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Antitumor Activity of Arylacetylshikonin Analogues

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Twenty one phenylacetylshikonin analogues were synthesized from various substituted phenyl acetic acids and their cytotoxicity values against A549, K562 and L1210 cell lines and antitumor action in mice bearing S-180 cells were measured. All of phenylacetylshikonin analogues expressed a potent cytotoxicity (ED_{50}, 0.1-1.80 μg/ml) against L1210 and K562 cells. L 1210 cells were the most sensitive to shikonin analogues among these cells. Except 4-methoxyphenylacetylshikonin (0.098 μg/ ml) and α-acetoxyphenylacetylshikonin (0.10 μg/ml), all other shikonin derivatives showed higher ED_{50} values than phenylacetylshikonin (0.13 μg/ml) in L 1210. In K562 cell, α-substitution of phenylacetylshikonin (0.1 μg/ml), while other substitutions increased it slightly; 4-methoxyphenylacetylshikonin (0.033 μg/ml) showed a exceptionally good cytotoxicity against K562 cell. 4-Halogenation tended to decrease the cytotoxic effect on L1210 cells, while it enhanced the effect on K562; 4-bromophenylacetyl [ED_{50} (L1210)=1.76 μg/ml, ED_{50} (K 562)=0.32 μg/ml] and 4-chlorophenylacetyl shikonin [ED_{50} (L 1210)=1.64 μg/ml, ED_{50} (K562)=0.32 μg/ml]. In contrast, A549 cells were much less sensitive to these shikonin analogues which showed ED_{50} values of 1.5-13.5 μg/ml. Most of phenylacetylshikonin derivatives showed good antitumor activity in mice bearing S-180 cells. α-Acetoxyphenylacetylshikonin and 4-dimethylaminophenylacetylshikonin showed highest T/C value (192-195%), implying that introduction of α-acetyl or of 4-dimethyl amino group gave a positive effect on the antitumor activity. Introduction of 4-dimethylamino group enhanced the antitumor activity as shown for 4-dimethylaminophenylacetylshikonin (T/C, 192%). It might be due to improvement of water solubility by dimethylamino group in the molecule.

Key words : Arylacetylshikonin, Synthesis, Cytotoxicity, Antitumor activity

INTRODUCTION

Shikonin, isolated from the root of *Lithospermum erythrorhizon*, has a wide spectrum of pharmacological activity (Hayashi, 1977a; 1977b; Shukla *et al.*, 1971; Papa georgiou, 1978) such as antiinflammatory, antibacterial (Kyogoku *at al.*, 1973; Papageorgiou *et al.*, 1979), or antimutagenic activity (Tikkanen, 1983), and are also used as raw materials for dyes and cosmetics (Papageorgiou *et al.*, 1979). Acetylshikonin showed a good cytotoxic activity against L1210 cells and prolonged the life span of ICR mice bearing Sarcoma 180 cells in the peritoneal cavity (Sankawa *et al.*, 1977; 1981; Kim and Ahn, 1990). Ahn and coworkers (Ahn and Baik, 1995) isolated substances from the *Lithospermi* root showing strong inhibitory effect on DNA topoisomerase-I and identified the structure as shikonin and acetylshikonin. It was found that acetylshikonin showed a better inhibitory effect than shikonin. Therefore, they synthesized a number of acylshikonin analogues and analyzed the structure-activity relationship. As a result, it was concluded that the introduction of acyl groups with chain lengths of C_2-C_5 potentiated the inhibitory effect. In a further study (Ahn *et. al.*, 1996), it was found that acylshikonin analogues with long acyl chain such as hexanoyl and decanoyl group showed much higher T/C values than acetylshikonin in mice bearing S-180 cell. These observations stimulated us to synthesize arylacetylshikonin derivatives and to evaluate the role of phenyl group for their antitumor activity. For this purpose, we have synthesized 21 phenylacetylshikonin derivatives and measured their cytotoxicity against L1210, K562 and A549 cells, and antitumor action in animals bearing S-180 ascites tumor cells.

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MATERIALS AND METHODS

Chemical reagents were obtained from Aldrich Chemical Company. All other solvents were of reagent grade and used without further purification. L

1210, K562 and A549 cells were obtained from Korea Institute for Chemical Technology. RPMI 1640, Fetal bovine serum and other reagents used for cell culture were purchased from Gibco Co.

Melting point was determined on an Electrothermal melting point apparatus and was not corrected. IR spectra were recorded on a Jasco Report-100 IR spectrometer. Proton NMR spectra were recorded on a Varian-Gemini 200 MHz spectrometer using tetramethylsilane as an internal standard. Analytical thin layer chromatography was performed on plastic sheet (0.2 mm) precoated with silica gel 60 F254 (E. Merck). Silica gel 60 (70-230 mesh, E. Merck) was used for column chromatography.

