CHAPTER III

EXPERIMENTS

3.1 Fungal sample

Dry spore print specimens of *Psilocybe samuiensis*, collected from Koh Samui, Surat Thani Province, Thailand were provided from Allen, J.W. in July 2004. The spore print were used for cultivation in this study.

3.2 Culture media

Media used for cultivation were Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Yeast Extract Agar (YEA), Corn Meal Agar (CMA) and Sabouraud Dextrose Agar (SDA). They were used to study the growth rate of *Psilocybe samuiensis* for selection optimal growth media. The medium of cultivation for study metabolites was malt extract medium. Nutrient medium was used for growing bacteria. Yeast-malt extract medium (agar and broth) was used for growing yeasts.

The formula for media is shown in Appendix A.

3.3 Chemicals

3.3.1 Solvents

All commercial grade solvents used in this research, such as hexane, chloroform, dichloromethane, ethyl acetate and methanol, were distillated prior to use. The reagent grade solvents were used for thin-layer chromatography and crystallization.

3.3.2 Other chemcals

3.3.2.1 Merck's silica gel 60 Art. 1.09385.9025 (230-400 mesh ASTM) were used as adsorbent for normal column chromatography.

3.3.2.2 Merck's TLC aluminium sheet, silica gel $60F_{254}$ Art. 1.05554.0001 procoated 25 sheets, 20x20 cm^2, layer 0.2 mm was used to monitor the fractions and preparative TLC.

3.4 Instruments and equipments

3.4.1 Melting point apparatus

The melting points were recorded on a Fisher-Johns melting point apparatus.

3.4.2 Optical Rotation

The optical rotation values were measured by a Perkin-Elmer 341 polarimeter.

3.4.3 Ultraviolet - visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in chloroform and methanol.

3.4.4 Fourier Transform-Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 spectrophotometer. Spectra of solid samples were recorded as KBr pellets.

3.4.5 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 400 and 100 MHz, respectively, on a Varian Model Mercury 400 MHz in deuterated chloroform ($CDCl_3$), dimethylsulfoxide-d_6 (DMSO-d_6), methanol-d_4 (CD_3OD) and deuterium oxide (D_2O). Chemical shifts were reported relative to residual solvents peaks.

3.4.6 Mass Spectrometer (MS)

The high resolution mass spectra were performed by Mass Spectrometer LCT, Micromass UK Limited.

3.4.7 Rotary Evaporator

The Buchi rotary evaporator was used for the rapid removal of large amounts of volatile solvents.

3.5 Cultivation of *Psilocybe samuiensis*

The spores of *Psilocybe samuiensis* from spore print were streaked in Petri dishes containing Potato Dextrose Agar (PDA). The Petri dishes were incubated at room temperature (25-30°C) and examined for fungal mycelium germinates from spores. Outgrowing mycelia were purified by subculturing onto PDA plates. Pure mycelial cultures were checked under light microscope.

3.6 Characteristics of *Psilocybe samuiensis*

3.6.1 Macroscopic structure examination

Characters such as shape, size, color and others were observed.

3.6.2 Microscopic structure examination

3.6.2.1 Preparation of specimen for light microscope

Materials preparation for slide culture technique was done onto V-shape glass rod onto filter paper in Petri dishes. The materials was sterilized in autoclave. Malt Extract Agar was poured plate in sterile Petri dishes and the agar was cut into 1 x 1 x 0.3 cm size, then aseptically put on prepared slide glass and inoculated the culture of *Psilocybe samuiensis* on middle of four edges of agar, after that covered a piece of agar with cover slide and poured sterile distilled water for moisture inside the Petri dish, incubated at room temperature (25-30°C) until fungi grew onto glass slide and cover slip. Picture of slide culture preparation is shown in Figure 3.1.

Semipermanent slides (from slide culture) for light microscopy were mounted in lactophenol-cotton blue for observations of clamp connection. The microscopic mycelial structure was examined under light microscope model Olympus CH_2.

