Research Article

Transformation of artemisinin to deoxyartemisinin by *Aspergillus terricola* TISTR 3019

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Abstract

Artemisinin, a sesquiterpene lactone endoperoxide compound, was transformed using *Aspergillus terricola* TISTR 3019. The transformed product was isolated from the culture broth and identified as deoxyartemisinin (yield 21%). Investigation of the biological activities of deoxyartemisinin was carried out *in vitro* for both antimicrobial and cytotoxic activities. Deoxyartemisinin showed more effective than artemisinin in inhibitory of cell growth against human mouse melanoma B16F10 (ATCC-CRL-6475) cell line. However, the transformed product derived from *A. terricola* TISTR 3019 did not exhibit antimicrobial activities against *Salmonella typhimurium* TISTR 292, *Escherichia coli* TISTR 780, *Candida albicans* BCC 5390 and *Aspergillus niger* TISTR 3254.

Keywords: artemisinin, transformation, *Aspergillus terricola*, deoxyartemisinin, cytotoxicity, antimicrobial activity

Introduction

Artemisinin and its derivatives are well-known as antimalarial agents that are effective against multidrug-resistant *Plasmodium falciparum* (Meshnick, 2002). Artemisinin is a sesquiterpene lactone endoperoxide compound occurring in the herb *Artemisinin annua.* It has low solubility in both water and oil, leading to certain limitations in its clinical application (Meshnick et al., 1996). Microbial transformation has become the focus of attempts to modify structures either to enhance their biological activities or to develop new bioactive substances. This transformation has the ability to overcome the inaccessibility through chemical reaction, requires mild conditions, and produces less pollution (Loughlin, 2000). Additionally, this approach can also assist in predicting how drugs are metabolized in mammal species (Asha & Vidyavathi, 2009). Derivatives of artemisinin have been developed successfully via the microbial transformation approach in various studies. A number of these derivatives show considerable potential, particularly those transformed using fungi. For example, *Mucor polymorphosporus, Cunninghamella elegans* ATCC 9245, *Umbelopsis ramanniana,* and *Aspergillus niger* VKM F-1119 are able to transform artemisinin into 9β-hydroxyartemisinin which cannot be prepared

through a chemical approach (Parshikov et al., 2006; Parshikov et al., 2005; Parshikov et al., 2004; Zhan et al., 2002; Zhan et al., 2017). The hydroxygenated compounds, 9β-hydroxyartemisinin and 10β-9α-hydroxyartemisinin, demonstrate better anti-malarial activity and water solubility when compared with artemisinin (Avery et al., 1999; Parshikov, et al., 2005; Zhan, et al., 2017). Goswami et al. (2010) also revealed that *Penicillium simplissimum* transforms artemisinin into 3β-acetoxyartemisinin. This artemisinin derivative shows particularly inhibitory tendency toward colon HCT-15, lung A549, and neuroblastoma IMR-32 cell lines. Several fungi, such as *Penicillium chrysogenum* ATCC 9480, *M. polymorphosporus*, *Aspergillus flavus, A. niger* and *Rhizopus stolonifer*, show the ability to deoxygenate artemisinin (Parshikov, et al., 2005; Gaur et al., 2014; Lee et al., 1989; Srivastava et al., 2009). The deoxygenated artemisinin was found to exhibit higher antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Streptococcus mutans* compared to artemisinin (Srivastava, et al., 2009).

In this study, the transformation of artemisinin into its derivatives using *Aspergillus terricola* was assessed. *A. terricola* is a typical soil filamentous fungus, which can produce a wide range of enzymes such as inulase, diastase, invertase, maltase, alcoholoxydase, lipase, protease, amidase and xylanase (Michelin et al., 2011; Scales, 1914). *A. terricola* is a bioconverter in the transformation of bicyclo[3.2.0]hept-2-en-6-one to (−)-(1*S*, 5*R*)-lactone which is used for the synthesis of prostaglandins (Borges et al., 2009), and it is also capable of degrading endosulfan from contaminated soil (Hussain et al., 2007). *A. terricola* was selected for this study on account of the characteristics described. The first stage of the study involved the artemisinin transformation and was followed by *in vitro* tests for cytotoxicity and antimicrobial activities in order to evaluate the effect of modified-artemisinin on biological activities.

Materials and methods
Chemicals and reagents

Chemicals and reagents used for biotransformation process were methanol (Merck, Germany), ethyl acetate (Fisher Scientific, England), dichloromethane (Fisher Scientific, England), hexane (Carlo Erba, Italy). For *in vitro* cytotoxicity and antimicrobial assays involved the use of RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, trypsin-EDTA (Invitrogen, England), 4)-3,-5dimethylthiazol--2yl)-2,-5 diphenyltetrazolium bromide or MTT, dimethylsulfoxide (Sigma-Aldrich, USA), agar, nutrient agar or NA, Mueller Hinton agar or MHA (Criterion, USA), Luria-Bertani or LB, Sabouraud dextrose broth or SDB (Scharlau, Spain), potato dextrose broth or PDB (Difco, France), ampicillin and amphotericin B (Applichem, Germany).

