Phytochemistry 107 (2014) 135–140

Contents lists available at ScienceDirect

Phytochemistry

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

In vitro antimalarial studies of novel artemisinin biotransformed products and its derivatives

Rashmi Gaur [a], Mahendra P. Darokar [b], P.V. Ajayakumar [c], Ram Sajiwan Shukla [d], Rajendra Singh Bhakuni [a,*]

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

ARTICLE INFO

Article history:
Received 2 May 2014
Received in revised form 28 July 2014
Available online 30 August 2014

Keywords:
Antimalarial
Artemisinin
Metabolism
In-vitro

ABSTRACT

Biotransformation of antimalarial drug artemisinin by fungi *Rhizopus stolonifer* afforded three sesquiterpenoid derivatives. The transformed products were 1α-hydroxyartemisinin (**3**), 3.0%, a new compound, 10β-hydroxyartemisinin, 54.5% (**4**) and deoxyartemisinin (**2**) in 9% yield. The fungus expressed high-metabolism activity (66.5%). The chemical structures of the compounds were elucidated by 1D, 2D NMR spectrometry and mass spectral data. The major compound 10β-hydroxyartemisinin (**4**) was chemically converted to five new derivatives **5**–**9**. All the compounds **3**–**9** were subjected for *in vitro* anti-malarial activity. 10β-Hydroxy-12β-arteether (**8**), IC_{50} at 18.29 nM was found to be 10 times better active than its precursor **4** (184.56 nM) and equipotent antimalarial with natural drug artemisinin whereas the α-derivative **9** is 3 times better than **4** under *in vitro* conditions. Therefore, the major biotransformation product **4** can be exploited for further modification into new clinically potent molecules. The results show the versatility of microbial-catalyzed biotransformations leading to the introduction of a hydroxyl group at tertiary position in artemisinin in derivative (**3**).

1. Introduction

Artemisinin (**1**) derivatives have achieved great importance as potential antimalarials for their action against multi-drug resistant *Plasmodium falciparum* malaria which cannot be treated with quinoline based and antifolate drugs (Sriram et al., 2004). In order to discover more potent anti-malarial leads than the existing lead molecules, artemisinin was transformed into value-added products by chemical and microbial methods in the last decade and resulted in the development of more effective anti-malarial drugs (Bhakuni et al., 2002; Goswami et al., 2010; Liu et al., 2006).

Microbial transformations can be considered as "green chemistry" for their ecological technology using mild conditions such as an aqueous medium, moderate pH and low temperature (Vézina, 1987). They can be used to modify chemical structures of biologically active products (Zhang et al., 2011), to prepare products which are difficult to obtain by conventional chemical methods (Adelin et al., 2012), to study the metabolism of xenobiotics (Abourashed et al., 1999) as well as to develop structure–activity relationship (SAR) models (Zhang et al., 2007). Till now, there are several reports on microbial transformations of sesquiterpene lactones (SLs) by fungi (Galal et al., 1999; Kumari et al., 2003; Lamare and Furstoss, 1990; Parshikov et al., 2006; Rocha et al., 2012). Some microorganisms are well known for their hydroxylation of artemisinin producing active metabolites (Khalifa et al., 1995). The main enzymatic reactions of SLs, catalyzed by *Rhizopus stolonifer*, are simple hydrogenations, hydroxylations, acetylations and reductions. Based on the potential of this fungus, metabolism of artemisinin (**1**) was investigated in order to obtain new potentially bioactive derivatives and, if possible, to study their structure–activity relationships.

In the continuation of our earlier biotransformation studies on bioactive compounds (Patel et al., 2010, 2011; Gaur et al.,

Abbreviations: DEPT, distortionless enhancement by polarization transfer; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple-bond correlation spectroscopy.

* Corresponding author. Address: Medicinal Chemistry Division, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O.-CIMAP, Kukrail Picnic Spot Road, Lucknow 226015, U.P., India. Tel.: +91 522 2718622 (O), +91 522 2359628; fax: +91 522 2342666.

E-mail addresses: bhakunirs2000@yahoo.com, rs.bhakuni@cimap.res (R.S. Bhakuni).

URL: http://www.cimap.res.in (R.S. Bhakuni).

http://dx.doi.org/10.1016/j.phytochem.2014.08.004
0031-9422/

2014a,b), five fungi *Alternaria alternata*, *Aspergillus flavus, Aspergillus niger, Penicillium citrinum* and *R. stolonifer* were used for the metabolism of **1** in the present study. *R. stolonifer* was selected for detailed study because of its high metabolic activity. Three microbial metabolites deoxyartemisinin **2**, 1α-hydroxyartemisinin **3** and 10 β-hydroxyartemisinin **4** of **1** were isolated (Fig. 1). 1D and 2D-NMR data showed that the yield of the new metabolite **3** was 4.6%. The major compound (54.5%), metabolite **4**, was chemically converted to five new derivatives (**5**–**9**). The isolates and derivatives were studied for *in vitro* activities against *P. falciparum*. The *in vitro* results indicated that 10β-hydroxy-12β-arteether (**8**) is ten times better active than its precursor **4**, and equipotent antimalarial with natural drug artemisinin whereas the α-derivative **9** is three times better than **4**.