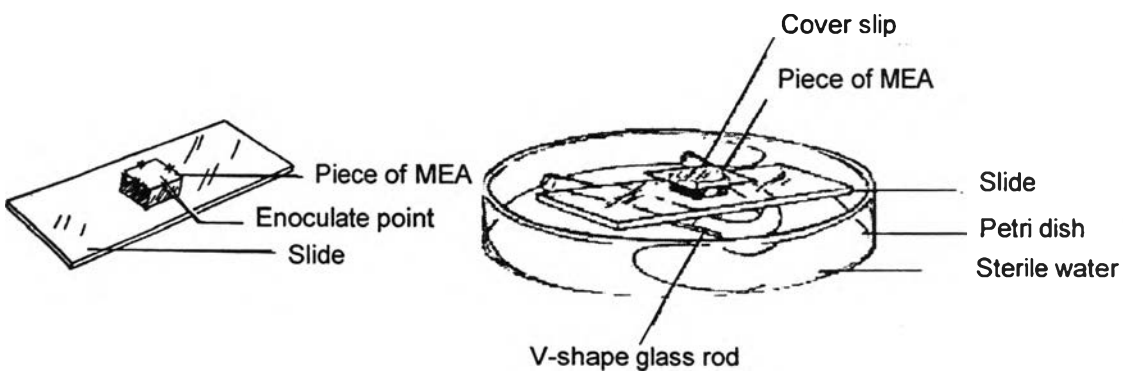


Figure 3.1 Slide culture preparation

3.6.2.2 Preparation of basidiospore for scanning electron microscope

The basidiospores print of *Psilocybe samuiensis* were fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodelate buffer (pH 7.2) for 2 hrs. The samples were then dehydrated under the series of ethanol concentration (70-95%) within 15 minutes. The sample were dried under critical point dried and coated with gold under sputter coater model. Changes of each fine immersed in absolute ethanol for 30 minutes for each twice and observed and photographed with a JSM-5410 LV scanning electron microscope.

3.6.3 Preparation of DNA *Psilocybe samuiensis* analysis

Genomic DNA was prepared from the fresh mycelial culture of *Psilocybe samuiensis* and extracted with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 50 µl which comprised approx. 100 ng genomic DNA, 1x PCR Master Mix (fermentas, Califonia, USA), and the primer ITS1f (Grades and Bruns, 1993) and ITS4 (White et al., 1990). The amplification was performed in a thermocycler (TGradient; Biometra, Germany) with 94°C for 5 min, followed by 38 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, with final extension of 72°C for 5 min. PCR Product was purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and sequenced externally by Macrogen (Seoul, Korea) using the same primer as for amplification.

The ITS sequence of *Psilocybe samuiensis* was submitted to GenBank with accession number AB257586. This sequence was also searched or compared with other fungal sequences using BLAST 2.1 at the web site http://ncbi.nml.nih.gov/BLAST/Blast.cgi.

3.7 Determination of media effect on growth rate

The mycelial culture of *Psilocybe samuiensi* were cut into 8 mm diameter of small cylinder by a flamed cork borer, and then placed onto follwing media; Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Yeast Extract Agar (YEA), Sabouraud Dextrose Agar (SDA) and Cornmeal Agar (CMA). The Petri dishes were

incubated at room temperature (25-30°C) for 2 weeks and recorded daily to compared size of colony of fungal in various media.

After the determination of suitable media for cultivation the mycelia of *Psilocybe samuiensis* were culture on MEA and incubated at room temperature for 2 weeks. Then the culture were cut at 5 mm from the edge of colony only one row around the colony by cork hole borer and inoculated 5 pieces of cultures into 100 ml Malt Extract Broth (MEB) contained in 250 ml Erlenmeyer flasks, incubated at room temperature. Two fermentation broth of *Psilocybe samuiensis* were filtered through filter paper (Whatman No. 93) every one week. The fungal mycelia were dried at 50°C for 48 hours in the oven and determined dry weight.