Cytotoxic activity against L1210 and K562 tumor cells

Cytotoxicity was measured against L1210 and K562 cells *in vitro* using the known method (Thayer *et al*, 1971). Fisher's medium supplemented with horse serum in 10% was used for the proliferation of L1210 cells. RPMI medium enriched with fetal bovine serum in 5% was used for the proilferation of K562 cells. Cell numbers were counted using a hemacytometer, and ED_{50} value was defined as the concentration of drug to produce a 50% reduction in viability relative to the control in three independent experiments.

Cytotoxic activity against A549 tumor cells

The *in vitro* cytotoxic activity against A549 tumor cells were determined using sulforhodamine B (SRB) method (Rubinstein *et al.*, 1990; Skehan *et al.*, 1990); cultures fixed with trichloroacetic acid were stained with 0.4% sulforhodamine B dissolved in 1% acetic acid, unbound dye was removed with acetic acid, and protein-bound dye was extracted with 10 mM Tris base for the determination of optical density at 520 nm. The ED_{50} value (μg/ml), the concentration of compound which inhibits the growth of tumor cells by 50%, was calculated using a data regressive function of the optical density in LOTUS program. Each value is the average ±SEM of triplicate experiments.

Antitumor activity in ICR mice bearing Sarcoma 180 cells

Sarcoma 180 cells (0.1 ml per mouse) suspended in saline (1×10^7 cells/ml) were inoculated intraperitoneally (National Cancer Institute USA,1972). After 24 hours from the transplantation, mice were divided so that each group contains 8 mice. The test sample dissolved in a predetermined amount of dimethylsulfoxide was stored at 4°C. The schedule for injection comprises a total of 7 injections in the manner that after the transplantation of cancer cells 0. 1 ml of the test sample per a day is administered for 2 days followed by the rest for one day. The survival rate was calculated on the basis of the date on which all of one (control group died after approximately 17 days). The survival rate (T/C, %) was calculated by the following equation as proposed in NCI's protocol;

$$\text{Survival rate (T/C, \%)} = \frac{\text{Average survival period in the test group}}{\text{Average survival period in the control group}} \times 100$$

General synthetic method

Shikonin (1 mmole), dicyclohexylcarbodiimide (1.1 mmole) and 4-dimethylaminopyridine (0.25 mmole) were dissolved in 3 ml of dichloromethane, and the solution was put into a 25 ml flask and cooled to 0°C. Organic acid (1 mmole) was added in small portions under stirring, and the mixture was stirred for 30 min. After further stirring for 3 h at room temperature, 20 ml of hexane was added, and insoluble part was filtered off. The hexane solution was evaporated to a residue, which was recrystallized or column chromatographed to give a pure substance (Fig. 1).

Shikonin; 2-(1-hydroxy-4-methylpent-3-enyl)-5,8-dihydroxy-1,4-naphthoquinone: mp 135-137°C, IR V_{max} (KBr); 3400 (OH), 2905, 1600 (C=O), 1580 (C=C), 1450 ($-CH_3$), ^{1}H-NMR ($CDCl_3$) δ; 12.06 (1H, s, OH at C-8), 12.49 (1H, s, OH at c-5), 7.20 (2H, s, H-6,H- 7), 7.17 (1H, d, J=1.1 Hz, H-3), 5.21 (1H, dd, J=8.6 & 8.0 Hz, H-3'), 4.92 (1H, m, H-1'), 2.64 (1H, m, H-2'), 2.38 (1H, s, OH at C-1'), 2.36 (1H, m, H-2'), 1.76 (3H, s, $-CH_3$ at C-5'), 1.66 (3H, s, $-CH_3$ at C-6').

^{13}C-NMR ($CDCl_3$) δ; 180.4 (C-4), 179.6 (C-1), 165.6 (C-5), 164.9 (C-8), 151.5 (C-2), 137.2 (C- 4'), 132.6 (C-6, C-7), 131.9 (C-3), 18.5 (C-3'),112.0 (C-9), 111.5 (C-10), 68.3 (C-1'), 35.6 (C-2'), 25.9 (C-6'), 18.0 (C-5')

Phenylacetylshikonin; 2-[(1-phenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2990, 2960, 1730 (O-C=0), 1605 (C=O), 1445 (CH_3). ^{1}H-NMR ($CDCl_3$) δ 12.55 (1H, s, -OH at C-8), 12.38 (1H, s, -OH at C-5), 7.45-7.30 (5H, m, -phenyl), 7.17 (2H, s, H-6, H-7), 6.78 (1H, s, H-3), 6. 02 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.69 (2H, s, H-2''), 2.48 (2H, m, H-2'), 1.64 (3H, s, $-CH_3$ at C-5'), 1. 53 (3H, s, $-CH_3$ at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.2 (C-4), 175.7 (C-1), 170.2 (C-1''), ^{13}C-NMR ($CDCl_3$) δ;