2. Material and methods

2.1. General experimental procedures

Melting points obtained with a Toshniwal melting point apparatus are uncorrected. Infrared spectra of the samples were performed with a Perkin Elmer Bx infrared spectrophotometer and recorded in KBr pellets. Specific rotations, $[\alpha]_D$ were measured with a polarimeter (SEPA-300, HORIBA). ^1H and ^{13}C NMR spectra were determined on a Bruker Avence-300 instrument at 300 MHz and 75 MHz, respectively in $CDCl_3$ using TMS as an internal standard. The chemical shift values are in ppm units, and Hz denotes the coupling constants. The NMR signals abbreviations are: s (singlet), d (doublet), dd (double doublet), br (broad), t (triplet), dt (double triplet), q (quartet), dq (double quartet), m (multiplet). COSY, DEPT experiments were performed using standard pulse sequence. Electrospray ionization (ESI) mass spectra, obtained from Shimadzu LC–MS system were used to determine the molecular weight or molecular formula of the metabolites. HRMS spectra were recorded on JEOL-AccuTOF JMS-T100LC mass instrument using dry Helium for ionization. Artemisinin, used for the metabolism study was isolated from *Artemisia annua* (Tandon et al., 2003).

2.2. Chromatographic conditions

For the detection and quantification of the isolates, CAMAG HPTLC system with WINCATS software was used. Samples of 10 µl of bio-transformed products, except metabolite **3** 20 µl were spotted using ATS-4 CAMAG, Switzerland on HPTLC plates from Merck and scanned the plates at 620 nm using CAMAG TLC scanner 3, Switzerland. HPLC data were obtained, to check the purity of compounds, from a Shimadzu LC-10 instrument using an SPD-10A UV–Vis detector and a CBM- 10A interface module. The data were analyzed using class LC-10 Work Station. The sample analysis was carried out through reverse phase chromatography (RP 18 column, 250 mm, 5 µm, SymmetryShield®TM) using acetonitrile and water mobile phase (75:25), flow rate 1 ml/min at λ 220 nm.

2.3. Microorganisms and culture media

Alternaria alternate, *A. flavus*, *A. niger*, *P. citrinum* (all lab isolates, confirmed from Commonwealth Mycological Institute, Kew, Surrey, England.) and *Rhizopus stolonifer* (confirmed by the Indian Agricultural Research Institute, New Delhi) (Shukla et al., 2006) were used in this experiment. All the cultures and biotransformation experiments using filamentous fungi were performed in potato medium consisting of peeled potato extract (200 g), dextrose (10 g) and distilled H_2O (1000 ml).

2.4. Biotransformation procedures

The media for preliminary screening and scale-up experiments contained potato infusion 200 g, dextrose 10 g and appropriate amount of distilled water to make the final volume of 1 l, pH 7.0. The screening experiments using five fungi were conducted in conical flasks (125 ml each) containing 25 ml of sterilized potato dextrose broth (PDB) medium. The metabolism experiments were performed by inoculation and incubation of the cultures on orbital shaker (model 3020, GFL mbH, Germany) at 160 rpm and 23 ± 1 °C using literature procedures (Elmarakby et al., 1986; Shukla et al., 1997). Usually, 6% solution of the substrate in acetone was added to the 48 h old stage II culture media at 0. 2 mg/ml concentration. The substrate controls contained only sterile media, and the culture controls were experimented under similar conditions in the absence of substrate.

2.5. Bioconversion of artemisinin

R. stolonifer was cultured in 50 ml of PDB medium inside 250 ml conical flasks. For ascertain the toxicity tolerance of the fungus against artemisinin (**1**), the substrate was added at different concentrations, i.e. from 0.2 mg/ml to 2 mg/ml (Table 1), to the 48 h old stage II culture in triplicates. After five days, the bioconversion mixtures were monitored by TLC analysis. The higher amounts of bio-products with the highest biomass were obtained up to 0.8 mg/ml concentration of artemisinin, but its 1.6 mg/ml and 2 mg/ml concentrations produced less fungal biomass. Percent increase/decrease over the initial inoculum on dry weight basis is denoted as growth index (GI). The bioconversion mixtures of three flasks from 3rd to 10th day of inoculation were mixed to observe the time course of biotransformation. All the experiments were performed two times each in three repetitions/treatments. In the scale up experiment, artemisinin (1 g) acetone (6.2 ml) solution was distributed equally in twenty-five 250 ml culture flasks, each containing 50 ml PDB of 48 h old *R. stolonifer* cultures. After seven days of incubation, the total mixtures were filtered, washed the mycelia mates with water and combined. The combined aqueous filtrate (1.2 l) was extracted three times with an equal volume of ethyl acetate. The ethyl acetate layer was dried (using Na_2SO_4), filtered and evaporated under reduced pressure to yield a dark brown residue (1.1 g).

2.5.1. Isolation and characterization of deoxyartemisinin ***2***

The residue showed a major and two minor spots on TLC in hexane–ethyl acetate (70:30). The residue (1.1 g) was subjected to chromatography on a silica-gel column (12.5 g, 17 .5 × 0.8 cm), eluting with different proportions of hexane:ethyl acetate. Fractions of 15 ml each were collected and monitored by TLC. Hexane–ethyl acetate (85:15) fractions 28–36, which showed two spots on TLC were pooled and evaporated to dryness (305 mg); 100 mg from that was purified by preparative TLC in hexane–ethyl acetate (70:30) to yield 30 mg of metabolite **2** (9.1%, Rf = 0.43) and 65 mg of unreacted artemisinin (19.8%, Rf = 0.38). Metabolite **2**, mp 110–111 °C, $[\alpha]_D^{23} = -131.6$ ($c = 0.6$ DCE); IR (KBr) λ_{max}, cm^{-1} 2938, 2886, 1748(CO), 1595, 1458, 1386, 1138, 1021; ESI-MS (positive): m/z 267 $[M+H]^+$, 289 $[M+Na]^+$, (Calc. for $C_{15}H_{23}O_4$); ^1H NMR: δ 0.94 (3H, d, $J = 3.3$ Hz, H_3-14), 1.02 (1H, m, H-8), 1.13 (2H, m, H_2-9), 1.19 (3H, d, $J = 7.2$ Hz, H_3-13), 1.21 (2H, m, H_2-2), 1.23 (1H, m, H-10), 1.26 (1H, m, H-1), 1.52 (3H, brs, H_3-15), 1.64 (1H, m, H-3), 1.75–1.83 (2H, m, H-3, H-9), 1.94 (1H, m, H-2, H-8), 2.01 (1H, m, H-7), 3.18 (1H, m, H-11), 5.70 (1H, s, H-5); ^{13}C NMR data (see Table 2). It was identified as deoxyartemisinin by comparison of its spectral data with literature (Lee et al., 1989) and Co-TLC with an authentic sample.

