42 (C-13), 20.37 (C-14), 65.79 (C-15). ES-MS (positive): m/z 289[M + Na]$^+$, 267[M + H]$^+$ ($C_{15}H_{22}O_4$ for **3**).

2.7. Diacetate of 3β, 15-dihydroxyartemisinic acid (*4*)

Acetylation of metabolite **3** (0.149 g, 0.50 mmol) with acetic anhydride (0.5 ml) in pyridine (2 ml) afforded colorless crystals of diacetate **5**, 0.156 g, 92% yield, mp 70–71 °C, IR ν^{max} (KBr): 1735 cm^{-1} (ester CO); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.48 (1H, brs, Ha-13), 5.67 (1H, s, H-5), 5.57 (1H, brs, Hb-13), 5.29 (1H, brs, H-3), 4.56 (1H, d, J = 12.3 Hz, Ha-15), 4.33 (1H, d, J = 12.3 Hz, Hb-15), 2.79 (1H, m, H-7), 2.63 (1H, brs, H-6), 2.27 (1H, dd, J = 2.5, 15.0 Hz, Ha-2), 1.86 (1H, m, H-10), 1.71–1.79 (2H, m, Hb-2, Ha-9), 1.37–1.49 (1H, m, H-1), 1.19–1.29 (2H, m, H_2-8), 1.06 (1H, m, Hb-9), 0.84 (3H, d, J = 6.0 Hz, H_3-14). ^{13}C NMR (CDCl3, 75 MHz), δ 40.20 (C-1), 31.54 (C-2), 66.26 (C-3), 131.95 (C-4), 132.53 (C-5), 38.83 (C-6), 41.88 (C-7), 26.71 (C-8), 35.63 (C-9), 29.08 (C-10), 142.52 (C-11), 169.83 (C-12), 127.25 (C-13), 20.03 (C-14), 65.77 (C-15) and 21.26, 171.18 ($COCH_3$), 21.25, 171.28 ($COCH_3$). ES-MS (positive): m/z 351[M + H]$^+$($C_{19}H_{26}O_6$ for **4**).

2.8. Time course of substrate and metabolic products

The culture media were extracted with ethyl acetate several times at different time intervals. The crude extracts were analyzed by TLC and HPLC analysis. The ratios between the substrate and metabolic products were determined on the basis of HPLC (Figs. 2 and 3) and proton NMR analysis.

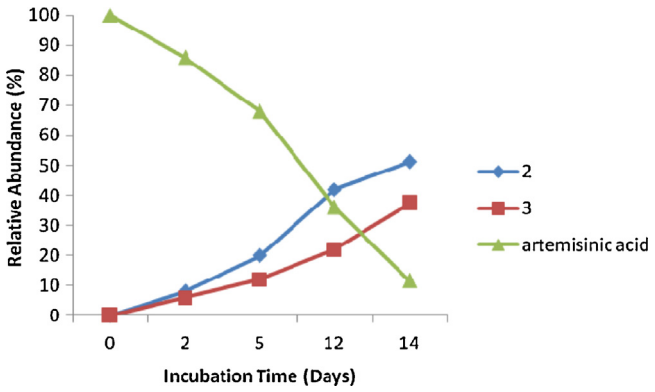

Fig. 3. Time course of biotransformation of artemisinic acid (**1**) by fungus by *T. Roseum.*

2.9. Fungal identification

For the identification fungal isolates, reaction mixture was prepared by adding 2 μl genomic DNA template, 20 pmol of each primer (ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) encoding the 5.8s rDNA fragment, 50 μM of each deoxynucleoside triphosphate, 0.7 U of Taq DNA polymerase (Genie), 10 μl of 10x Taq DNA polymerase buffer (Genie) and the reaction volume was made up to 100 μl with milli-Q water. PCR temperature cycle comprised of 35 cycles at 94 °C for 40 s, 55 °C for 1 min and 72 °C for 2 min, followed by an additional 10 min at 72 °C. An aliquot (10 μl) of the PCR product was analyzed on 0.8% horizontal agarose gels. PCR products were cleaned using a QIAquick spin column (Qiagen) according to the manufacturer's instructions. The PCR products were sequenced by ABI3130 XL (Applied Biosystems, USA). Further the nucleotide sequence was subjected to homology search using the Blast tool (www.ncbi.nlm.nih.gov/org) and was submitted to Sequin (GenBank, NCBI) as the accession number JQ713566. The phylogenetic analysis was performed by neighbour-joining in MEGA5, using 1,000 bootstrap replicates. For the Culture preservation and further use the fungal culture, it was deposited to IMTECH Chandigarh.

2.10. Biological activity

2.10.1. In vitro antioxidant assay

Natural molecules play vital role in free radical scavenging activities. Keeping in the view all the compounds **1–4** were evaluated for their potential *in vitro* antioxidant activity.

2.10.2. DPPH (1,1-diphenyl-2-picryl hydrazyl) assay

The free radical scavenging activity of **1** and metabolites **2, 3** (Table 1) was measured by protocols of [35,36] with minor modifications. The *in vitro* analyses (various concentrations) of each test compound (10, 50, 100, 250 μM/ml) were performed with 100 mM Tris–HCl buffer (pH 7.4) and 100 μM of DPPH. The reaction mixture was incubated for 30 min in the dark at room temperature and measured at 517 nm, using Spectra Max M2 multimode micro plate reader (Molecular Devices) against the blank.

Table 1
Description of compounds.