Microorganism methods, maintenance, and preservation

Aspergillus terricola TISTR 3019, *Candida albicans* BCC 5390 and *Aspergillus niger* TISTR 3254 were maintained on potato dextrose agar (PDA) slants, while a slant of LB was

used for *Escherichia coli* TISTR 780 and a NA slant was used for *Salmonella typhimurium* TISTR 292.

Cell lines and cultivation

The mouse melanoma B16F10 and normal mouse fibroblast L929 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured at 37°C in an atmosphere of 5% carbon dioxide (Sanyo, Japan) in RPMI 1640 containing 10% (v/v) FBS and 1% penicillin (100 IU/mL) and streptomycin (100 mg/mL).

Biotransformation technique

The spore suspension of *A. terricola* TISTR 3019 was inoculated to SDB at final concentration 2×10^6 spore/mL and incubated at 37°C for 48 h on the 160 rpm rotary shaker (N-Biotek, Taiwan). The artemisinin at concentration 25 mg/mL was then introduced to the culture flask to achieve a final concentration of 0.5 mg/mL. The culture was grown for a further period of 96 h at 37°C on the rotary shaker. In the experiments, a transformed medium without the fungal culture was also prepared to use as the control. In the meantime, the substrate control was prepared under the same experimental conditions without adding artemisinin.

Isolation and identification of the transformed product

The cultured medium and mycelia were separated by filtration. The filtrate was extracted with ethyl acetate and evaporated *in vacuo* (Buchi, Switzerland). The residue was purified using column chromatography eluted with increasing proportions of methanol, which is, 1, 3 and 5% (v/v), respectively, in dichloromethane. Fractions containing the transformed product were collected and detected on TLC plates (Merck, Germany) using ethyl acetate/hexane (1:1 v/v) as the mobile phase. The TLC plate was developed with artemisinin reagent at a high temperature. The purified transformed product was further characterized by Mel-Temp melting point apparatus, IR, ESI-MS, ^{1}H-NMR, and ^{13}C-NMR. Deoxyartemisinin (yield = 21%), mp 110-111 °C, IR (KBr): 2941, 1748, 1385, 1140, 1016 cm^{-1}; ESI-MS *m/z*: 267.1617 $[M]^+$, calcd. for $C_{15}H_{23}O_4$: 267.3407; ^{1}H-NMR (400 MHz, CDCl$_3$) δ_H: 0.96 (3H, J = 5.6 Hz, H-14), 1.03 (1H, m, H-8a), 1.15 (1H, m, H-9a), 1.22 (3H, d, J = 7.2 Hz, H-13), 1.27 (1H, m, H-2a), 1.29 (1H, m, H-10), 1.30 (1H, m, H-1), 1.55 (3H, s, H-15), 1.65 (1H, m, H-3a), 1.77 (1H, m, H-3b), 1.78 (1H, m, H-9b), 1.92 (1H, m, H-2b), 1.95 (1H, m, H-8b), 2.03 (1H, m, H-7), 3.20 (2H, dq, H-11), and 5.71 (1H, s, H-5); ^{13}C-NMR (100 MHz, CDCl$_3$) δ_C: 12.8(C-13), 18.8(C-14), 22.3(C-2), 23.7(C-8), 24.2(C-15), 33.0(C-11), 33.7(C-9), 34.2(C-3), 35.6(C-10), 42.7(C-7), 44.9(C-1), 82.6(C-6), 99.9(C-5), 109.4(C-4), 172.1(C-12).

In vitro cytotoxicity assay

The effect of deoxyartemisinin on the growth of mouse melanoma B16F10 and normal mouse fibroblast L929 cell lines were determined by using MTT assay (Mosmann, 1983). The process required the seeding of cell lines at a 10,000 cell/well in 96-well plates (Corning, USA) followed by culturing in a 5% carbon dioxide atmosphere for 48 h at 37°C before washing in

phosphate buffer saline (PBS) at pH 7.4. The RPMI 1640 which contained concentrations of 0.0001 to 2.5 mg/mL artemisinin or deoxyartemisinin was introduced and incubated for a further 48 h, while 20 μL of MTT solution (100 μg/mL in PBS) was introduced to the individual well for incubation extending an additional 4 h. The formazan product was solubilized by the addition of 100 μL of dimethylsulfoxide to the medium containing sample. The reduction in the amount of formazan product by the mitochondrial dehydrogenase was determined by measuring in a microplate reader (Biotek, Taiwan) at absorbance at 550 nm. The cytotoxic dose (CD_{50}) of the transformed product was calculated and then compared with the value obtained from the untreated sample. All experiments were performed in triplicate and repeated two times in an independent experiment.