Table 1
Effect of artemisinin concentration on fungal biomass and percent transformation.

Artemisinin concentration (mg/ml PDB)		Fungal biomass (a) Dry weight (mg)	Growth measurements % Biomass increment (GI on dry weight basis)		% Transformation (b)
			Over 0 day inoculums	After artemisinin addition	
Control	0.0	284.0	42.00	2	–
	0.2	290.0	45.00	3	50
	0.4	293.3	46.65	3	50
	0.8	293.3	46.65	3	50
	1.6	255.0	27.50	−10	40
	2.0	225.0	12.50	−20	25

a = All values represent mean of triplicates, b = by HPTLC analysis, c = statistics used to compare means between fungal biomass of different concentrations of artemisinin. Initial inoculum dry weights were 200 mg.

Table 2
^{13}C NMR (δ) delta of artemisinin and its derivatives.

Carbons	**1**	**2**	**3**	**4**	**5** (isomeric)	**6**	**8**
C-1	49.90	44.99	75.82	51.56	53.10, 53.97	54.02	54.03
C-2	24.70	22.40	33.92	19.07	19.83, 19.89	19.92	19.95
C-3	35.70	34.35	33.70	35.55	36.28, 36.38	36.49	36.51
C-4	105.22	109.61	105.32	105.22	104.46, 104.72	104.35	104.31
C-5	93.62	100.04	93.66	94.4	88.91, 92.48	88.90	89.05
C-6	79.38	82.80	81.06	79.94	81.40, 81.98	81.96	82.05
C-7	44.80	42.79	40.41	45.30	44.87, 45.94	45.08	45.08
C-8	23.32	23.91	23.48	19.57	20.68, 18.29	20.55	20.49
C-9	33.45	33.83	29.14	39.97	40.04, 40.63	40.63	40.61
C-10	37.42	35.74	40.76	72.18	72.64, 72.77	72.71	72.71
C-11	32.78	33.15	33.42	32.90	35.25, 31.33	31.44	31.39
C-12	171.92	172.28	172.46	172.49	95.09, 96.68	103.62	101.86
C-13	12.47	13.03	13.00	12.6	13.61, 13.12	13.38	13.43
C-14	19.74	18.98	15.14	25.25	30.09, 29.96	30.21	30.21
C-15	25.10	24.37	25.41	25.25	26.42, 26.30	26.59	26.62
O$\underline{C}H_3$/O$\underline{C}H_2$						56.28	63.88
OCH$_2\underline{C}H_3$							15.61

*2.5.2. Isolation and characterization of 1α-hydroxyartemisinin **3***

Hexane–ethyl acetate (85:20) fractions 49–53 showed a single spot with Rf = 0.23 (hexane–ethyl acetate – 70:30) on TLC. Removal of the solvent provided 46 mg (4.6%) of metabolite **3**. It was crystallized from hexane–ethyl acetate to yield white crystals, mp 109–110 °C, $[\alpha]_D^{23}$ = +66.6 (*c* = 0.5 DCE), IR (KBr) λ_{max}, cm^{-1}: 3448(OH), 2930, 2863, 1752(ester CO), 1578, 1560, 1476, 1378, 1253, 1038; ESI-MS (positive): *m/z* 321 [M+Na]$^+$ and (negative): 297 [M−H]$^-$; HRMS (*m/z*): [M+H]$^+$ 299.1478 (Calc. for $C_{15}H_{23}O_6$ 299.1494); ^{1}H NMR: δ 0.97 (3H, d, J = 6.6 Hz, H_3-14), 1.01 (1H, m, H-8β), 1.14 (3H, d, *J* = 7.2 Hz, H_3-13), 1.40 (3H, s, H_3-15), 1.46 (2H, m, H_2-9), 1.57 (1H, m, H-10), 1.77 (1H, m, H-8α), 1.83 (2H, m, H_2-3), 1.99 (1H, m, H-2β), 2.13 (1H, dt, *J* = 9.6, 4.8 Hz, H-7), 2.32 (1H, m, H-2α), 3.24 (1H, dq, *J* = 7.2, 5.7 Hz, H-11), 5.86 (1H, s, H-5); ^{13}C NMR data (see Table 2); COSY (^{1}H–^{1}H correlations): H-2α (2.32) with H-2β (1.99) and H_2-3 (1.83), H-7 (2.13) with H-8α (1.77), H-8β (1.01) and H-11 (3.24), H-8α (1.77) with H-8β (1.01), H-10 (1.57) with H_2-9 (1.46) and H_3-1 4 (0.97); HMBC: C-1 (75.82), C-9 (29.14) and C-10 (40.76) with H_3-14 (0.97), C-3 (33.70) and C-4 (105.32) with H_3-15 (1.40), C-4 (105.32), C-6 (81.06) and C-12 (172.46) with H-5 (5.86), C-7 (40.41), C-11 (33.42) and C-12 (172.46) with H_3-13 (1.14) (Fig. 2).