Compounds	Short forms
Artemisinic acid (**1**)	AA
3β-hydroxyartemisinic acid (**2**)	3β,HAA
3β, 15-dihydroxyartemisinic acid (**3**)	3β,15HAA
Diacetate 3β, 15-dihydroxyartemisinic acid (**4**)	Diacetate 3β,15 HAA

2.10.3. Total antioxidant capacity estimation assay

The total antioxidant capacity of compounds **1–4** (Table 1) was performed using methods of [36,37]. The total antioxidant capacity of different test compounds (10, 50, 100, 250 μM/ml) was measured with ascorbic acid as the standard. The absorbance of the samples was taken at 695 nm against a blank using Spectra Max M2 multimode micro plate reader (Molecular Devices).

2.10.4. Reducing power assay

Reducing power assay of test compounds **1–4** was performed with slight modifications of protocols reported in the literature [36,38]. Different concentrations (10, 50, 100, 250 μM/ml) was analyzed using 0.2 M phosphate buffer (pH 6.6), 1% K_4FeCN_6, 10% TCA (tri-carboxylic acid) and 0.10% $FeCl_3$. The absorbance was taken at 700 nm with a reagent blank using Spectra Max M2 multimode micro plate reader (Molecular Devices).

2.10.5. In vivo toxicity and antioxidant assay

N2 Bristol (Wild type) strains of *Caenorhabditis elegans* and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, USA, routinely cultured and maintained at 20 °C on nematode growth medium (NGM), seeded with *E. coli* OP50 bacteria [39]. The different concentrations (5, 50, 100 μM/ml) of test compounds were used for toxicity assay and *in vivo* antioxidant assay on *C. elegans* model system.

2.10.6. Toxicity assay

Test compounds were prepared in different (5, 50, 100 μM/ml) concentrations with acetone as solvent (0.001%) system. The age synchronized wild type (N2) worms (L4) were transferred to 24-well plate (tissue culture Axygen-India) containing defined concentrations. Worms were observed after every hour for their mortality/survival by touch provoked method [40].

2.10.7. Antioxidant capacity/ROS detection assay

The assay was conducted as described in literature [41–43]. Adult day 2 worms were used for intracellular ROS determination. Age synchronized worms (30 in number) were collected in 300 μl of 0.1% phosphate buffer saline with Tween 20 (PBST). Worms were washed thrice with PBST and the lysate was prepared by equally timed homogenization and sonication (Branson Sonifer 250; VWR Scientific, Suwannee, GA, USA). The homogenized samples were transferred to 96 wells plates with 1.5 μl of 10 mM H_2DCF-DA which was added to the final concentration of 50 μM before reading of the plates. Fluorescent readings were measured using Spectra Max M2 multimode micro plate reader (Molecular Devices) at 485 nm excitation and 530 nm emission. All observations were recorded at every 20 min for 2 h and 30 min at 37 °C.

2.10.8. Antibacterial assay

The test compounds **1–4** were tested for their antibacterial activity (Table 2) against selected pathogenic microbes through disc diffusion assay with slight modifications [44,45]. The minimum inhibitory concentration (MIC) of compounds **1–4** was determined as per McFarland Standard 0.5 against the selected bacteria [Gram positive–*Staphylococcus aureus* (ATCC2940), *S. epidermidis* (ATCC435), *Streptococcus mutans* (ATCC890), Gram negative–*Enterobacter aerogenes* (ATCC111), *E. coli* (ATCC739), and acid fast staining bacteria *Mycobacterium smegmatis* ($MC^2$155 (Delhi University)]. All the bacterial strains were procured from American type culture collection (ATCC). The impregnated paper disc (Whattman) with different test concentrations (100, 200, 500 μg/disc) were placed over the seeded plates and incubated for 24 h at 37 °C. The activity was measured in terms of the zone of microbial growth inhibition (Hi Antibiotic Zone Scale) through Himedia scale.

Table 2
Disc diffusion assay of compounds showing net zone of inhibition (in mm) against test bacteria.

Compounds	SA ATCC.No.-2940	SE ATCC.No.-435	SM ATCC.No.-890	EC ATCC.No.-739	EA ATCC.No.-111	MS MC²155 (Delhi University)
AA (**1**)	–	–	–	–	–	√
3β,HAA (**2**)	–	–	–	–	–	–
3β,15HAA (**3**)	–	–	–	–	–	–
Diacetate 3β,15 HAA (**4**)	–	–	–	–	–	–

SA = *Staphylococcus aureus*, SE = *S. epidermidis*, SM = *Streptococcus mutans*, EA = *Enterobacter aerogenes*, EC = *E. coli*, MS = *Mycobacterium smegmatis*.

2.10.9. BACTEC radiometric susceptibility assay

To assess the impact of test compounds **1**–**4** on *M. tuberculosis* H37Rv (ATCC 27294) strain, it was scraped from maintained slants (Löwenstein-Jansen media). The bacterial strain was then transferred in 1.0 ml of BACTEC diluting fluid and made the complete homogenized suspension by vortexing with glass beads (2 mm diameter) [46]. In continuation for further preparation of the test strain and to access the efficacy of compounds on *M. tuberculosis* the protocol of our group was followed with slight modifications [46].

3. Results and discussion

The endophytic fungal isolates, obtained from *A. annua* were screened for their biotransformation potentials. Among them four isolates showed positive results of which CIMAPN1 isolate showed maximum activity with intense TLC bands and thus was selected for scale up study. The isolate CIMAPN1 after the 14th day of incubation with 400 mg artemisinic acid yielded two metabolites **2** and **3** (Fig. 1). HPLC analysis of the ethyl acetate extract of culture broth (Fig. 2) clearly showed the metabolites **2** and **3** with traces of artemisinic acid. The time course study of reaction was also measured by TLC and HPLC (Fig. 2)/proton NMR analysis. It was found that metabolite **2,** R_f = 0.55 (hexane–acetone 1:1) appeared as a major compound (51.1%) with 97.2% HPLC purity. IR spectrum showed a broad band at 3445 cm^{-1}, indicating the presence of hydroxyl group in **2**. It was identified as 3β-hydroxyartemisinic acid (Fig. 1) by comparison of its spectral data (IR, NMR, MS) with reported in the literature [14,15,17].