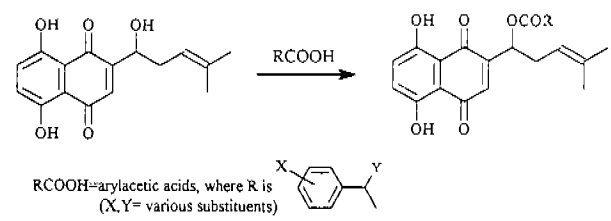
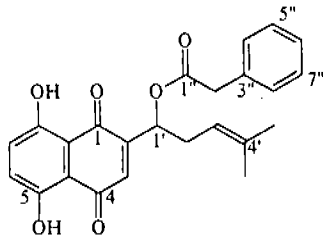


Fig. 1. Synthesis of Arylacetylshikonin Analogues



177.9 (C-4), 176.5 (C-1), 170.2 (C-1"), 167.4 (C-5), 166.9 (C-8), 147.9 (C-2), 136.0 (C-4'), 133.4 (C-3"), 132.8 (C-6), 132.6 (C-7), 131.3 (C-3), 129.2 (C-5", C-7"), 128.6 (C-4", C-8"), 127.3 (C-6"), 117.5 (C-3'), 111.7 (C-9), 111.5 (C-10), 69.7 (C-1'), 41.5 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 57%

3,4,5-Trimethoxyphenylacetylshikonin; 2-[1-(3,4,5-trimethoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2920, 1725 (O-C=O), 1600 (C=O), 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.55 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.17 (2H, s, H-6, H-7), 6.78 (1H, s, H-3), 6.49 (2H, s, H-4", H-8"), 6.02 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.81 (9H, s, -OCH_3 at C-5", C-6", C-7"), 3.60 (2H, s, H-2"), 2.48 (2H, m, H-2'), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) d; 177.2 (C-4), 175.7 (C-1), 170.2 (C-1"), 168.2 (C-5), 167.7 (C-8), 153.3 (C-2), 147.7 (C-5", C-7"), 137.1 (C-4'), 136.1 (C-3"), 133.2 (C-8), 132.9 (C-7), 131.1 (C-3), 128.9 (C-6"), 117.5 (C-3'), 111.7 (C-9), 111.4 (C-10), 106.2 (C-4", C-8"), 69.9 (C-1'), 60.8 (C-9", C-11"), 56.1 (C-10"), 41.7 (C-2"), 32.7 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 49%

4-Biphenylacetylshikonin; 2-(2-biphenylacetoxy-4-methylpent-3-enyl)-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2920, 1730, 1600, 1445, ^{1}H, -NMR ($CDCl_3$) δ; 12.56 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.7-7.55 (4H, m, H-4", H-5", H-7", H-8"), 7.5-7.4 (2H, m, H-3"', H-5"'), 7.4-7.3 (3H, m, H-2" ', H-4"', H-6"'), 7.17 (2H, s, H-6, H-7), 6.78 (1H, s, H-3), 6.02 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.62 (2H, s, H-2"), 2.48 (2H, m, H-2'), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.7 (C-4), 176.2 (C-1), 170.2 (C-1"), 167.8 (C-5), 167.2 (C-8), 147.8 (C-2), 140.7 (C-3"), 140.3 (C-1"'), 136.1 (C-4'), 132.9 (C-6), 132.7 (C-7), 132.4 (C-5", C-7", C-3"', C-5" '), 131.3 (C-3), 129.6 (C-2"', C-5"'), 128.7 (C-4", C-8"), 127.4 (C-4"'), 127.1 (C-6"), 117.6 (C-3'), 111.7 (C-9), 111.5 (C-10), 69.8 (C-1'), 41.1 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 41%

4-Brophenylacetylshikonin;2-[1-(4-bromophenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: mp: 99-102.5°C, Red crystaline precipitate from Ethylacetate-Hexane, IR V_{max} (KBr); 2950, 2910, 1720, 1600, 1440, ^{1}H-NMR ($CDCl_3$) δ; 12.55 (1H, s, -OH at C-8), 12.38 (1H, s, -OH at C-5), 7.52-7.20 (4H, m, H-4", H-5", H-7", H-8"), 7.16 (2H, s, H-6, H-7), 6.84 (1H, s, H-3), 6.04 (1H, m, H-1'), 5.02 (1H, m, H-3'), 3.65 (2H, s, H-2"), 2.56-2.40 (2H, m, H-2'), 1.66 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) d; 177.3 (C-4), 175.7 (C-1), 170.1 (C-1"), 168.2 (C-5), 167.7 (C-8), 147.6 (C-2), 136.1 (C-1'), 133.1 (C-1), 132.9 (C-7), 132.0 (C-3"), 131.6 (C-5", C-7"), 131.1 (C-3), 131.0 (C-4", C-8"), 121.4 (C-6"), 117.5 (C-3'), 111.7 (C-9), 111.5 (C-10), 69.9 (C-1'), 40.4 (C-2"), 32.7 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 56%