*2.5.3. Isolation and characterization of 10β-hydroxyartemisinin **4***

Hexane–ethyl acetate (80:20) fractions 60–72, single spot on TLC plate, Rf = 0.13 (hexane–ethyl acetate – 70:30) were mixed together and evaporation of solvent to dryness provided 545 mg of metabolite **4** (54.5%) which upon crystallization furnished white needles, mp 160-62 °C, $[\alpha]_D^{23}$ = +31.3 (*c* = 0.5 DCE), IR(KBr) λ_{max} cm^{-1}: 3396(OH), 1724 (ester CO), 1558, 1381, 1032, 992, 962, 875; ESI-MS (positive): *m/z* 619 [2 M+Na]$^+$, 337 [M+K]$^+$, 321 [M+Na]$^+$ and (negative): 595 [2 M−H]$^-$, 297 [M−H]$^-$; HRMS (*m/z*): [M+H]$^+$ 299.1482 (Calc. for $C_{15}H_{23}O_6$ 299.1494). ^{1}H NMR: δ 1.23 (3H, d, *J* = 7.2 Hz, H_3-13), 1. 31 (3H, s, H_3-14), 1. 46 (3H, s, H_3-15), 1.50 (1H, m, H-8β), 1.54 (1H, m, H-9β), 1.63 (1H, m, H-1), 1.66 (1H, m, H-7), 1.69 (1H, m, H-8α), 1.78 (1H, m, H-9α), 1.81, m, H-2β), 2.03 (1H, m, H-2α), 2.10 (1H, m, H-3β), 2.46 (1H, ddd, 13.8, 13.5, 4.5, H-3α), 3. 36 (1H, m, H-11), 6 .50 (1H, s, H-5); ^{13}C NMR data (see Table 2); It was identified as 10β-hydroxyartemisinin **4** by comparison of its mp, NMR, and mass spectral data with literature (Parshikov et al., 2004).

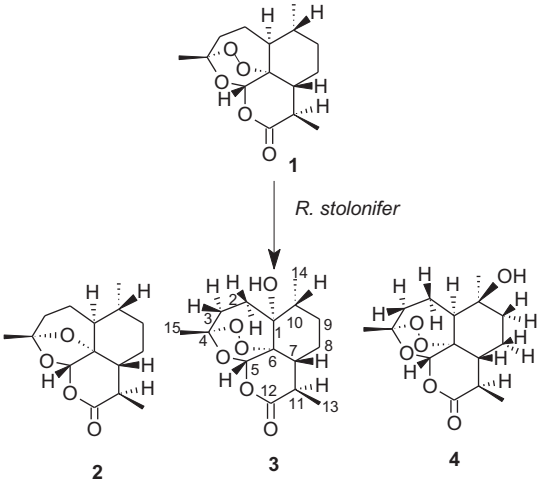


Fig. 1. Biotransformation of artemisinin.

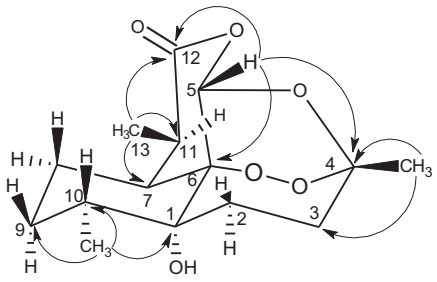


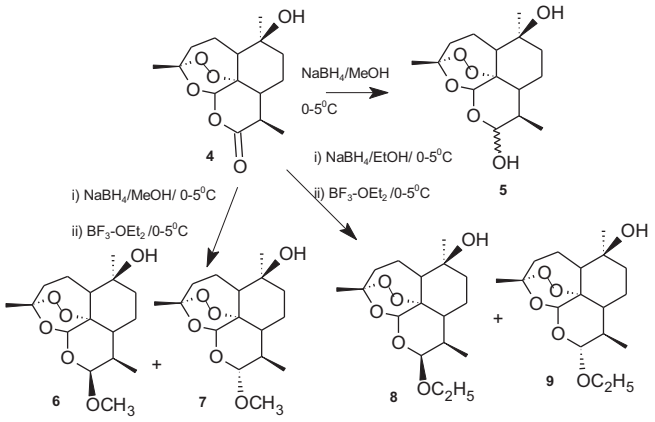
Fig. 2. HMBC correlation of metabolite **3**.

*2.5.4. Reduction of **4** to product **5***

As per Scheme 1, to the stirring solution of metabolite **4** (0.21 mmol) in methanol (10 ml) NaBH4 (1.17 mmol) was added and stirred at 0 to −5 °C for 1 h. Thereafter upon addition of cold water in the reaction mixture a white precipitate was obtained. The precipitate was filtered, washed twice with cold water and dried. The dried precipitate was crystallized to yield colorless crystals of **5** (yield = 91.5%), single on TLC with Rf = 0.23 (hexane–ethyl acetate – 70:30), mp 109-110^{0}C but found as an isomeric mixture of α- and β-products in the ratio of 46:54 from the ^{1}H NMR data. The isomeric mixture **5** was inseparable. $[\alpha]_D^{23}$ = +122.4 (c = 0.5 DCE), IR (KBr) λ_{max}, cm^{-1}: 3498(OH), 1207, 1138, 1099; HRMS (m/z): [M+H]$^+$ 301.2019 (Calc. for $C_{15}H_{25}O_6$ 301.2015). ^{1}H NMR: δ 0.96 (6H, d, J = 7.2 Hz, 2 × H_3-13), 1.27 (6H, s, 2 × H_3-14), 1.43, 1.44 (3H each, s, 2 × H_3-15), 2.58 (2H, m, 2 × H-11), 4.77 (1H, d, J = 9.3 Hz, H-12β), 5.29 (1H, d, J = 3 Hz, H-12α), 5.79 (1H, s, H-5 of β-isomer), 6.00 (1H, s, H-5 of α-isomer); ^{13}C NMR data (Table 2). Comparison of spectral data with α- and β-dihydroartemisinin (DHA) (Wei et al., 2010) the reduced product **5** was identified as 10β-hydroxy-12α/β-dihydroartemisinin.