Metabolite **3,** R_f = 0.36 (hexane–acetone 1:1), mp 91–92 °C was obtained as colourless crystals with 37.3% yield having 98.7% HPLC purity. It showed IR absorption bands at ν^{max} 3430 cm^{-1} for hydroxyl, 2925, 2856, 1686 cm^{-1} for α, β unsaturated CO and 1623 cm^{-1} for a double bond functions. ESI (positive and negative) mass spectral analysis showed a molecular weight of 266, indicated two hydroxyl groups in the molecule. The ^{13}C NMR spectrum of metabolite **3** showed two new peaks at δ 64.95 and 65.79 ppm which confirmed it to be a dihydroxylated product. The DEPT 135 spectra showed that the number of methyl carbons had decreased from two to one and the number of methine carbons increased from five to six, but the methylene carbons remain same, five in number which designated that one methyl and one methylene group of the substrate must be hydroxylated as CH_2OH and CHOH groups. A strong cross peak of H-5 broad singlet, δ 5.44 ppm with the new methine carbon signal at δ 64.95 ppm in the HMBC spectrum of metabolite **3** assigned the position of one hydroxyl group at C-3 (Fig. 4). The H-5 broad singlet showed another strong cross peaks with other new methylene carbon signal at δ 65.79 ppm indicated the presence of second hydroxyl at C-15 in **3**. Similarly the H-3, doublet at δ 4.21 ppm and H_2-15, singlet at δ 4.10 ppm showed cross peaks with C-4 at δ 140.40 ppm and C-5 at δ 124.37 ppm signals also confirmed the positions of the hydroxyls. Comparing

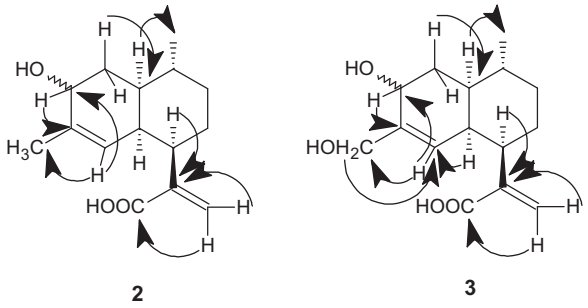

Fig. 4. HMBC Correlation diagram of compounds **2** and **3**.

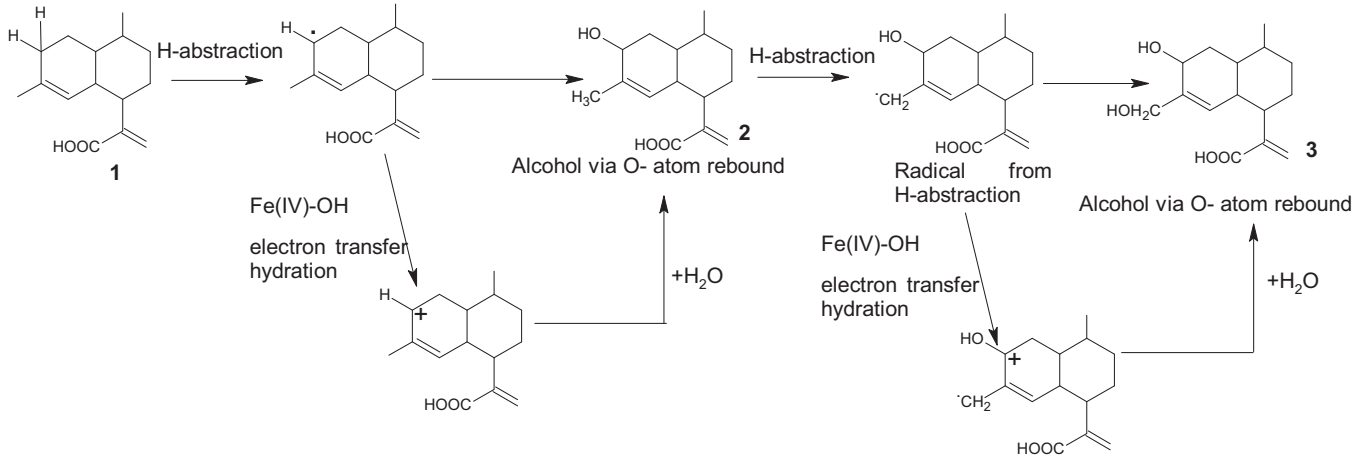

Fig. 5. Proposed mechanism for the bioconversion of artemisinic acid (**1**) to metabolites **2** and **3.**

