CHAPTER 3

MATERIALS AND METHODS

3.1 CHEMICALS

All chemicals used in this study were listed as follows:

Chemicals	Companies
Acetic acids	Carlo Erba
Acetone (Commercial grade)	
Agar	Criterion
Ampicillin	T.P Drug Laboratories
p-Anisaldehyde	Merck
Artemisinin	
Chloroform-D1	Merck
Dichloromethane (Commercial grade)	
Diethyl ether (analyzed grade)	RCI labscan limited
Dimethyl sulfoxide (DMSO)	Riedel-de Haën
3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	Sigma
Doxorubicin-HCl	EBEWE Pharma
Ethanol (Commercial grade)	
Ethyl acetate (Commercial gade)	
Fetal bovine serum	Gibco

Chemicals	**Companies**
Glucose	Fisher Scientific
Hexane (Commercial grade)	
[^{3}H] Hypoxanthine	Amersham
Methanol (Commercial grade)	
Mueller Hinton Agar (MHA)	Criterion
Nutrient Broth (NB)	Criterion
Penicillin-G	Sigma - Aldrich
Peptone	Becton Dickinson
Potassium bromide (KBr)	Fluka
Roswell Park Memorial Institute-1640 (RPMI-1640)	Gibco
Siliga gel 60 (230-400 mesh ASTM)	Merck
Sodium chloride	Lab-Scan
Sodium sulfate anhydrous	Fisher Scientific
Sulfuric acid	Merck
Tryptic soy broth (TSB)	Scharlau
Yeast extract	Criterion

3.2 MATERIALS AND INSTRUMENTS

All materials and instruments used in this study were listed as follows:

Materials and instruments	**Companies**
Antibiotic disc	Macherey - Nagel
Anti-pump	PYREX
Autoclave	All American
Beaker	ISOLAB
Evaporator	Bűchi
Freezer	Sanyo
Funnel	
Hot plate	Fisher - Scientific
Fourier transform infrared (FT-IR) spectrometer	Parkin Elmer
Incubator shaker	N -Biotek
Mass spectrometer	Bruker
Melting points apparatus	Laboratory device, inc.
Microplate reader spectrophotometer	N-Biotek
Micropipette	Eppendorf
Nuclear magnetic resonance (NMR) spectrometer	Bruker
NMR tube	Aldrich
Petri dish	Pyrex
pH meter	Trans Instruments
Separating funnel	Witeg

Materials and instruments	Companies
Thin Layer Chromatography plate	Merck
Top Count microplate scintillation counter	Packard
Vernior caliper	Macoh
Vortex	Scientific industries

3.3 MICROORGANISM STRAINS AND MAINTENANCE CONDITIONS

The fungal strains used for identifying the ability to transformed artemisinin were *Aspergillus niger* TISTR 3254, *Aspergillus usamii* TISTR 3258, *Aspergillus terricola* TISTR 3109 *Aspergillus melleus* TISTR 3128 and *Aspergillus oryzae* (Ozykat-1). All fungal strains were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), except *Aspergillus oryzae* (Ozykat-1) which was obtained from the Department of Biotechnology, Faculty of Science, Mahidol University. These fungal cultures were maintained on potato dextrose agar (PDA). After the fungi were inoculated, slant cultures were incubated at 37°C for 2 days and subsequently stored at 4°C.

The microorganisms used in the study of antimicrobial activity consisted of five pathogenic microbes. *Staphylococcus aureus* TISTR 1466, *Salmonella typhimurium* TISTR 292, *Escherichia coli* TISTR 780 and *Aspergillus niger* TISTR 3254 were purchased from the Thailand Institute of Scientific and Technological Research (TISTR). *Candida albicans* BCC 5390 was purchased from the BIOTEC Culture Collection of the National Center for Genetic Engineering and Biotechnology, Thailand. The bacteria were grown and maintained on nutrient agar (NA) slants for *S.*

aureus TISTR 1466 and on Luria Bertani agar (LB agar) slants for *S. typhimurium* TISTR 292 and *E. coli* TISTR 780, *C. albicans* BCC 5390 was cultured on Yeast-extract Malt-extract agar (YM agar) slants. The inoculated slants were incubated at 37^{o}C overnight before subsequently stored at 4°C.