3.8 Cultivation and metabolite extraction of *Psilocybe samuiensis*

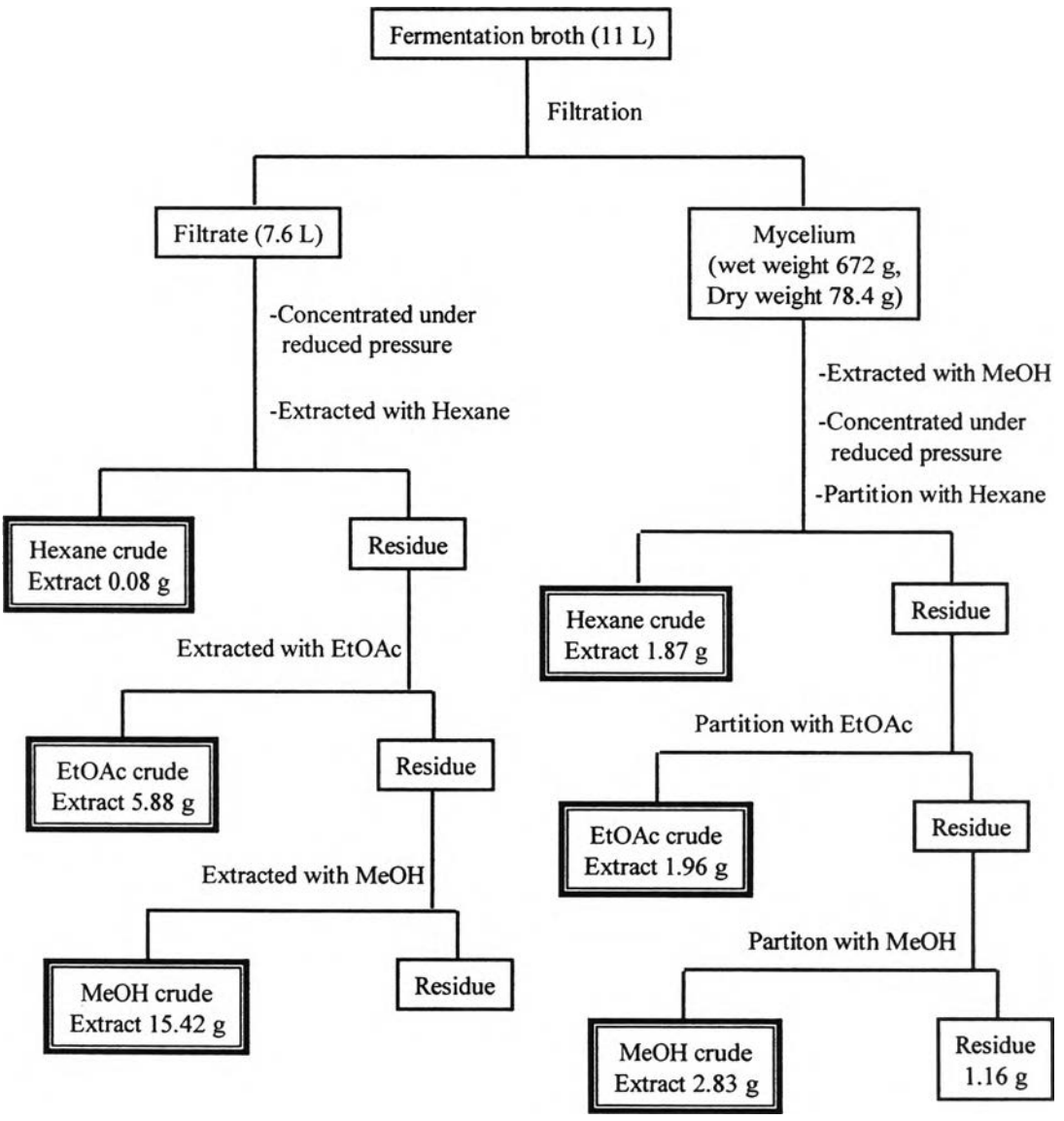
3.8.1 Cultivation

Psilocybe samuiensis inoculum was grown on MEA at room temperature (25-30°C) for 2 weeks. The agar was then cut into 8 mm diameter with a flamed cork hole borer. Five pieces of agar cultures were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MEB (x 110), and then statically incubated at room temperature for 11 weeks.