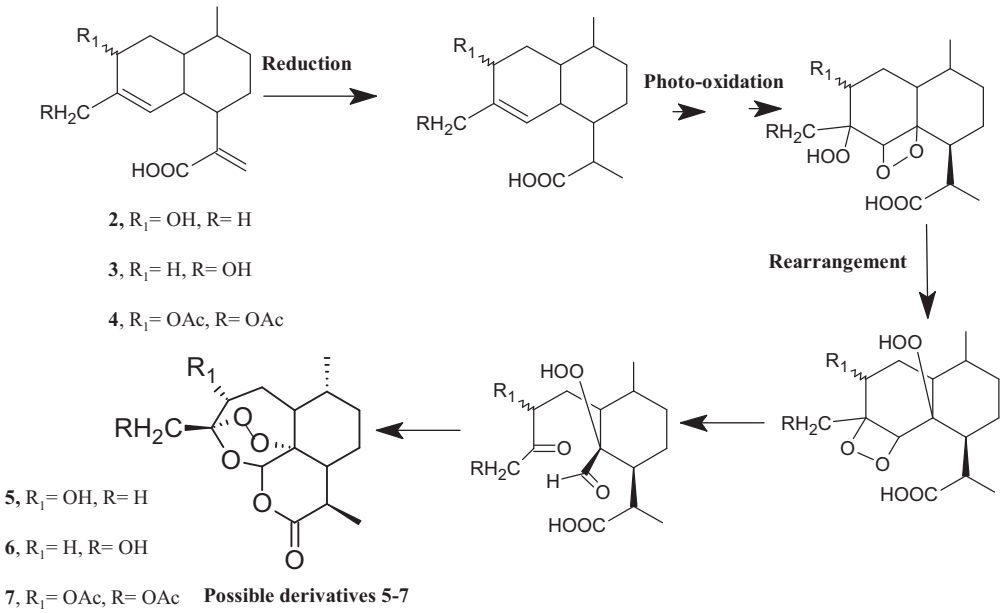

Fig. 6. Plausible mechanism for the synthesis of possible artemisinin analogues (**5**–**7**) from metabolites **2, 3** and diacetate **4.**

the coupling constant, J = 5.1 Hz of H-3 with the H-3, J = 5.4 Hz of metabolite **2** suggested a β-configuration of the hydroxyl group at C-3 in metabolite **3**. Acetylation of **3** by pyridine-acetic anhydride afforded a diacetate **4** (Fig. 1). IR band at ν^{max} 1735 cm^{-1} for ester CO, $^{1}H/^{13}C$ NMR signals, δ 6.07, brs/21.26, 171.18 ppm for CHOAc and 5.38, 5.11 ppm, doublets, J = 12.3 Hz/21.25, 171.28 ppm for CH_2OAc of **4** further confirmed that the metabolite is a dihydroxylated product of artemisinic acid. All the protons and carbon signals of **3** were assigned by analyzing the ^{1}H, ^{13}C DEPT, COSY, HMBC and HSQC data of the metabolite and by comparison of the spectral data reported for artemisinic acid [47,48]. Thus, the metabolite **3** was characterized as 3β, 15-dihydroxyartemisinic acid (Fig. 1) and is a new report. As in both metabolites **2** and **3** the configuration at C-3 is β, the results indicated that the fungus CIMAPN1 have the abilities to hydroxylate artemisinic acid in stereoselective manner.

The proposed stereoselective hydroxylation reaction mechanism pathway of artemisinic acid to metabolites **2** and **3** by the fungus is mentioned in Fig. 5. The reaction involve direct oxidation of the –CH–H and –CH_2–H bonds at C-3 and C-15 poisons, respectively to corresponding alcohols which is difficult chemically due to inertness of carbon–hydrogen bonds of the saturated hydrocarbon moieties. Such stereoselective hydroxylation reactions, catalyzed by microorganisms are well studied. The enzymes responsible for these transformations belong to oxygenases family. Oxygenases using only one molecular oxygen atom as an oxidant to oxidize a substrate and are called monooxygenases. One of the most thoroughly studied monooxygenases are cytochrome P450 enzymes [49]. CYP enzymes belong to a group of proteins (hemoproteins) that contain a prosthetic iron-heme cofactor. They consist of an Fe(III)-protoporphyrin-IX covalently linked to the protein by the sulfur atom of cysteine. In the presence of a substrate molecule the enzymatic hydroxylation involves several oxidation-reduction steps of the iron atom in porphyrin binding site, resulting in elimination of complexed water molecule. The next step is the reduction of Fe(III) to Fe(II) by activation of oxygen molecule in situ to form complexed peroxide radical which is subsequently reduced to peroxide anion. Two protonations of oxygen molecule in peroxo complex result in loss of water and formation of Fe(IV)+ complex which is responsible for hydroxylation of the substrate R–H (**1**) (Fig. 5). [50].

Thus, the utilization of endophytic microbes for the production of novel C-3 β, C-15 products from existing compounds opens a new arena for acquiring novel compounds which are impossible to produce through chemical methods. Advances in photochemistry and flow chemistry have proved to be an efficient chemical method for the synthesis of artemisinin from artemisinic acid, which is found to be in plentiful amount [9].

Artemisinic acid production by engineered yeast cells in higher yields [11] demonstrated an efficient and a viable alternative supply for artemisinin and new analogues. The novel, stereo-selective artemisinic acid metabolites **2** and **3** produced in higher yield (total 88.4%) in our study have great importance to be converted into new clinically potent artemisinin analogues. A plausible mechanism for the synthesis of new artemisinin analogues **5**–**7** viz. reduction and photo-oxidation/rearrangement, etc. [51] from metabolites **2, 3** and diacetate **4** is depicted in Fig. 6.

3.1. *Identification of the endophytic fungus*

The potent endophytic fungi showing best biotransformation activity was identified through molecular techniques. The PCR product was sequenced and BLAST analysis of 18S rRNA gene (CIMAPNI) was performed. Sequences having maximum similarities were retrieved, aligned with that of isolate JQ713566 (Fig. 7). The alignment was subjected to neighbour-joining analysis indicated the fungus as *Trichothecium roseum* (Fig. 7). The fungal strain CIMAPN1/CIM1 (JQ713566) has been deposited at IMTECH, Chandigarh with reference number "MTCC No.–11569" for preservation and future use.