4-Chlorophenylacetylshikonin; 2-[1-(4-chlorophenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2910, 1720, 1600, 1440, ^{1}H-NMR ($CDCl_3$) δ; 12.46 (1H, s, -OH at C-8), 12.29 (1H, s, -OH at C-5), 7.20-7.17 (4H, m, H-4", H-5", H-7", H-8"), 7.08 (2H, s, H-6, H-7), 6.76 (1H, s, H-3), 5.95 (1H, m, H-1'), 4.95 (1H, m, H-3'), 3.58 (2H, s, H-2"), 2.41 (2H, m, H-2'), 1.58 (3H, s, -CH_3), 1.47 (3H, s, -CH_3), ^{13}C-NMR ($CDCl_3$) d; 177.3 (C-4), 175.7 (C-1), 169.8 (C-1"), 168.1 (C-5), 167.6 (C-8), 147.6 (C-2), 136.2 (C-4'), 133.3 (C-6"), 133.1 (C-6), 132.9 (C-7), 131.8 (C-3"), 131.2 (C-3), 130.6 (C-4", C-8"), 128.8 (C-5", C-7"), 117.5 (C-3'), 111.7 (C-9), 111.5 (C-10), 69.9 (C-1'), 40.7 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 52%

4-Ethoxyphenylacetylshikonin; 2-[1-(4-ethoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2960, 2910, 1730, 1600, 1450, 1235, 1040, ^{1}H-NMR ($CDCl_3$) δ; 12.54 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.264-6.78 (4H, m, H-4", H-5", H-7", H-8"), 7.14 (2H, s, H-6, H-7), 6.88 (1H, s, H-3), 6.00 (1H, m, H-1'), 5.05 (1H, m, H-3'), 4.03 (2H, q, J=6.32 Hz, -OCH_2CH_3) 3.62 (2H, s, H-2"), 2.48 (2H, m, H-2'), 1.65 (3H, s, -CH_3 at C-5'), 1.54 (3H, s, -CH_3 at C-6'), 1.41 (3H, t, J=6.32 Hz, -OCH_2CH_3), ^{13}C-NMR ($CDCl_3$) δ; 177.9 (C-4), 176.5 (C-1), 170.5 (C-1"), 167.5 (C-5), 166.9 (C-8), 158.3 (C-6"), 148.0 (C-2), 135.9 (C-4'), 133.1 (C-6), 132.8 (C-7), 131.4 (C-3), 130.4 (C-4", C-8"), 125.3 (C-3"), 117.6 (C-3'), 114.7 (C-5", C-7"), 111.8 (9-C), 111.5 (10-C), 69.6 (C-1'), 63.4 (C-9"), 40.2 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), 14.0 (C-10"), Yield;47%

2,6-Dichlorophenylacetylshikonin; 2-[1-(2,6-dichlorophenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2905, 1735, 1600, 1440, ^{1}H-NMR ($CDCl_3$) δ; 12.54 (1H, s, -OH at C-8), 12.40 (1H, s, -OH at C-5), 7.42-7.19 (3H, m, H-5", H-6", H-7"), 7.16 (2H, s, H-6, H-7), 6.90 (1H, s, H-3), 6.05 (1H, m, H-1'), 5.05 (1H, m, H-3'), 4.10 (2H, s, H-2"), 2.50 (2H, m, H-2'), 1.67 (3H, s, -CH_3 at C-5'), 1.52 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.8 (C-4), 176.3 (C-1), 168.1 (C-1"), 167.6 (C-5), 167.1 (C-8), 147.8 (C-2), 136.0 (C-4'), 135.9 (C-4", C-8"), 132.8 (C-6), 132.6 (C-7), 131.4 (C-3"), 130.8 (C-3), 129.1 (C-6"), 128.1 (C-5", C-7"), 117.5 (C-3'), 111.7 (C-9), 111.5 (C-10), 70.2 (C-1'), 36.8 (C-2"), 32.8 (C-

2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 49%

4-Methoxyphenylacetylshikonin; 2-[1-(4-methoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2920, 1730, 1600, 1445, 1240, 1030, ^{1}H-NMR ($CDCl_3$) δ; 12.54 (1H, s, -OH at C-8), 12.38 (1H, s, -OH at C-5), 7.26-6.76 (4H, m, H-4",H-5", H-7", H-8"), 7.16 (2H, s, H-6, H-7), 6.89 (1H, s, H-3), 6.00 (1H, m, H-1'), 5.04 (1H, m, H-3'), 3.81 (3H, s, -OCH_3 at C-6"), 3.63 (2H, s, H-2"), 2.51 (2H, m, H-2'), 1.66 (3H, s, -CH_3 at C-5'), 1.54 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.9 (C-4), 176.4 (C-1), 170.5 (C-1"), 167.5 (C-5), 167.0 (C-8), 158.9 (C-6"), 147.9 (C-2), 135.9 (C-4'), 132.8 (C-6), 132.6 (C-7), 131.4 (C-3), 130.2 (C-4", C-8"), 125.5 (C-3"), 117.7 (C-3'), 114.1 (C-5", C-7"), 111.8 (C-9), 111.5 (C-10), 69.7 (C-1'), 55.3 (C-9"), 40.6 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 52%