*2.5.5. One pot conversion of **4** to produce **6–7***

Metabolite **4** was converted to alkylated derivatives **6–7** by one pot process (Bhakuni et al., 2004a,b) as per Scheme 1. To a stirring solution of metabolite **4** (63 mg, 0.21 mmol) in methanol (10 ml) at 0 to −5 °C, $NaBH_4$ (9 mmol) was added up to 30 min and the stirring was continued for 3 h. After completion of the reduction of **4–5**, monitored by TLC, BF_3-Et_2O (15 mmol) was added, and the mixture was stirred for another 3 h. After completion of metherification of the reduced product **5**, water (40 ml) was added and extracted the aqueous mixture with dichloromethane (3 × 40 ml). The collective dichloromethane extract was washed with saturated NaCl solution (30 ml) followed by water (40 ml) and dried (anhydrous Na_2SO_4). Removal of the solvent provided a crude product which was purified by flash column chromatography to afford 38 mg of 10β-hydroxy-12-β-artemether **6**) and 11 mg of 10β-hydroxy-12α-artemether **7** in the ratio of 73:27. Derivative **6**, mp 92–93 °C, $[\alpha]_D^{23}$ = +116.4 (c = 0.5 DCE), IR (KBr) λ_{max}, cm^{-1}: 2928, 1476, 1378, 1138, 1036; HRMS (m/z): [M+H]$^+$ 315.1954 (Calc. for $C_{16}H_{27}O_6$ 315.1960). ^{1}H NMR: 0.92 (1H, d, J = 7.2 Hz, H_3-13), 1.26 (1H, s, H_3-14), 1.45 (1H, s, H_3-15), 2.62 ((1H, m, H-11), 3.43 (3H, s, OCH_3), 4.08 (1H, d, J = 6.6 Hz, H-12α), 5.76 (1H, s, H-5); ^{13}C NMR data (Table 2). Derivative **7**, viscous, $[\alpha]_D^{23}$ = +11.2 (c = 0.5 DCE), IR (KBr) λ_{max}, cm^{-1}: 2927, 1444, 1379, 1280, 1023; HRMS (m/z): [M+H]$^+$ 315.1970 (Calc. for $C_{16}H_{27}O_6$). ^{1}H NMR: ^{1}H NMR: 0.90 (1H, d, J = 7.2 Hz, H_3-13), 1.27 (1H, s, H_3-14), 1.46 (1H, s, H_3-15), 2.48 (1H, m, H-11), 3.51 (3H, s, OCH_3), 4.35 (1H, d, J = 9.0 Hz, H-12β), 5.73 (1H, s, H-5).



Scheme 1. Synthesis of derivatives of metabolite **4**.

*2.5.6. One pot conversion of 4 to produce **8–9***

Arteethers **8** and **9** were synthesized and purified as per procedure, Scheme 1 followed for the production of **6** and **7** from metabolite **4** (63 mg, 0.21 mmol) except the solvent (reactant) methanol was replaced by ethanol. 10β-hydroxy-12β-arteether **8**, 41 mg, viscous, $[\alpha]_D^{23}$ = +214.7 (c = 0.5 DCE), IR(KBr) λ_{max}, cm^{-1}: 2928, 1497, 1385, 1138, 1037. HRMS (m/z): [M+H]$^+$ 329.2055 (Calc. for $C_{17}H_{29}O_6$ 329.2058). ^{1}H NMR: 0.92 (1H, d, J = 7.2 Hz, H_3-13), 1.18 (3H, t, J = 6.9 Hz, $OCH_2C\underline{H}_3$), 1.26 (1H, s, H_3-14), 1.45 (1H, s, H_3-15), 2.60 ((1H, m, H-11), 3.46, 3.87 (1H each, m, $OC\underline{H}_2CH_3$), 4.79 (1H, d, J = 3.3 Hz, H-12α), 5.81 (1H, s, H-5); ^{13}C NMR data (Table 2). 10β-hydroxy-12α-arteether **9**, 12 mg, viscous, $[\alpha]_D^{23}$ = +37.2 (c = 0.5 DCE), IR(KBr) λ_{max}, cm^{-1}: 2930, 1476, 1378, 1139, 1057; HRMS (m/z): [M+H]$^+$ 329.2054 (Calc. for $C_{17}H_{29}O_6$ 329.2058). ^{1}H NMR: ^{1}H NMR: 0.89 (1H, d, J = 7.5 Hz, H_3-13), 1.21 (3H, t, J = 6.9 Hz, $OCH_2C\underline{H}_3$), 1.27 (1H, s, H_3-14), 1.45 (1H, s, H_3-15), 2.39 ((1H, m, H-11), 3.50, 3.98 (1H each, m, $OC\underline{H}_2CH_3$), 4.43 (1H, d, J = 9.3 Hz, H-12β), 5.71 (1H, s, H-5).

2.6. In vitro anti-malarial activity

The *in vitro* experiments were performed using *P. falciparum* (NF54 strain) in human blood as per the established protocol (Makler and Hinrichs, 1993). Compounds were dissolved in DMSO and then diluted. The *Pf*-NF54 cultures and 2% hematocrit with 1–1.5% parasitemia were subjected to incubation with compounds in multiple concentrations in 96 well microtitre plates at 37 °C for 48 h. The inhibitory action of the compounds on parasite growth was determined by measuring the pLDH activity in control and test experiments through spectrophotometer. After completion of incubation time, the cultures were carefully resuspended, and 20 µl samples were taken up and added to 0.1 ml of Malstat reagent in a 96 well microplate. Added of 25 µl solutions of 1.9 µM NBT and 0.24 µM PES. The NBT was reduced to APADH, a blue formazan product which was measured by a spectrophotometer at 650 nm. IC_{50}, (50% inhibitory concentration), used to show the anti-malarial action of the test compounds.