3.2. *In vitro antioxidant assay*

Scavenging activity of compounds **1**–**4** was assessed at different concentrations by reduction of the stable radical DPPH to colored diphenylpicrylhydrazine. The interpretation of the scavenging effect of **1**–**4** with the DPPH radical depicts that biotransformed compounds were better scavengers than the parent **1** ($p < 0.001$) in Fig. 8A. *In vitro* analysis also reveals that with an increase in the concentration of compounds, the scavenging potentials also increased (Fig. 8A).

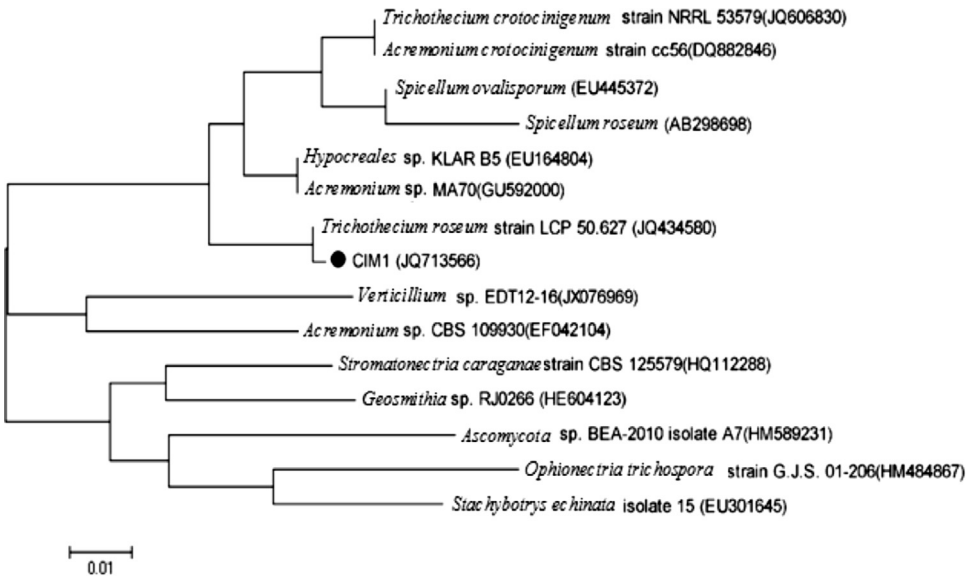

Fig. 7. Phylogenetic tree constructed from the 18S rRNA gene of strain CIMAPN1/CIM-1 and related organisms using neighbour-joining algorithm. Accession numbers of corresponding sequences are given in parenthesis and scale bar corresponds to nucleotide substitutions per nucleotide position.

Table 3
p-Values depicting the statistical significant difference of test compounds (*in vitro*).

S. no.	Antioxidant assay	250 μM	100 μM	50 μM	10 μM
1	DPPH	<0.001	<0.001	<0.001	<0.001
2	TAC	0.0757	<0.001	<0.001	<0.001
3	RP	<0.001	<0.001	>0.050	0.0621

** Significative at a level of 1% of probability ($p < 0.01$).
* Significative at a level of 5% of probability ($p < 0.05$).
ns, Non-significative ($p \geq 0.05$).

While having the assessment of total antioxidant capacity of **1**–**4** (Table 1) in terms of ascorbic acid equivalence, the results obtained thus, suggested that compounds showed a significant increase ($p < 0.001$, Table 3) in antioxidant capacity (**3 > 1 > 2 > 4**) at different concentrations. It was also observed that the antioxidant capacity followed the same trends of antioxidant elevation with the increase in concentration of compounds (Fig. 8B).

The test compounds for analysis of their reducing power activity showed significant ($p < 0.0001$, Table 3) increment in the activity as the concentration rises. The results for compound **1** and biotransformed derivatives (**2, 3, 4**) depicted that derivatives were quite better reducers than comparison to parent compound (**1**) (Fig. 8C).

3.3. Toxicity and in vivo antioxidant assay

Toxicity is an important factor for utility of any compound towards drug development. The above tested concentrations were showing no toxic effects against *C. elegans* even after 72 h exposure period (24 well plates).

Antioxidants scavenging free radicals play an important role in managing aging and age related diseases in all living systems [42,43,52] and thus, have enormous demands from pharmaceutical and food industries. It was observed that, in comparison to the untreated control compound **1** supplementation, along with derivatives **2** and **3** at 5, 50 and 100 μM concentration rendered 5.20 to 58.50% reduction of ROS generation respectively in wild type worms (Fig. 9). The best ROS reduction was observed in derivative **3** with effective concentration 50 μM (57.21%) followed by **2** (47.84%) and **1** (13.72%), respectively, as depicted in Fig. 9.

Table 4
Antimicrobial activity of compounds against *M. tuberculoses* H37 RV strain by BACTEC assay.

S. no.	Test compound	MIC (μg/ml)
1	AA	100
2	3β,HAA	>100
3	3β,15HAA	>100
4	Diacetate 3β,15 HAA	>100
5	Rifampicin	2.0
6	Streptomycin	2.0

3.4. Anti-bacterial assay

The minimum inhibitory concentration (MIC) of compound **1** along with derivatives **2**–**4** against the tested pathogenic bacteria was found to be negative with all the tested concentrations indicating no possible antibacterial activity of the same, except against *M. smegmatis* MC215 (DU) which showed little activity at higher concentrations as shown in Table 2. Similarly, an assessment of antitubercular activities on compound **1** and its derivatives against *M. tuberculosis* H37Rv strain in BACTEC-460 assay. The results thus, obtained were calculated and expressed as minimum inhibitory concentrations (MIC) in mg/ml (Table 4). Compound **1** exhibited positive antitubercular activity at MIC 100 μg/ml while its derivatives **2**–**4** possessed antitubercular activity showing MIC >1000 μg/ml.