2,5-Dimethoxyphenylacetylshikonin; 2-[1-(2,5-dimethoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: mp 101-104°C, IR V_{max} (KBr); 2950, 2920, 1735, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.55 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.17 (2H, s, H-6, H-7), 6.78 (1H, s, H-3), 6.84-6.75 (3H, m, H-4", H-6", H-7"), 6.02 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.76 (3H, s, -OCH_3 at C-4"), 3.75 (3H, s, -OCH_3 at C-10"), 3.65 (2H, s, H-2"), 2.48 (2H, m, H-2'), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 178.6 (C-4), 177.1 (C-1), 170.4 (C-1"), 166.9 (C-5), 166.4 (C-8), 153.3 (C-7"), 151.6 (C-4"), 148.4 (C-2), 135.8 (C-4'), 132.6 (C-6), 132.5 (C-7), 131.5 (C-3), 123.4 (C-3"), 117.7 (C-3'), 117.3 (C-5"), 112.8 (C-8"), 111.8 (C-9), 111.5 (C-10), 111.1 (C-6"), 69.7 (C-1'), 55.8 (C-10"), 55.7 (C-9"), 36.4 (C-2"), 32.7 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 49%

α-Methoxyphenylacetylshikonin; 2-[1-(α-methoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: mp 70-72°C, IR V_{max} (KBr); 2960, 2920, 1750, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ: 12.40 (1H, s, -OH at C-8), 12.22 (1H, s, -OH at C-5), 7.45-7.36 (5H, m, H-4", H-5", H-6", H-7", H-8"), 7.14 (2H, s, H-6, H-7), 6.26 (1H, s, H-3), 6.05 (1H, m, H-1'), 5.06 (1H, m, H-3'), 4.84 (1H, s, H-2"), 3.43 (3H, s, -OCH_3 at C-6"), 2.45 (2H, m, H-2'), 1.59 (3H, s, -CH_3 at C-5'), 1.49 (3H, s, -CH3 at C-6'), ^{13}C-NMR ($CDCl_3$) **d**: 177.7 (C-4), 176.3 (C-1), 169.2 (C-1"), 167.5 (C-5), 166.9 (C-8), 147.3 (C-2), 136.2 (C-3"), 135.7 (C-4'), 132.9 (C-6), 132.6 (C-7), 131.0 (C-3), 129.2 (C-5"), 128.8 (C-6"), 127.2 (C-4"), 117.5 (C-3'), 111.7 (C-9), 111.4 (C-10), 82.4 (C-2"), 69.9 (C-1'), 57.3 (C-9"), 32.8 (C-2'), 25.7 (C-6'), 17.9 (C-5'), Yield; 45%

4-Isobutyl-α-methylphenylacetylshikonin; 2-[1-(4-isobtyl-α-methylphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2945, 2910, 1730, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ: 12.44 (1H, s, -OH at C-8), 12.26 (1H,s, -OH at C-5), 7.18-7.02 (4H, m, H-4", H-5", H-7", H-8"), 7.06 (2H, s, H-6, H-7),.6.37 (1H, s, H-3), 5.90(1H, m, H-1'), 5.00 (1H, m, H-3'), 3.68 (1H, q, J=7.14 Hz, H-2"), 2.45 (2H, d, J =6.94 Hz, H-10"), 2.48 (2H, m, H-2'), 1.96 (1H, m, H-11"), 1.60 (3H, s, -CH_3 at C-5'), 1.48 (3H, s, -CH_3 at C-6'), 1.44 (3H, d, J=5.35 Hz, -CH_3), 0.83 (6H, d, J=6.38 Hz, -CH_3, at C-11, C-12), ^{13}C-NMR ($CDCl_3$) δ: 178.1 (C-4), 176.8 (C-1), 173.3 (C-1"), 167.2 (C-5), 166.6 (C-8), 148.0 (C-2), 141.0 (C-6"), 136.9 (C-3"), 135.9 (C-4'), 132.7 (C-6), 132.4 (C-7), 131.2 (C-3), 129.5 (C-5", C-7"), 127.2 (C-4", C-8"), 117.8 (C-3'), 111.7 (C-9), 111.4 (C-10), 69.4 (C-1'), 45.17 (C-2"), 45.03 (C-9"), 32.8 (C-2'), 30.1 (C-10"), 25.7 (C-6'), 22.38 (C-11", C-12"), 18.06 (C-13"), 17.9 (C-5'), Yield; 43%