Antimicrobial assays

Agar disc methods were used to determine the antimicrobial activity of the transformed product (Bizani & Brandelli, 2002). The purified transformed product 18 mg was dissolved in 3 mL of 5% (v/v) dimethylsulfoxide, diluted at the final concentration of 1, 2 and 3 mg/mL, respectively. The diluted transformed product was applied on discs on MHA plates previously inoculated with a swab of *S. typhimurium* TISTR 292, *E. coli* TISTR 780 and *C. albicans* BCC 5390 which corresponded to 0.5 McFarland turbidity standard solution or a spot of *A. niger* TISTR 3254. All plates were incubated at 37°C for 24 h. The clear zones of inhibition were observed and the diameter of the clear zones were measured and compared with that of ampicillin and amphotericin-B.

Statistical analysis

Data values of cytotoxicity were given as mean ± standard deviation (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) carried out using SPSS 11.5 for windows (SPSS Inc, Chicago, USA). Scheffe and Tamhane tests were used as a post-hoc method to determine differences among the groups of LD_{50} or IC_{50}. Differences were considered statistically significant when $p < 0.05$.

Results and discussion
The isolation and identification of transformed product in the culture broth of *A. terricola* TISTR 3019

A. terricola TISTR 3019 was used in the production of artemisinin derivatives. The resulting transformed product was then extracted and purified from the culture broth for determining the chemical structure. The ESI-MS, ^{1}H-, and ^{13}C-NMR spectra suggesting that an oxygen atom had been removed from the endoperoxide position of artemisinin molecule. Further examination through the FT-IR spectrum found the important absorptions band of the transformed product were 2941 (C-H), 1748 (C=O), 1385 (C-H), 1140 and 1017 (C-O-C) cm^{-1}, which revealed the existence of lactone carbonyl group or C=O at υ_{max} 1748 cm^{-1} and absence of the C-O-O-C at υ_{max} 1117 cm^{-1}, which was presented in artemisinin. The resulting non-endoperoxide derivative had a melting point within the range from 110-111 °C. On the whole,

the transformed product was identified as deoxyartemisinin (Figure 1) which was reported to be the transformed product of artemisinin by *Nocardia coralline* ATCC 19070, *P. chrysogenum* ATCC 9480, *M. polymorphosporus*, *A. niger, A. flavus*, and *R. stolonifer* (Gaur, et al., 2014; Lee, et al., 1989; Parshikov, et al., 2005; Srivastava, et al., 2009). The yield of purified transformed product was 21% that was higher than deoxyartemisinin production by *N. coralline* ATCC 19070, *P. chrysogenum* ATCC 9480, *M. polymorphosporus*, *A. niger* and *R. Stolonifer* (Gaur, et al., 2014; Lee, et al., 1989; Parshikov, et al., 2005).
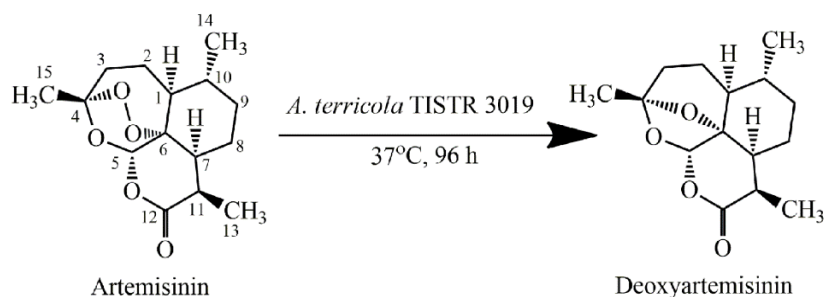
Figure 1. The biotransformation of artemisinin to deoxyartemisinin by *A. terricola* TISTR 3019.

In vitro cytotoxicity

To date, cytotoxicity for artemisinin and deoxyartemisinin towards mouse melanoma B16F10 and normal mouse fibroblast cell lines remain undetermined. Accordingly, the cytotoxicity of artemisinin and the deoxygenated artemisinin against mouse melanoma B16F10 and normal mouse fibroblast cell lines was evaluated by determining the reduction in mitochondrial dehydrogenase of the treated cells. An *in vitro* cytotoxicity study, deoxyartemisinin showed the cell growth inhibition at the CD_{50} value of 1.621 ± 0.095 mg/mL for normal mouse fibroblast L929 and 0.585 ± 0.058 mg/mL for mouse melanoma B10F16 cells (Table 1). Notably, deoxyartemisinin was shown to be 1.5 times less cytotoxic towards normal mouse fibroblast L929 cell lines than artemisinin, but considerably more effective in inhibiting the growth of mouse melanoma B16F10 cells (Table 1). These findings suggest that the endoperoxide bridge might not be essential for cytotoxicity in mouse melanoma B16F10 cell lines. This confirms the work of Beekman, who found that the presence of the endoperoxide in artemisinin and its derivatives have a minimal influence upon cytotoxicity in either the CFU-GM progenitor or EAT cells (Beekman et al., 1998). However, the cytotoxic mechanisms of artemisinin and deoxyartemisinin have not yet been precisely determined.