3.4 CELL LINES AND CULTURE CONDITIONS

Mouse fibroblast L929 (ATCC-CCL-1), mouse melanoma B10F16 (ATCC-CRL-6475) and human lung carcinoma A549 (ATCC-CCL-185) cell lines were purchased from the Amarican Type Collection Culture of America (ATCC). Human colorectal adenocarcinoma Caco-2 and human colorectal adenocarcinoma HT-29 were kindly provided by Dr. Jeffrey Penny, School of Pharmacy and Pharmaceutical Science, Faculty of Medical and Human Sciences, The University of Manchester, England.

3.5 METHODS

3.5.1 Growth of fungi in the transformed medium

A transformed medium was prepared and autoclaved at 121^{o}C, 1.5 bar for 20 minutes before inoculation with a well-developed fungal mycelia. All cultures were incubated at 37^{o}C on a rotary shaker at a speed of 160 rpm. The cultures were harvested by filtration at 0, 12, 24, 36, 48, 72, 96, 120, 144, 168 and 192 hours. The pH of each filtrate was immediately determined by a pH meter. The fungal pellets were dried in a hot air oven to measure contents of the dried matter.

3.5.2 Biotransformation procedures

The fungi was grown on PDA slants and incubated at 37°C for 2 days. The well developed mycelia were inoculated against 50 ml of transformed medium in Erlenmeyer flasks. The pH of the media was adjusted to 5.6 before autoclaving. All cultures were incubated at 37°C for 2 days on the rotary shaker at a speed of 160 rpm. The fungal culture was used to transform artemisinin in one of the two following biotransformation procedures.

3.5.2.1 Single-stage biotransformation

Artemisinin 25 mg was dissolved in 1 ml methanol (MeOH) and added to a fungal culture, at the final concentration of 0.5 mg/ml. The fungal culture was further cultivated at 37°C at a speed of 160 rpm on the rotary shaker for 4 days. The transformation medium was separated by filtration through filter cloth supported with cotton, extracted with the equal volume of EtOAc, and then washed twice with half of the volume of DI water and saturated solution of sodium chloride (NaCl), respectively. The extract was dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated until dried in a vacuum. The extract was subjected to TLC using ethyl acetate and hexane (1:1) as mobile phase to preliminarily detect the transformed products. The spots were visualized by dipping in *p*-anisaldehyde and charring at higher temperature. The substrate control was run by adding the same amount of artemisinin to the flasks containing only the transformed medium. For the culture control, inoculation of fungal stains was added into the transformed medium without adding artemisinin. All controls were incubated under the same condition throughout the experiment.

3.5.2.2 Two-stage biotransformation

The fungi were cultured and incubated in 50 ml of transformation medium under the same condition as described in the section above. After 2 days of pre-incubation, the fungal pellets were separated and then transferred to 50 ml of 0.05 M phosphate buffer, pH 7.0. Artemisinin was added at the final concentration of 0.5 mg/ml to the cultures. The methods of extraction and detection of transformed products were the same as in section 3.5.2.1.

3.5.3 Purification of the transformed product

The ethyl acetate extract was purified by chromatography using a packed column of silica gel 60 (230-400 mesh ASTM) and then eluted by increasing proportion of MeOH from 1 to 5% in dichloromethane (DCM). The mobile phase was applied with a flow rate of 1 ml per minute. Then 3 ml fractions were collected and analyzed by TLC. The analysis was to identify fractions if any of them contain the purified transformed products. Fractions containing purified transformed products were pooled and dried *in vacuo*.

3.5.4 Chemical characterization of the transformed product

The purified transformed product was further identified and characterized by NMR, IR, high resolution electron spray ionization-mass spectrometer (HRESI-MS) and melting point apparatus.

3.5.4.1 Nuclear Magnetic Resonance (NMR) Spectra

^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Bruker AVANCE 400 NMR spectrometer operating at 400 and 100 MHz, respectively. Samples were dissolved in chloroform-D ($CDCl_3$).

3.5.4.2 Infrared (IR) Spectra

Infrared (IR) spectra were recorded using a Tender 27 FT-IR spectrometer (Perkin Elmer). Spectra of the transformed product were recorded as potassium bromide (KBr) pellets.