3. Results and discussion

A. alternata, *A. flavus*, *A. niger*, *P. citrinum* and *R. stolonifer* were experimented to find their ability to metabolize artemisinin. Out of them *R. stolonifer* was selected for scale up transformation because of its high metabolic activity (68.2%). A very poor transformation was exhibited by *A. flavus* at 0.2 mg/ml concentration, whereas the other fungi and control did not show any conversion of the substrate on TLC even after 4 days of the study. *R. stolonifer* was found to tolerate artemisinin up to 0.8 mg/ml without any significant adverse effect on microbial transformation capacity and

growth of fungal biomass (Fungal biomass = 293.3 g, GI = 3). Higher concentrations, 1.6 and 2.0 mg/ml of artemisinin, was toxic to the fungus and the fungal biomass was reduced significantly to 255 g and 225 g respectively (Table 1) whereas *Nocardia corallina*, *Penicillium chrysogenum* (Lee et al., 1989), *Cunninghamella echinulata*, *A. niger* (Zhan et al., 2002) can tolerate artemisinin up to 0.2 mg to 0.3 mg/ml with 15 and 50% transformed products, respectively. Growth index (GI = −10 and −20 respectively) of *R. stolonifer* showed decline when harvested after 5 days of substrate addition both at 1.6 and 2 mg/ml level respectively (Table 1). We have employed a very simple and economically viable medium i.e. 1% PDB for *R. stolonifer* culturing, whereas Lee et al., 1989 have used a complex and costly medium containing yeast extract, peptone, NaCl and KH_2PO_4 for *N. corallina* and *P. chrysogenum* fermentation processes. The time course of biotransformation studies indicated maximum accumulation (54.5%) of metabolite **4**, 9.1% of metabolite **2** and 4.6% of metabolite **3** after 7 days incubation of *R. stolonifer* stage II culture while Zhan et al., 2002 have reported 50% recovery of transformed products after 4 days of *C. echinulata* stage II culture. Thus, this is the first report on *R. stolonifer* as a highly potent fungus for the microbial conversion of artemisinin. The metabolite **4** was produced first time by *Cunninghamella elegans* (Parshikov et al., 2004) but in low yield (6.5%) from artemisinin. Thus, we established that *R. Stolonifer* is very useful, particularly in the microbial production of metabolite **4** of artemisinin.

Preparative scale metabolism of artemisinin **1** provided metabolites **2**, **3**, and **4**. Metabolite **3**, molecular formula $C_{15}H_{22}O_6$ suggesting the introduction of a hydroxyl group in artemisinin. A new peak at δ 75.82 ppm in the ^{13}C NMR spectrum and a strong IR band at 3448 cm^{-1} supported our conclusion that **3** is a hydroxyartemisinin derivative. Analysis of its ^{13}C NMR (Table 2) and DEPT spectra revealed that the number of quaternary carbons increased from three to four and subsequently achieved a decrease in the number of tertiary carbons from five to four which explained the hydroxylation of a methine group. The H-5 singlet, H_3-13, and H_3-14 doublets indicated the position of the hydroxyl group is either at C-1 or C-7. A strong cross peak of 14-methyl doublet (δ 0.97 ppm) with the new carbon signal at δ 75.82 ppm depicted in the HMBC spectrum assigned the position of the hydroxyl group at C-1. Comparison of the ^1H and ^{13}C NMR data of compound **3** with the same reported for artemisinin showed promising downfield shifts at C-2 (1.47 αH, 2.01 βH, 24.7 ^{13}C, to 1.99, 2.32, 33.92), C-6 (79.38 to 81.06), C-10 (1.42 βH, 37.42 ^{13}C to 1.57, 40.76) and 1, 3 axial-axial interactions in H-7 (1.87–2.13) and H-9 (1.08–1.46) because of closer (1, 2) H–O and C–O interactions. The same spectral comparison also showed upfield shifts in ^{13}C values at C-7 (44.80–40.41) and C-9 (33.45–29.14) that because of far away (1, 3) C–O interactions between the oxygen atom of C-1 OH and the C-7 and C-9 nuclei. This all NMR spectral comparison suggested α orientation of the hydroxyl group at C-1 position in **3**. Further comparison of the ^{13}C NMR data of the metabolite **3** with those reported for 1- α-hydroxyarteether (Hufford et al., 1995), the hydroxyl group was supported to be α oriented. All NMR signals were assigned by analyzing its ^1H, ^{13}C, COSY, HSQC and HMBC (Fig. 2) spectral data. Finally, metabolite **3** was characterized as 1 α-hydroxyartemisinin and reported for the first time.

Metabolites **2** and **4,** were identified as deoxyartemisinin and 10β-hydroxyartemisinin by comparing their mp, IR, NMR and MS data with their reported values, respectively (Lee et al., 1989; Parshikov et al., 2004).

The major metabolite **4** was chemically converted to five new derivatives (**5**–**9**). 10β-Hydroxy-dihydroartemisinin **5**, an acetal was obtained by the reduction of metabolite **4** with $NaBH_4$, which converts into a mixture of anomers in the solution state. Instead alkylation of **5**, the other four methyl and ethyl ether derivatives (**6**–**9**) were synthesized directly from metabolite **4** in one pot by its reduction with $NaBH_4$ followed by alkylation with methanol or ethanol in the presence of Lewis acid, BF_3-etherate as per Scheme 1. The β/α- isomers (**6**/**7**, **8**/**9**) were separated by column chromatography. The α- and β-compounds were identified by analyzing their H-12 couplings (*J* values) with H-11. The higher H-12 *J* values, 9.0 Hz in the derivative **7** and 9.3 Hz in **9** showed a trans diaxial relationship of H-12 with H-11, therefore, identified them as α-products, whereas the H-12 *J* value of 6.6 Hz (derivative **6**) and 3.3 Hz (**8**) indicated a gauche relationship of H-12 with H-11, therefore identified as β-derivatives.