4-Trifluoromethylphenylacetylshikonin; 2-[1-(4-trifluoromethylphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: mp 95-96°C, IR V_{max} (KBr); 2950, 2910, 1720, 1600, 1425, 1320, ^{1}H-NMR ($CDCl_3$) δ 12.55 (1H, s, -OH at C-8), 12.37 (1H, s, -OH at C-5), 7.62-7.35 (4H, m, H-4", H-5", H-7", H-8"), 7.15 (2H, s, H-6, H-7), 6.81 (1H, s, H-3'), 6.02 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.70 (2H, s, H-2"), 2.49 (2H, m, H-2'), 1.62 (3H, s, -CH_3 at C-5'), 1.51 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 176.8 (C-4), 175.3 (C-1), 169.5 (C-1"), 168.6 (C-5), 168.1 (C-8), 147.4 (C-2), 137.4 (C-4'), 136.2 (C-3"), 133.3 (C-6), 133.1 (C-7), 131.1 (C-3), 130.4 (C-6"), 129.9, 129.8, 129.6, 129.1, 128.8 (C-5", C-7"), 125.8 (C-4", C-8"), 125.64, 125.59, 125.54, 125.49, 122.24, 118.6, 117.4 (C-3'), 111.7 (C-9), 111.5 (C-10), 70.1 (C-1'), 41.1 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.9 (C-5'), Yield; 40%

α-Bromophenylacetylshikonin; 2-[1-(α-bromophenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2910, 1740, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.44 (1H, s, -OH at C-8), 12.27 (1H, s, -OH at C-5), 7.50-7.29 (5H, m, H-4", H-5", H-6", H-7", H-8"), 7.06 (2H, s, H-6, H-7), 6.74 (1H, s, H-3), 5.99 (1H, m, H-1'), 5.33 (1H, s, H-2"), 4.99 (1H, m, H-3'), 2.49 (2H, m, H-2'), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C NMR ($CDCl_3$) δ: 176.8 (C-4), 175.3 (C-1), 168.4 (C-1"), 167.0 (C-5), 166.8 (C-8), 146.7 (C-2), 136.4 (C-4'), 135.1 (C-3"), 133.2 (C-6), 132.9 (C-7), 131.1 (C-7), 129.4 (C-4"), 128.8 (C-5"), 128.6 (C-6"), 117.3 (C-3'), 111.7 (C-9), 111.5 (C-10), 71.4 (C-1'), 46.8 (C-2"), 32.7 (C-2'), 25.7 (C-6'), 17.9 (C-5'),Yield; 56%

α-Acetoxyphenylacetylshikonin; 2-[1-(α-acetoxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoqunone: mp 121-122°C, IR V_{max} (KBr); 2960, 2900, 1740, 1600, 1445, 1225, 1195, 1H-NMR ($CDCl_3$) δ; 12.47 (1H, s, -OH at C-8), 12.28 (1H, s, -OH at C-5), 7.53-5.37 (5H, m, H-4", H-5", H-6", H-7", H-8"), 7.14 (2H, s, H-6, H-7), 6.29 (1H, s, H-3), 6.03 (1H, m, H-1'), 6.00 (1H, s, H-2"), 5.10 (1H, m, H-3'), 2.55 (2H, m, H-2'), 2.21 (3H, s, -O=CCH_3 at C-2"), 1.69 (3H, s, -CH_3 at C-5'), 1.57 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.1 (C-4), 175.6 (C-1), 170.2 (C-1"), 168.0 (C-5), 167.5

(C-8), 146.8 (C-2), 136.4 (C-4'), 133.2 (C-3"), 133.0 (C-6), 132.7 (C-7), 130.9 (C-3), 129.6 (C-5"), 128.9 (C-6"), 127.6 (C-4"), 117.2 (C-3'), 111.6 (C-9), 111.4 (C-10), 74.1 (C-2"), 70.6 (C-1'), 32.6 (C-2'), 25.7 (C-6'), 20.6 (C-10"), 17.9 (C-5'), Yield; 55%

4-Methylphenylacetylshikonin; 2-[1-(4-methylphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: mp 96°C, IR V_{max} (KBr); 2950, 2905, 1730, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.45 (1H, s, -OH at C-8), 12.29 (1H, s, -OH at C-5), 7.15-7.0 (6H, m, H-4", H-5", H-7", H-8", H-6, H-7), 6.70 (1H, s, H-3), 6.00 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.56 (1H, m, H-2"), 2.55 (2H, m, H-2'), 2.35 (3H, s, -CH_3 at C-6"), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 178.1 (C-4), 176.6 (C-1), 170.5 (C-1"), 167.4 (C-5), 166.9 (C-8), 148.0 (C-2), 136.9 (C-4'), 135.9 (C-6"), 132.8 (C-6), 132.6 (C-7), 131.4 (C-3), 130.4 (C-3"), 129.3 (C-5", C-7"), 129.0 (C-4", C-8"), 117.6 (C-3'), 111.7 (C-9), 111.5 (C-10), 69.7 (C-1'), 41.0 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 21.0 (C-9"), 17.8 (C-5'), Yield; 52%