Antimicrobial assays

The capacity of deoxyartemisinin to inhibit Gram-positive bacteria, *S. aureus*, *S. epidermidis* and *S. mutans*, represented an improvement over artemisinin (Srivastava, et al., 2009). However, there remains a gap in the literature regarding the inhibitory effects of deoxyartemisinin against Gram-negative bacteria.   It was therefore of interest to assess the
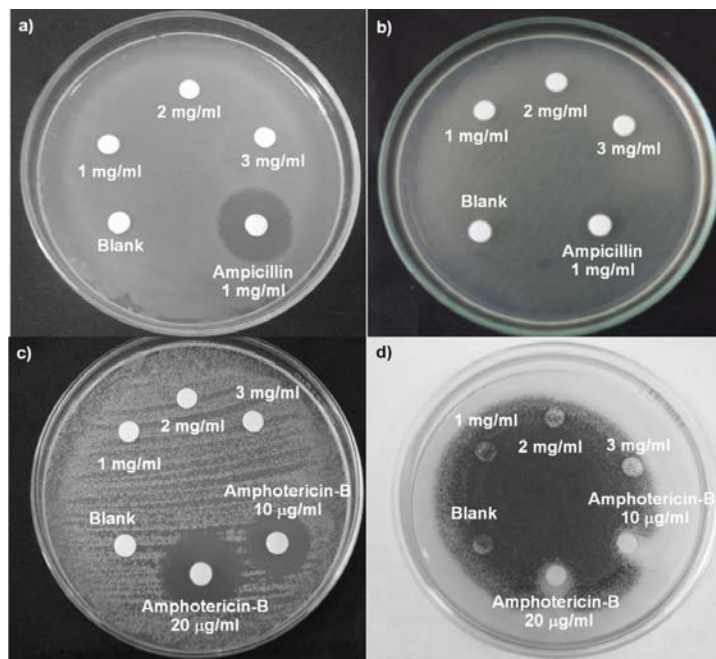
antimicrobial activity of deoxyartemisinin towards *S. typhimurium* TISTR 292 and *E. coli* TISTR 780 which is pathogenic Gram-negative bacteria. The results from this research demonstrated that deoxyartemisinin could not inhibit of *S. typhimurium* TISTR 292, *E. coli* TISTR 780, *C. albicans* BCC 5390 and *A. niger* TISTR 3254 for all concentrations up to 3 mg/mL (Figure 2).

Table 1. The *in vitro* cytotoxicity of deoxyartemisinin, artemisinin and doxorubicin against normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines

Compounds	CD_{50} (mg/mL)*	
	L929	**B16F10**
Artemisinin	1.108 ± 0.130^a	0.871 ± 0.070^a
Deoxyartemisinin	1.621 ± 0.095^b	0.585 ± 0.058^b
Doxorubicin	0.002 ± 0.000^c	0.001 ± 0.000^c

*Data are representative of mean±SD of triplicate experiments.

[a-c] Means within a column with different superscript are considered significantly different ($p < 0.05$).

Figure 2. Antimicrobial activity of deoxyartemisinin determined by agar disc diffusion assay against, *E.coli* TISTR 780 (a), *S. typhimurium* TISTR 292 (b), *C. albicans* BCC 5390 (c), and *A. niger* TISTR3254 (d).

Conclusion

The findings demonstrate that *A. terricola* TISTR 3109 was able to produce derivatives of artemisinin. The transformed product extracted from the culture broth was identified as deoxyartemisinin. The *in vitro* biological activity of deoxyartemisinin in terms of cytotoxicity activity indicated that deoxyartemisinin was significantly effective than artemisinin in B16F10 cell line. It was also found that the deoxygenation of artemisinin served to alter the cytotoxic capabilities of artemisinin towards the normal mouse fibroblast L929 and mouse melanoma B10F16 cell lines. Furthermore, the assessment of deoxyartemisinin on antimicrobial activity did not exhibit antimicrobial activities against *S. typhimurium* TISTR 292, *E. coli* TISTR 780, *C. albicans* BCC 5390 and *A. niger* TISTR 3254. In conclusion, the findings of this study have provided insights into a number of areas regarding the production and biological applications of deoxyartemisinin which the authors sincerely hope others will find to be of use and interest.

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