3.5.4.3 Mass Spectra (MS)

High resolution electron spray ionization (HRESI) mass spectra were recorded on a Bruker micrOTOF™ in positive mode using methanol as the solvent.

3.5.4.4 Melting point (m.p.)

To determine the melting point, a small amount of the purified transformed product was placed into a thin-welled capillary tube that was sealed at one end. The capillary was inserted into Mel-Temp II apparatus. Two temperatures were recorded: the temperature at which the substance start to liquefy and the temperature at which it had completely liquefied. The observed melting-point range was the interval between these two temperatures.

3.5.5 Screening of ability of fungi in biotransformation

A. niger TISTR 3254, *A.usamii* TISTR 3258, *A. terricola* TISTR 3109, *A. melleus* TISTR 3128 and *A. oryzae* (Ozykat-1) were used for screening their ability to transform artemisinin. The methods of biotransformation, extraction and detection of transformed products were described above in section 3.5.2 and 3.5.3. The fungi which were able to transform artemisinin were selected for further studies.

3.5.6 Optimization of biotransformation conditions

Biotransformation of artemisinin was conducted to determine the effect of temperature, transformed medium, artemisinin concentration, fungal pre-incubated period and incubation time on production of the transformed product. The methods of biotransformation, extraction and detection of the transformed products were described in section 3.5.2 and 3.5.3.

3.5.6.1 Effect of temperature on growth of fungi and production of the transformed product

The effect of temperature on biotransformation of artemisnin was carried out at three different temperatures, room temperature (26^{o}C), 30^{o}C and 37^{o}C using single-stage biotransformation. Artemisinin was added to a 2-day pre-incubated fungal culture. The conversion of artemisinin was monitored after 4 days. The suitable temperature achieved by this step was applied to subsequent experiments.

3.5.6.2 Effect of the transformed medium on production of the transformed product

Potato dextrose broth (PDB) and sabouraud dextrose broth (SDB) were used to study the effect of the transformed medium on the transformed product in single-stage biotransformation. For two-stage biotransformation, 0.05 M phosphate buffer with pH value of 7.0 was used for studying the effect on the transformed product. All procedures were described above in section 3.5.2. The optimum transformed medium achieved by this step was applied to subsequent experiments.

3.5.6.3 Effect of artemisinin concentration on growth of fungi and production of transformed product

To evaluate the effect of artemisinin concentration on growth of fungi in the transformed medium, artemisinin was dissolved in methanol and added to the transformed medium at varying concentrations of 0.5, 0.75 or 1.0 mg/ml. All procedures were similar to those described in section 3.5.1.

3.5.6.4 Effect of pre-incubation period on production o the transformed product

The different pre-incubation periods (lag, log and stationary phase) were studied for the biotransformation of artemisinin, other conditions were maintained at their obtained optimum conditions. The cultures of *Aspergillus* sp. were grown at 37 °C on SDA slants until conidial spores had developed. The spores were washed from the mycelia with the sterilized water and counted using haemacytometer under the microscope. The spore suspension was inoculated to the transformed medium at the final concentration 2×10^6 spore/ml. Artemisinin was added to the fungal cultures at lag, log or stationary phase of growth. The cultures were harvested every other day.

The methods of extraction and detection of the products were similar to those described in section 3.5.1 and 3.5.2.

3.5.7 Biological activity assays

The biological activity of transformed product was assayed according to antimalarial activity, antimicrobial and cytotoxicity assays.

3.5.7.1 Antimalarial activity assays

The multidrug resistant *P. falciparum* K1 strain was cultivated *in vitro* condition as described by Trager and Jensen, 1976. *P. falciparum* K1 was maintained in RPMI-1640 (Roswell Park Memorial Institute-1640) medium containing the mixtures of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 32 mM $NaHCO_3$ and 10% heat-activated human serum with 3% erythrocytes, in humidified 37°C incubator with 3% CO_2 (Trager and Jensen, 1976).