Table 3
In vitro antimalarial results of artemisinin and its derivatives.

Test compounds	*In-vitro* IC_{50} (nM)
β-Artemether	15.10
α-Artemether	16.77
Artemisinin (Sigma) (**1**)	18.43
3	3355.70
4	184.56
5	1666.66
6	127.38
7	3184.71
8	18.29
9	60.97

3.1. In vitro antimalarial activity

Metabolites **3, 4** and derivatives **5**–**9** were screened for *in vitro* antimalarial activity (Table 3). Among these, 10β-hydroxy-12β-arteether **8** showed promising antimalarial activity. It showed IC_{50} value 18.29 nM, 10 times better active than its precursor **4** (184.56 nM) and equipotent antimalarial with natural drug artemisinin whereas the α-derivative **9** is 3 times better than **4**. Perhaps the ethyl ether group of **8** made it more lipophilic as compared to artemether **6**, contained similarly oriented methyl ether group would be a possible advantage for the accumulation of metabolite **8** in brain tissue (Brossi et al., 1988).

4. Conclusions

The biotransformation of artemisinin using *R. stolonifer* has resulted in 10β-hydroxyartemisinin **4** as the major (54.5%), promising metabolite along with a new metabolite, 1α-hydroxyartemisinin **3**. Based on *in vitro* studies, we concluded that compound 10β-hydroxy-12β-arteether **8** derived from metabolite **4** has significant anti-malarial activity than the natural drug artemisinin and is ten times better than the parent compound **4**. Thus, the major biotransformation product, **4** can be exploited for further modification into new clinically potent molecules. Since the metabolite **4** was obtained as a novel compound in low production (6.5%) through microbial metabolism of artemisinin by *C. elegans* (Parshikov et al., 2004) consequently, the fungus *R. stolonifer* is highly useful for microbial production of **4** from artemisinin. Thus, the study suggested that *R. stolonifer* could be useful in the microbial production of new lead molecules from artemisinin in future.

Acknowledgements

We thank the Director, CIMAP, and AcSIR-CIMAP Lucknow for providing the necessary research facilities. The work was carried out under a supra-institutional project, Chem-Bio–BSC-0203-Chemical biology of Ocimum and other aromatic plants. We are also thankful to Dr. H.P. Singh for helping in statistical analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.004.

References

Abourashed, E.A., Clark, A.M., Hufford, C.D., 1999. Microbial models of mammalian metabolism of xenobiotics: an updated review. Curr. Med. Chem. 6, 359–374.

Adelin, E., Martin, M.T., Bricot, M.F., Cortial, S., Retailleau, P., Ouazzani, J., 2012. Biotransformation of natural compounds: unexpected thio conjugation of Sch-642305 with 3-mercaptolactate catalyzed by *Aspergillus niger* ATCC 16404 cells. Phytochemistry 84, 135–140.

Bhakuni, R.S., Jain, D.C., Sharma, R.P., 2002. Phytochemistry of *A. annua* and development of artemisinin derived anti-malarials. In: Wright, C.W. (Ed.), Artemisia-MAPs – industrial profiles. Taylor & Francis, London & NY, pp. 211–248.

Bhakuni, R.S., Singh, T., Kahol, A.P., Tewari, A., Tandon, S. Khanuja, S.P.S., 2004. Single pot conversion of Artemisinin to Artemether, US Patent No 6683193(2004), MY 129031-A(2007), filed-Indonesia, Thiland and African Countries, NF-230/2002.

Bhakuni, R.S., Singh, T., Tewari, A., Khanuja, S.P.S., 2004. A novel one pot conversion of Artemisinin into Arteether US Patent No 6750356(2004), Chinese ZL 02829967.1(2007), EP 1567530(2006), ZA 2005/04162(2006), DE 60211566(2007), field -PCT, NF 318/2002.

Brossi, A., Venugopalan, B., Dominguez Gerpe, L., Yeh, H.J.C., Flippen-Anderson, J.L., Buchs, P., Luo, X.D., Milhous, W., Peters, W., 1988. Arteether, a new antimalarial drug: synthesis and antimalarial properties. J. Med. Chem. 31, 645–650.

Elmarakby, S.A., Clark, A.M., Baker, J.K., Hufford, C.D., 1986. Microbial metabolism of bornaprine, 3-(diethylamino) propyl-2-phenyl bicyclo[2,2.1] heptane-2-carboxylate. J. Pharm. Sci. 75, 614–618.

Galal, A.M., Ibrahim, A.S., Mossa, J.S., El-Feraly, F.S., 1999. Microbial transformation of parthenolide. Phytochemistry 51, 761–765.

Gaur, R., Patel, S., Verma, R.K., Mathur, A., Bhakuni, R.S., 2014a. Biotransformation of artemisinin derivatives by *Glycyrrhiza glabra*, *Lavandula officinalis*, and *Panax quinquefolium*. Med. Chem. Res. 23 (3), 1202–1206.

Gaur, R., Tiwari, S., Jakhmola, A., Thakur, J.P., Verma, R.K., Gupta, N., Pandey, R., Bhakuni, R.S., 2014b. Novel biotransformation processes of artemisinic acid to their hydroxylated derivatives 3β-hydroxyartemisinic acid and 3β, 15-dihydroxyartemisinic by fungus *Trichothecium roseum* CIMAPN1 and their biological activity. J. Mol. Catal. B: Enzym. 106 (2014), 46–55.