3.8.2 Metabolite extraction of *Psilocybe samuiensis*

The culture broth (11 L) was filtered through filter paper (Whatman No. 93). The filtrate was evaporated under reduced pressure at 35°C. The concentrated broth was extracted with 200 ml of hexane 5 times. The hexane layers were collected, dried over anhydrous sodium sulfate and then evaporated under reduced pressure at 35°C to yield 0.08 g of hexane extract as yellow viscous liquid. The residue broth was extracted again with 200 ml of ethyl acetate (EtOAc) 5 times. The ethyl acetate layers were collected, dried over anhydrous sodium sulfate and then evaporated under reduced pressure at 35°C to yield 5.88 g of EtOAc extract as yellowish brown viscous liquid. Finally the residue broth was extracted with 200 ml of methanol (MeOH) 5 times. The methanol layers were collected and then evaporated under reduced pressure at 35°C to yield 15.42 g of MeOH extract as a dark brown viscous liquid.

The mycelia were blended and extracted with 500 ml of MeOH (x 6) in ultrasonic bath. The methanol extract was partitioned with 100 ml of hexane (x5) followed by evaporation to yield 1.87 g of hexane crude extract as brown liquid. The residue of crude extract was partitioned again with 100 ml of EtOAc (x 5) and MeOH (x 5), to yield 1.96 g and 2.83 g of brown viscous liquid, respectively. The extraction of the culture broth and mycelium of *Psilocybe samuiensis* is shown in Scheme 3.1.



Scheme 3.1 Diagram of extraction of the fermentation broth and mycelia of *Psilocybe samuiensis*

3.9 Isolation of the chemical constituents from fermentation broth of *Psilocybe samuiensis*

3.9.1 Isolation of ethyl acetate crude extract

The ethyl acetate crude extract of *Psilocybe samuiensis* fermentation broth was obtained as yellowish brown viscous liquid. The crude extract (4.97 g) was chromatographed on silica gel using a mixture of dichloromethane and methanol (97:3 to 0:100). Fractions with similar components were combined according to the TLC profile. The results was shown in Table 3.1.

Table 3.1. The results from column chromatography of ethyl acetate extract of fermentation broth from *Psilocybe samuiensis*.

Eluents	Fraction No.	Appearance	Weight (g)
3% MeOH in CH_2Cl_2	1-6	Yellow viscous liquid	0.02
3% MeOH in CH_2Cl_2	7-8	Yellow viscous liquid	0.03
3% MeOH in CH_2Cl_2	9-20	Yellow viscous liquid	0.02
3% MeOH in CH_2Cl_2	21-23	Yellow viscous liquid	0.01
3% MeOH in CH_2Cl_2	24-41	Brown viscous liquid	0.17
3% MeOH in CH_2Cl_2	42-55	Yellow viscous liquid	0.04
3% MeOH in CH_2Cl_2	56-59	Brown viscous liquid	0.18
3% MeOH in CH_2Cl_2	60-100	Brown viscous liquid	0.11
3% MeOH in CH_2Cl_2	101-125	Yellow viscous liquid	0.02
3% MeOH in CH_2Cl_2	126-151	Brown viscous liquid	0.21
3% MeOH in CH_2Cl_2	152-164	Brown viscous liquid	0.13
3% MeOH in CH_2Cl_2	165-171	Yellowish Brown viscous liquid	0.05
3% MeOH in CH_2Cl_2	172-628	Yellow viscous liquid mix with white solid (containing compound **1** and Compound **2**)	0.96
3% MeOH in CH_2Cl_2	629-650	Brown viscous liquid	0.33
10% MeOH in CH_2Cl_2	651-692	Brown viscous liquid	0.52
20% MeOH in CH_2Cl_2	693-728	Brown viscous liquid	0.55
50% to 100% MeOH in CH_2Cl_2	729-735	Brown viscous liquid	0.60