Antimalarial activity was determined by the method of Desjardins *et al*., 1979 using radioisotope techniques. A mixture of 200 µl of 1.5% erythrocytes suspension with 1% parasitemia at the early ring stage was added to 25 µl of the diluted transformed product or controls (the maximum concentration was 10 µg/ml) dissolved in 1% DMSO (0.1% final concentration). After 24 hours of incubation, 25 µl of [^{3}H] hypoxanthine (Amersham, USA) in the culture medium (0.5 µCi) was added to each well and then plates were incubated for another 24 hours. Levels of incorporated radioactive labeled hypoxanthine, indicating parasite growth, were determined using the microplate scintillation counter (Top Count, Packard,USA). The percentage of

parasite growth is calculated using the signal count per minute of treated (CPM_T) and untreated conditions (CPM_U), by the formula (1);

$$\text{Parasite growth (\%)} = \frac{CPM_T}{CPM_U} \times 100 \qquad (1)$$

The percentage of parasite growth was calculated as inhibition concentration (IC_{50}) which indicated 50% reduction in parasite growth. The positive controls were dihydroartemisinin and mefloquine and the negative control was 0.1% DMSO (Desjardins *et al*., 1979).

3.5.7.2 Antimicrobial activity assays

The antimicrobial activity of the transformed products was determined by the modified agar disc and well diffusion methods (Bizani and Brandelli, 2002; Kaushik and Chauhan, 2008). The transformed product was dissolved in 5% v/v DMSO, diluted at the final concentration 1, 2 and 3 mg/ml. For agar disc assay, the diluted transformed product was applied on discs on Mueller Hinton agar (MHA) plates previously inoculated with a swab of *S. aureus* TISTR 1466, *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. For agar well diffusion assay, the indicator strains, *S. aureus* TISTR 1466 and *E.coli* TISTR 780 which are corresponded to 0.5 McFarland turbidity standard solutions were inoculated to the sterile petri dishes and poured with Tryptic Soy Agar (TSA). After solidification, plates were punched to prepare the 6 mm well. The transformed product concentrations ranging from 1 to 3 mg/ml was added into each well. All plates were incubated at 37^{o}C for 24 hours. The clear zones of inhibition were

observed and the diameter of the clear zone were measured and compared with that of penicillin G, ampicilin and amphotericin-B.

3.5.7.3 Cytotoxicity assays

Cell viability was measured using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) which was based on the reduction of the soluble MTT by mitrochondrial reductase activity inside living cells (Carmichael *et al*., 1987). The effects of the transformed product on the growth of mouse fibroblast L929 (ATCC-CCL-1, normal cell), mouse melanoma B10F16 (ATCC-CRL-6475), human colorectal adenocarcinoma HT-29, human adenocarcinoma Caco-2 and human lung carcinoma A549 (ATCC CCL-185) cell lines were determined by MTT assay. A doxorubicin-HCl solution and an equivalent amount of DMSO were added as positive and negative controls.

All cell lines were maintained in RPMI-1640 medium containing 10% v/v fetal bovine serum and penicillin 100 IU/ml and streptomycin 100 mg/ml in an atmosphere of 5% CO_2. Cells were harvested by trypsinization and the number of viable cells was determined using a hemocytometer. Cells were placed in 96-well plates in a density of 1 x 10^4 cells per well and incubated at 37°C with 5% CO_2. After 48 hours of incubation, the media was removed, the cells were washed with phosphate buffer saline (PBS) and treated with RPMI 1640 containing with the transformed product (concentrations between 0.0001 and 2.5 mg/ml) or without the transformed product as a control for 48 hours at 37°C with 5% CO_2. Then, 20 µl of MTT solution in PBS was added and incubated for a further 4 hours. The medium was removed from each well. The formazan product was dissolved by 100 µl of DMSO and optical

density was measured using a microplate reader spectrophotometer at 550 and 620 nm (Mosmann, 1983). The percentage of cells viability was calculated using the absorbance at 550 nm (A_{550}) and 620 nm (A_{620}), by the formula (2);

$$\text{Cell viability (\%)} = \frac{A_{550}\,(\text{sample}) - A_{620}\,(\text{sample})}{A_{550}\,(\text{control}) - A_{620}\,(\text{control})} \times 100 \qquad (2)$$

The concentration resulting in 50% growth inhibition (IC_{50}) was used as a parameter for cytotoxicity. Analysis of IC_{50} values was carried out by using BioDataFit 1.02 (www.changbioscience.com/stat/ec50.html).

3.5.7.4 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 $p < 0.05$.