Fungal cultures have been frequently used as efficient biocatalysts to carry out complex regiospecific or stereoselective chemical reactions which are difficult or very costly to perform through synthetic roots [53]. However, this is the first report of isolation of endophyte *T. roseum* from *A. Annua*, its use as a biocatalyst for

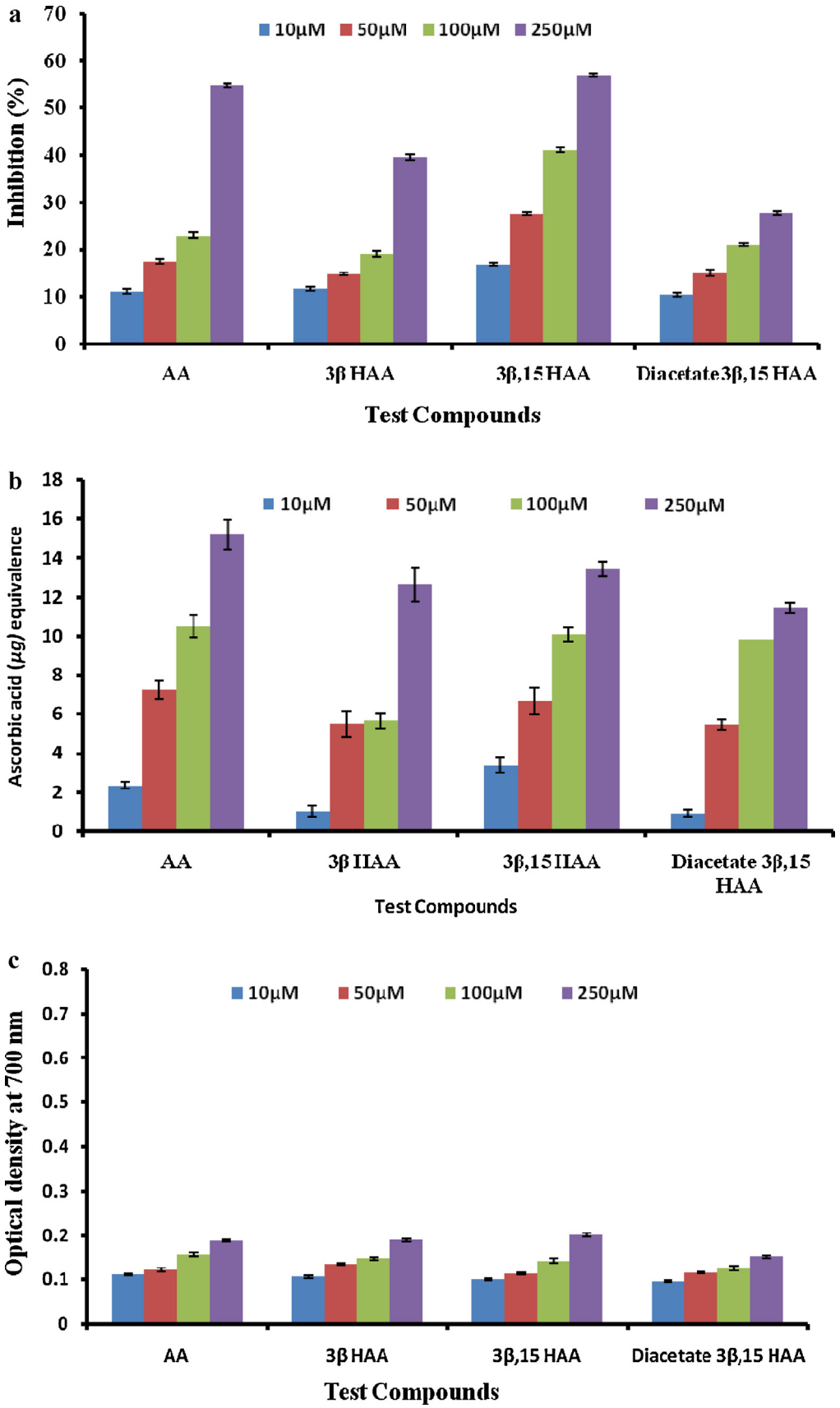

Fig. 8. (A) Graph is showing free radical scavenging (DPPH) activity of test compounds **1** (AA), **2** (3β, HAA), **3** (3β, 15HAA), and **4** (Diacetate 3β, 15 HAA). The values plotted are mean ± SE of three replicates. Results, showing significant (p **< 0.05**) variation in the tested compounds are depicted in Table 4. (B) Total antioxidant capacity of test compounds **1** (AA), **2** (3β, HAA), **3** (3β, 15HAA), and **4** (Diacetate 3β, 15 HAA) was estimated and provided as values are mean ± SE of three replicates. The p-values, depicting the statistical significance were tabulated in Table 4. (C) The graph depicting reducing power estimation of test compounds **1** (AA), **2** (3β, HAA), **3** (3β, 15HAA), and **4** (diacetate 3β, 15 HAA). Values are mean ± SE (error bars) of tested concentration taken from replicates of three. The p-values, depicting the statistical significance were tabulated in Table 4.