3,4-Methylenedioxyphenylacetyl Shikonin; 2-[1-(3,4-methylenedioxyphenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2910, 1730, 1600, 1245, 1035, ^{1}H-NMR ($CDCl_3$) δ; 12.55 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.17 (2H, s, H-6, H-7), 6.8-6.7 (4H, m, H-3, H-4", H-5", H-8"), 6.02 (1H, m, H-1'), 5.95 (2H, s, -OCH_2O-), 5.03 (1H, m, H-3'), 3.55 (2H, s, H-2"), 2.50 (2H, m, H-2'), 1.64 (3H, s, -CH3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) d; 177.8 (C-4), 176.3 (C-1), 170.3 (C-1"), 167.6 (C-5), 167.1 (C-8), 147.8 (C-5"), 146.9 (C-6"), 136.0 (C-4'), 132.8 (C-6), 132.6 (C-7), 131.3 (C-3), 126.9 (C-3"), 122.4 (C-8"), 117.6 (C-3'), 111.7 (C-9), 111.5 (C-9), 109.6 (C-7"), 108.4 (C-4"), 101.1 (C-9"), 69.7 (C-1'), 41.1 (C-2"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield; 40%

a-Methyl-(-trifluoromethylacetylshikonin; 2-[1-(a-methoxy-a-trifluoro methyl phenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinonoe: mp 120-122°C, IR V_{max} (KBr); 2950, 1745, 1610, 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.58 (1H, s, -OH at C-8), 12.36 (1H, s, -OH at C-5), 7.56-7.37 (5H, m, H-4", H-5", H-6", H-7", H-8"), 7.16 (2H, s, H-6, H-7), 6.94 (1H, s, H-3), 6.33 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.53 (2H, s, H-2"), 2.55 (2H, m, H-2'), 1.64(3H, s, -CH_3 at C-5'), 1.53(3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 175.4 (C-4), 173.7 (C-1), 169.9 (C-1"), 169.5 (C-5), 165.6 (C-8), 145.9 (C-2), 136.6 (C-4'), 133.8 (C-6), 133.5 (C-7), 131.6 (C-3"), 131.3 (C-3), 129.8 (C-5"), 128.5 (C-6"), 127.4 (C-4"), 124.2, 122.5, 116.9 (C-3'),111.7 (C-9), 111.5 (C-10), 85.1, 84.7, 84.5, 84.1, 71.5 (C-1'), 55.4 (C-9"), 32.8 (C-2'), 25.7 (C-6'), 17.8 (C-6'), Yield: 55%

4-Dimethylaminophenylacetylshikonin; 2-[1-(4-dimethylaminophenylacetoxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinone: IR V_{max} (KBr); 2950, 2910, 1730, 1600, 1445, ^{1}H-NMR ($CDCl_3$) δ; 12.54 (1H, s, -OH at C-8), 12.39 (1H, s, -OH at C-5), 7.2-6.6 (7H, m, H-4", H-5", H-7", H-8", H-6, H-7, H-3), 5.99 (1H, m, H-1'), 5.03 (1H, m, H-3'), 3.58 (2H, s, H-2"), 2.93 (6H, s, -$N(CH_3)_2$ at C-6"), 2.51 (2H, m, H-2'), 1.64 (3H, s, -CH_3 at C-5'), 1.53 (3H, s, -CH_3 at C-6'), ^{13}C-NMR ($CDCl_3$) δ; 177.2 (C-4), 175.7 (C-1), 172.0 (C-1"), 168.2 (C-5), 167.7 (C-8), 149.7 (C-6"), 147.7 (C-2), 136.1 (C-4'), 133.1 (C-6), 132.9 (C-7), 131.1 (C-3), 129.9 (C-4",C-8"), 121.8 (C-3"), 117.5 (C-3'), 113.2 (C-5",C-7"), 111.7 (C-9), 111.4 (C-10), 69.9 (C-1'), 40.8 (C-2"), 40.1 (C-9".10"), 32.7 (C-2'), 25.7 (C-6'), 17.8 (C-5'), Yield: 45%

RESULTS AND DISCUSSION

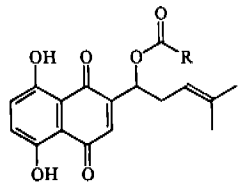
Chemistry

Acylation at C-1' side chain of shikonin was carried out according to Baik's method (Ahn and Baik, 1995) to give 21 phenylacetylshikonin analogues with a yield of 40-57%. In general, the presence of bulkier groups at α-C or benzene ring, or electron-releasing groups gave lower yields; biphenylacetylshikonin (41%), 4-isobutyl-methylphenyl acetylshikonin (43%) and, α-trifluoromethyl-4-methylphenyl acetylshikonin (40%) containing bulky groups, and 4-ethoxyphenylacetylshikonin (47%) and 3,4-methylenedioxyphenylacetylshikonin (40%) possessing electron releasing groups. Acylshikonin analogues with electron withdrawing group at the phenyl ring were produced in higher yields; 4-bromophenylacetylshikonin (56%) and 4-chlorophenyl acetylshikonin (52%). The yield of acylshikonin derivatives possessing electron withdrawing groups at α-C was elevated; α-bromophenylacetylshikonin (56%), α-acetyloxyphenylacetylshikonin (55%) and α-methoxy-α-trifluoromethylphenylacetyl shikonin (55%).