The compound **1** was obtained from elution of 3% methanol in dichloromethane. The solvent was removed by rotary evaporation and the residue was obtained as white solid mixed with yellow viscous liquid (0.96 g). The residue was purified by crystallization in dichloromethane-methanol to give colorless crystals of compound **1**. The filtrate was further purified by silica gel column chromatography (Merck's silica gel 60 Art. 1.09385.9025), and crystallization to give compound **1** as colorless crystals (736 mg, yield 66.9 mg/L);

R_f : 0.38 (10% MeOH in CH_2Cl_2);

mp. 91-92°C;

FT-IR (KBr) ν_{max}: 3425-3352 (s), 2960 (s), 2927 (s), 2872 (s), 1140 (s), 1102 (s), 1043 (s) and 1013 (s) cm^{-1} (Fig. B1, Table 4.1);

$[\alpha]_D^{20}$ -48 (*c* 0.25, MeOH);

^{1}H-NMR ($CDCl_3$, 400 MHz) δ : 0.83 (3H, d, *J*=7.2 Hz, H-15), 0.91 (1H, m, H-7), 0.93 (1H, t, *J*=10 Hz, H-6), 1.13 (3H, s, H-13), 1.34 (3H, s, H-14), 1.13 (3H, s, H-13), 1.53 (1H, m, H-8a), 1.55 (1H, dd, *J*=6.8, 14.4, H-9a), 1.69 (1H, m, H-8b), 1.94 (1H, brs, H-1), 2.01 (1H, m, H-4), 2.09 (1H, m, H-5), 2.11 (1H, dd, *J*=12.8, 14 Hz, H-9b), 3.27 (1H, d, *J*=10.8 Hz, H-12a), 3.42 (1H, dd, *J*=11.6, 4.4 Hz, H-3a), 3.55 (1H, d, *J*=10.4, H-12b), 3.87 (1H, dd, *J*=11.6, 11.6 Hz, H-3b), and 5.30 (1H, d, *J*=2 Hz, H-2) ppm, (Fig. B2, Table 4.2);

^{13}C-NMR ($CDCl_3$, 100 MHz) δ : 11.4 (C-13), 14.5 (C-15), 18.4 (C-8), 21.5 (C-6), 24.2 (C-7), 26.5 (C-11), 30.4 (C-5), 31.6 (C-14), 35.6 (C-4), 38.6 (C-9), 56.6 (C-1), 61.8 (C-3), 73.4 (C-10), 73.4 (C-12), and 92.7 (C-2) ppm, (Fig. B3, Table 4.2);

HR/ES-TOF MS *m/z*: 293.1732 $[M+Na]^+$; $C_{15}H_{26}O_4Na$ calc 293.1729, (Fig. B8).

The filtrate from purification of compound **1** was obtained as yellow viscous liquid (179 mg), after removal of solvent. The residue was purified by preparative TLC (Merck's TLC aluminium sheet, silica gel $60F_{254}$ Art. 1.05554.0001) to give compound **2** as colorless oil (19 mg). Compound **2** was combined with two isomers in ratio 4:1. The characteristic of compound **2** was described below;

R_f = 0.55 (10% MeOH in CH_2Cl_2);

$[\alpha]_D^{20}$ -51 (*c* 0.07, $CHCl_3$);

FT-IR (KBr) ν_{max} 3397 (s), 2967 (s), 2931 (s), 2878 (s), 1129 (s), 1103 (s) and 1045 (s) cm^{-1} (Fig. B9, Table 4.3);