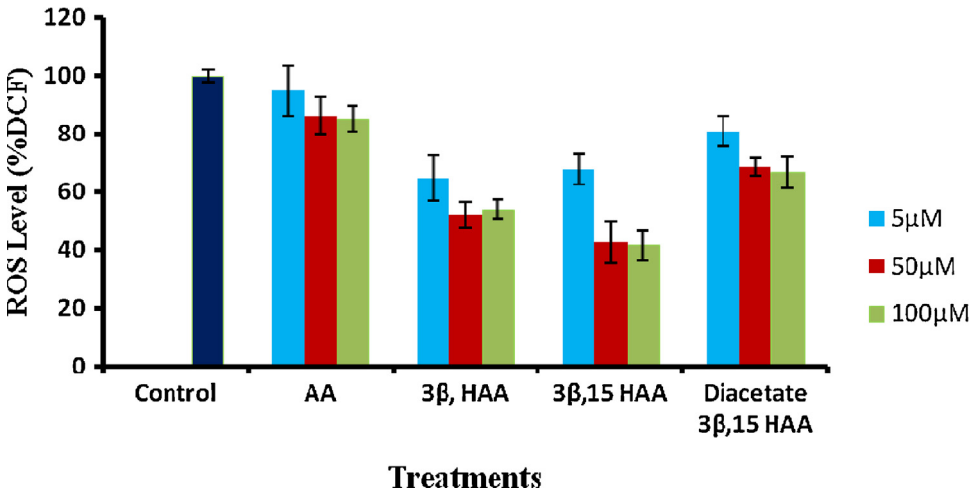

Fig. 9. Effect of **1** (AA), **2** (3β, HAA), **3** (3β,15HAA), and **4** (diacetate 3β,15 HAA) on ROS level in *C. elegans*. Graph was plotted as relative change in ROS compared to control as 100%. Errors bars represent the standard error of mean (SEM).

efficient conversion of artemisinic acid to major derivatives **2, 3** and showing their significant biological activities in this study.

4. Conclusion

The biotransformation study of artemisinic acid by endophytic fungus *T. roseum* resulted into two novel major metabolites 3β-hydroxyartemisinic acid and 3β, 15-di-hydroxyartemisinic acid. Endophytic fungus *T. roseum* possessed the potential to catalyze hydroxylation reaction at the position of allylic methylene and the main products were β-configurated. The study suggested that the fungus *T. roseum* will be highly important, economical and beneficial option in producing promising novel products from artemisinic acid in future. The biological activity results revealed that these derivatives can be a more dependable choice for other pharmacological actions and opens new arena towards management of other diseases related to ROS (aging, age related diseases). Thus, artemisinic acid derived metabolites **2** and **3** not only will serve as an option towards utilization in terms of starting material for chemical synthesis of new potent artemisinin derivatives but it will also serve as a potential lead for the generation of novel compounds targeting other severe diseases.

Acknowledgements

Authors are grateful to Prof. Anil K. Tripathi, Director CSIR-CIMAP, Lucknow, for providing the necessary facilities and constant encouragement to carry out this study. The work was carried out under a supra-institutional project, Chem-Bio–BSC-0203—chemical biology of *Ocimum* and other aromatic plants. Thanks are also due for Dr. Suaib Luqman towards his help in performing antioxidant activities and to Dr. Shivesh Pratap Singh, Editor In Chief, National Journal of Life Sciences for his editorial help.