Goswami, A., Saikia, P.P., Barua, N.C., Bordoloi, M., Yadav, A., Bora, T.C., Gogoi, B.K., Saxena, A.K., Suri, N., Sharma, M., 2010. Bio-transformation of artemisinin using soil microbe: direct C-acetoxylation of artemisinin at C-9 by *Penicillium simplissimum*. Bioorg. Med. Chem. Lett. 20, 359–361.

Hufford, C.D., Khalifa, S.I., Orabi, K.Y., Wiggers, F.T., Kumar, R., Rogers, R.D., Campana, C.F., 1995. 1α-hydroxyarteether, a new microbial transformation product. J. Nat. Prod. 58, 751–755.

Khalifa, S.I., Baker, J.K., Jung, M., McChesney, J.D., Hufford, C.D., 1995. Microbial and mammalian metabolism studies on the semisynthetic antimalarial deoxoartemisinin. Pharm. Res. 12, 1493–1498.

Kumari, G.N.K., Masilamani, S., Ganesh, M.R., Aravind, S., 2003. Microbial transformation of zaluzanin-D. Phytochemistry 62, 1101–1104.

Lamare, V., Furstoss, R., 1990. Bioconversion of sesquiterpenes. Tetrahedron 46, 4109–4132.

Lee, I.S., El-Sohly, H.N., Croon, E.M., Hufford, C.D., 1989. Microbial metabolism studies of the antimalarial sesquiterpene artemisinin. J. Nat. Prod. 52, 337–341.

Liu, J.H., Chen, Y.G., Yu, B.Y., Chen, Y.J., 2006. A novel ketone derivative of artemisinin biotransformed by *Streptomyces griseus* ATCC 13273. Bioorg. Med. Chem. Lett. 16, 1909–1912.

Makler, M.T., Hinrichs, D.J., 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am. J. Trop. Med. Hyg. 48, 205–210.

Parshikov, I.A., Muraleedharan, K.M., Avery, M.A., Williamson, J.S., 2004. Transformation of artemisinin by *Cunninghamella elegans*. Appl. Microbiol. Biotechnol. 64, 782–786.

Parshikov, I.A., Miriyala, B., Muraleedharn, K.M., Avery, M.A., Williamson, J.S., 2006. Microbial transformation of artemisinin to 5-hydroxyartemisinin by *Eurotium amstelodami* and *Aspergillus niger*. J. Ind. Microbiol. Biotechnol. 33, 349–352.

Patel, S., Gaur, R., Verma, P., Bhakuni, R.S., Mathur, A., 2010. Biotransformation of artemisinin using cell suspension cultures of *Catharanthus roseus* (L.) *Lavandula officinalis*. Biotechnol. Lett. 32, 1167–1171.

Patel, S., Gaur, R., Upadhyaya, M., Mathur, A., Mathur, A.K., Bhakuni, R.S., 2011. *Glycyrrhiza glabra* (Linn.) and *Lavandula officinalis* (L.) cell suspension cultures-based biotransformation of β-artemether. J. Nat. Med. 65, 646–650.

Rocha, B.A., Pupo, M.T., Antonucci, G.A., Sampaio, S.V., Paiva, R.M.A., Said, S., Gobbo-Neto, L., Da Costa, F.B., 2012. Microbial transformation of the sesquiterpene lactone tagitinin C by the fungus *Aspergillus terreus*. J. Ind. Microbiol. Biotechnol. 39, 1719–1724.

Shukla, R.S., Alam, M., Sattar, A., Khaliq, A., Singh, H.N., 2006. First report of *Rhizopus stolonifer* causing inflorescence and fruit rot of *Rauvolfia serpentina* in India. EPPO Bull. 36, 11–13.

Shukla, R.S., Sangwan, R.S., Singh, K.P., Singh, H.N., Kumar, S., 1997. Biotransformation of menthol by *Corynespora cassiicola* causing leaf spot of *Mentha arvensis*. In: National Symposium on Microbial technologies for environmental management and resource recovery, held at Univ. of Delhi on October 1–2, 80.

Sriram, D., Rao, V.S., Venkata, K., Chandrasekhar, G., Yogeeswary, P., 2004. Progress in the research of artemisinin and its analogues as antimalarials: an update. Nat. Prod. Res. 18, 503–527.

Tandon, S., Kahol, A.P., Jain, D.C., Bhakuni, R.S., Kumar, S., 2003. Pilot Plant processing data for isolation of Artemisinin from new variety of *Artemisia annua* "Jeevanraksha". J. Sci. Ind. Res. 62, 457–461.

Vézina, C., 1987. Biotransformations. In: Bu'lock, J., Kristiansen, J. (Eds.), Basic Biotechnology. Academic Press, London, pp. 461–483.

Wei, G.F., Liao, X.J., Pan, G.Y., Huang, Z.L., Cheng, J.S., He, Y.C., 2010. Facile one-pot conversion and characterization of dihydroartemisinin and artemether. J. Nat. Prod. 3, 161–164.

Zhan, J., Guo, H., Dai, J., Zhang, Y., Guo, D., 2002. Microbial transformation of artemisinin by *Cunninghamella echinulata* and *Aspergillus niger*. Tetrahedron Lett. 43, 4519–4521.

Zhang, J., Guo, H., Tian, Y., Liu, P., Li, N., Zhou, J., Guo, D., 2007. Biotransformation of 20(S)-protopanaxatriol by Mucor spinosus and the cytotoxic structure activity relationships of the transformed products. Phytochemistry 68, 2523–2530.

Zhang, X., Ye, M., Dong, Y.-H., Dong, Y., Hu, H.-B., Tao, S.-J., Yin, J., Guo, D.-A., 2011. Biotransformation of bufadienolides by cell suspension cultures of *Saussurea involucrata*. Phytochemistry 72, 1779–1785.