^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.77 (3H, d, *J*=6.8 Hz, H-15), 0.82 (1H, m, H-7), 0.84 (1H, m, H-6), 1), 1.08 (3H, s, H-13), 1.24 (3H, s, H-14), 1.47 (1H, m, H-8a), 1.47 (1H, m, H-9a), 1.64 (1H, m, H-8), 1.89 (1H, br s, H-1), 1.96 (1H, m, H-4), 1.96 (1H, m, H-5), 2.03 (1H, m, H-9b), 3.21 (1H, d, *J*=11.2 Hz, H-12a), 3.32 (1H, dd, *J*=11.6, 4.8 Hz, H-3a), 3.34 (3H, s, H-16), 3.51 (1H, d, *J*=11.2 Hz, H-12b), 3.64 (1H, dd, *J*=11.6, 11.6 Hz, H-3b) and 4.70 (1H, d, *J*=2.4 Hz, H-2) ppm, (Fig. B10, Table 4.4);

^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 11.5 (C-13), 14.4 (C-15), 18.45 (C-8), 21.9 (C-6), 24.3 (C-7), 26.3 (C-11), 30.8 (C-5), 31.7 (C-14), 35.6 (C-4), 38.6 (C-9), 55.3 (C-16), 56.6 (C-1), 61.8 (C-13), 73.4 (C-10), 73.9 (C-12) and 99.6 (C-2) ppm, (Fig.B11, Table 4.4);

HR/ES-TOF MS *m/z*: 307.1890 $[M+Na]^+$; $C_{16}H_{28}O_4Na$ calc 307.1885, (Fig. B16).

3.9.2 Methoxylation of compound 1

Solution of compound **1** (21.1 mg, 0.078 mmol) in methanol (3 ml) was added 1 mg of *p*-toluenesulfonic acid (0.005 mmol). The resulting solution was then stirred for 1 h at room temperature. Water was added to the reaction mixture, follow by extraction with dichloromethane (3 times). The organic layer was dried (Na_2SO_4) and evaporated. The crude extract was purified by preparative-TLC (Merck's TLC aluminium sheet, silica gel $60F_{254}$ Art. 1.05554.0001) to yield 14 mg of compound **2**. The spectral data (^{1}H and ^{13}C NMR spectra) of compound **2** from this reaction were similar to compound **2** from the filtrate of compound **1**.

3.10 Test of *Psilocybe samuiensis* chemical constituents on antimicrobial activity and cytotoxicity

3.10.1 Antimicrobial activity test

Microorganisms used for antimicrobial activity assay were *Bacillus subtilis* ATTC 6633, *Staphyllococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853. Pure colonies bacteria were inoculated into 5 ml of Nutrient Broth (NB) and inoculated at 37°C for 24 h. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFerland. *Candida albicans* ATTC 10231 were grown on Yeast-malt Extract Broth (YEM) and incubated at room temperature for 24 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFerland.

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess moisture. The agar was inoculated by streaking the swab across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. 2 mg of compound **1** was dissolved in 1 ml of 10% DMSO in sterile distilled water. Streptomycin and Captan (1μg/ml) were used as positive control for bacteria and yeast, respectively. Then sterile paper disc (size 6.0 mm,Whatman) were placed on microorganism test plate. 100 μl of compound **1** (2 mg/ml) was pipetted onto paper dics. Microoganism test plates were incubated at 37°C for 24 h, followed by observation the clear zone.

3.10.2 Cytotoxicity test

Cytotoxicity test were carried out at the Institute of Biotechnology and Genetic Engineering. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT colorimetric method (Carmichael *et al.*, 1987). In Principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubilization, and could be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed into replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37°C, 5% CO_2, 100% relative humidity, 100 μl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of

each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ ml PBS was sterilized and filtered through 0.45-μl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well, resulting in 50 μl MTT/ 250 μl total medium volumes; and cultures were incubated at 37 °C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms of mean (+/- 1 SD) absorbance units and / or percentage of control absorbance (+/- 1 SD %) following subtraction of mean "background" absorbance.

Samples were also tested for cytotoxic activity towards 5 cell lines, which contain HEP-G2 (hepatoma), SW620 (colon), Chago (lung), KATO-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cyototoxic activity.