References

[1] R.S. Bhakuni, D.C. Jain, R.P. Sharma, S. Kumar, Curr. Sci. 80 (2001) 35–48.
[2] R.S. Bhakuni, D.C. Jain, R.P. Sharma, ARTEMISIA, in: C.W. Wright (Ed.), Medicinal and Aromatic Plants Industrial Profiles, vol. 18, Taylor and Frances, Fetter lane, London, USA & Canada, 2002, pp. 211–248.
[3] J. Kong, Y. Yang, W. Wang, K. Cheng, P. Zhu, RSC Adv. 3 (2013) 7622–7641.
[4] G. Schmid, W. Hofheinz, J Am. Chem Soc. 105 (1983) 624–625.
[5] M.A. Avery, W.C. Jennings, W.K.M. Chong, Tetrahedron Lett. 28 (1987) 629–4632.
[6] R.J. Roth, N. Acton, J. Nat. Prod. 52 (1989) 1183–1185.
[7] R.K. Haynes, S.C. Vonwiller, Trans. Royal Soc. Trop. Med. Hyg. 88 (1994) 23–25.
[8] A.K. Bhattacharya, M.S. Siddiqui, R.S. Bhakuni, D.C. Jain, R.P. Sharma, Patent No. 184682 (2001).
[9] F. Lévesque, P.H. Seeberger, Angew. Chem. Int. Ed. 51 (2012) 1706–1709.
[10] D.K. Ro, E.M. Paradise, M. Ouellet, K.J. Fisher, K.L. Newman, et al., Nature 440 (2006) 940–943.
[11] C.J. Paddon, P.J. Westfall, D.J. Pitera, et al., Nature 496 (2013) 528–532.
[12] S.A. Elmarakby, F.S. El-Feraly, H.N. Elsohly, E.M. Croom, C.D. Hufford, Phytochemistry 27 (1988) 3089–3091.
[13] H. Kawamoto, Y. Asada, H. Sekine, T. Furuya, Phytochemistry 48 (8) (1998) 1329–1333.
[14] J.H. Zhu, R.M. Yu, L. Yang, Y.S. Hu, L.Y. Song, Y.J. Huang, W.M. Li, S.X. Guan, Process Biochem. 45 (2010) 1652–1656.
[15] J.H. Zhu, R.M. Yu, Zhongcaoyao 43 (6) (2012) 1065–1067.
[16] Y.S. Hu, J.H. Zhu, B. Jiang, R.M. Yu, Zhong Yao Cai 33 (2010) 662–665.
[17] L. Yang, J. Zhu, L. Song, X. Shi, X. Li, R. Yu, Nat. Prod. Res. 26 (15) (2012) 1388–1394.
[18] D. Wilson, Oikos 73 (1995) 274–276.
[19] M. Zikmundova, K. Drandarov, L. Bigler, M. Hesse, C. Werner, Appl. Environ. Microbiol. 10 (2002) 4863–4870.
[20] M. Saunders, L.M. Kohn, New Phytol. 182 (2009) 229–238.
[21] L. Zhang, R. An, J. Wang, N. Sun, S. Zhang, J. Hu, J. Kuai, Curr. Opin. Microbial. 8 (2005) 276–281.
[22] Y. Wang, C.C. Dai, Ann. Microbiol. 61 (2011) 207–215.
[23] J. Li, G.Z. Zhao, H.H. Chen, H.B. Wang, S. Qin, W.Y. Zhu, L.H. Xu, C.L. Jiang, W.J. Li, Lett. Appl. Microbiol. 47 (2008) 574–580.
[24] M.A. Penalva, R.T. Rowlands, G. Turner, Trends Biotechnol. 16 (1998) 483–489.
[25] R.X. Tan, W.X. Zou, Nat. Prod. Rep. 18 (2001) 448–459.
[26] S. Patel, R. Gaur, P. Verma, R.S. Bhakuni, A. Mathur, Biotech. Lett. 32 (2010) 1167–1171.
[27] S. Patel, R. Gaur, M. Upadhyaya, A. Mathur, A.K. Mathur, R.S. Bhakuni, J. Nat. Med. 65 (2011) 646–650.
[28] R. Gaur, S. Patel, R.K. Verma, A. Mathur, R.S. Bhakuni, Med. Chem. Res. 23 (2014) 1202–2120, 6.
[29] W. Yuan, L.P. Zhang, K.D. Cheng, P. Zhu, Q. Wang, H.X. He, H.X. Zhu, J. Nat. Prod. 69 (5) (2006) 811–814.
[30] S.A.A. Shah, S. Sultan, M. Zaimi bin Mohd Noor, J. Mol. Struct. (2013) 118–122.
[31] K.H.C. Baser, G. Buchbauer, Handbook of Essential Oils: Science Technology, and Applications, 2010, pp. 633.
[32] K. Zilbeyaz, M. Taskin, E.B. Kurbanoglu, N.I. Kurbanoglu, H. Hamdullah, Chirality 22 (2010) 543–547.
[33] T. Gong, L. Zheng, X. Zhen, H.X. He, H.X. Zhu, P. Zhu, J. Asian Nat. Prod. Res. 16 (2014) 383–386.
[34] I.A. Parshikov, K.M. Muraleedharan, M.A. Avery, J.S. Williamson, Appl. Microbiol. Biotechnol. 64 (2004) 782–786.
[35] Y.C. Chung, C.T. Chang, W.W. Chao, C.F. Lin, S.T. Chou, J. Agr. Food Chem. 50 (2002) 2454–2458.
[36] S. Luqman, S. Srivastava, R. Kumar, A.K. Maurya, D. Chanda, Evidence-Based Complement. Alt. Med. (2012), http://dx.doi.org/10.1155/2012/519084.
[37] P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem. 269 (1999) 337–341.
[38] G.C. Yen, H.Y. Chen, J. Agric. Food Chem. 43 (1995) 27–32.
[39] S. Brenner, Genetics (1974) 71–94.
[40] G.J. Lithgow, T.M. White, S. Melov, T.E. Johnson, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7540–7544.
[41] J.V. Smith, Y. Luo, J. Alzheimers Dis. 5 (2003) 287–300.
[42] V. Shukla, D. Yadav, S.C. Phulara, M.M. Gupta, S.K. Saikia, R. Pandey, Free Radic. Biol. Med. 53 (2012) 1848–1856.
[43] S. Tiwari, S. Singh, P. Pandey, S.K. Saikia, A.S. Negi, S.K. Gupta, R. Pandey, S. Banerjee, Protoplasma (2014), http://dx.doi.org/10.1007/s00709-014-0617-0.

[44] A.W. Bauer, M.M. Kirby, J.C. Sherris, M.A.M. Turck, J. Clin. Pathol. 36 (1966) 493–496.
[45] S. Goswami, R.S. Bhakuni, A. Chinniah, A. Pal, S.K. Kar, P.K. Das, Antimicrob. Agents Chemother. 56 (2012) 94594–94607.
[46] S. Singh, J.K. Kumar, D. Saikia, K. Shanker, J.P. Thakur, A.S. Negi, et al., Eur. J. Med. Chem. 45 (2010) 4379–4382.
[47] M. Jung, H.N. ElSohly, E.M. Croom, A.T. McPhail, D.R. McPhail, J. Org. Chem. 51 (26) (1986) 5417–5419.
[48] Y.Y. Tu, M.Y. Ni, Y. Zhong, L. Li, S. Cui, M. Zhang, X. Wang, Z. Ji, X. Liang, Planta Med. 44 (1982) 143–145.
[49] B. Meunier, S.I.P. Visser, S. Shaik, Chem. Rev. 104 (2004) 3947–3980.
[50] R.K. Adamusiak, D. Strub, S. Lochyński, Appl. Microbiol. Biotechnol. 95 (2012) 1427–1436.
[51] R.J. Roth, N. Action, J. Nat. Prod. 52 (1989) 1183.
[52] T. Finkel, N.J. Holbrook, Nature 408 (2000) 239–247.
[53] P. Langley, A. Shuttleworth, P.J. Sidebottom, S.K. Wrigley, P.J. Fisher, Mycol. Res. 94 (1990) 705–706.