Cytotoxicity

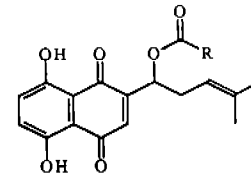
As demonstrated in Table 1, the solid cancer cell line A549 was more resistant to the acylshikonin derivatives, compared to the leukemic L1210 and K562 cell lines. Among phenylacetylshikonin derivatives, 4-methoxy-, α-methoxy-, 4-trifluoromethyl-, α-bromo-, α-acetoxy-, 4-methyl-, 3, 4-methylenedioxy-, α-methoxy-α-trifuoromethyl-, 4-dimethylamino-, 4-biphenyl-, 2, 6-dichloro-, and 2, 5-dimethoxyphenylacetylshikonin analogues showed a potenter cytotoxicity (ED_{50}, 1.0-5.0 μg/ml) than phenylacetyl shikonin (8.85 μg/ml) in A549 cells. In general, α-substituted phenylacetylshikonins possessed stronger cytotoxic activity. As a result, it was supposed that α-substituents might keep the acyl group away from the shikonin side chain, so that the resulting conformation would bind more suitably to a acceptors such as an active site of enzyme or a DNA of the cells. The electronic effect of 4-substituents seems to be un-

Table I. Cytotoxicity of arylacetylshikonin analogues in some cancer cells



RCO	ED_{50} (μg/ml) A549	K562	L1210
3,4,5-trimethoxyphenylacetyl	11.2	0.35	0.72
4-bromophenylacetyl	11.7	0.32	1.76
4-chlorophenylacetyl	8.0	0.22	1.64
4-ethoxyphenylacetyl	13.5	0.29	0.45
2,6-dichlorophenylacetyl	4.4	0.36	0.20
4-methoxyphenylacetyl	1.5	0.03	0.1
2,5-dimethoxyphenylacetyl	5.7	0.72	0.24
α-methoxyphenylacetyl	2.2	0.94	0.24
4-isobutyl-α-methylphenylacetyl	1.7	1.81	0.15
4-trifluoromethylphenylacetyl	2.9	1.47	0.40
α-bromophenylacetyl	1.9	1.26	0.18
α-acetoxyphenylacetyl	1.5	0.67	0.10
4-methylphenylacetyl	2.7	1.39	0.14
3,4-methylenedioxyphenylacetyl	2.9	0.53	0.20
α-methoxy-α-trifluofomethylphenylacetyl	2.5	2.93	0.27
4-(N,N-dimethylamino)phenylacetyl	2.2	0.44	0.14
biphenylacetyl	4.78	0.88	0.31

Table II. T/C values of arylacetylshikonin analogues in mice bearing S-180 cells



RCO	dose	T/C %>	50day
phenylacetyl	4.06	173.3	1
3,4,5-trimethoxyphenylacetyl	4.99	175.0	0
4-bromophenylacetyl	4.85	168.8	1
4-chlorophenylacetyl	4.40	160.0	0
4-ethoxyphenylacetyl	4.50	186.3	0
2,6-dichlorophenylacetyl	4.75	166.7	1
4-methoxyphenylacetyl	2.18	157.3	1
2,5-dimethoxyphenylacetyl	2.33	186.8	2
α-methoxyphenylacetyl	4.36	169.1	0
4-isobutyl-α-methylphenylacetyl	4.76	165.7	1
4-trifluoromethylphenylacetyl	2.37	150.5	1
α-bromophenylacetyl	2.43	142.2	2
α-acetoxyphenylacetyl	4.64	195.2	0
4-methylphenylacetyl	6.30	153.8	0
3,4-methylenedioxyphenylacetyl	2.25	93.3	0
α-methoxy-α-trifluoromethylphenylacetyl	5.04	141.6	1
4-(N,N-dimethylamino)phenylacetyl	6.74	192.1	0
biphenylacetyl	4.82	176.5	2

dose; mg/kg body weight. >50 day; survivors longer than 50 days

important for the cytotoxicity. Generally, electron-releasing groups such as methoxy or ethoxy at C-4 of the phenyl ring tended to increase the cytotoxicity, while electron-withdrawing group such as halogens reduced it. Contrary to the observation with A549 cell, α-substitution reduced the cytotoxicity against K562 cell; 4-isobutyl-α-methyl-(ED_{50}=1.80 μg/ml), α-bromo-(1.26 μg/ml) and α-methoxyl-α-trifluoromethylphenylshikonin (2.93 μg/ml) showed weaker cytotoxicity than phenylacetylshikonin (0.8 μg/ml).Thus, it is suggested that the structural requirement for binding of these compounds with K562 cell acceptors might be different from that for their interaction with A549 cell.

Prolongation of ICR mice bearing S-180 cells

All of synthetic phenylacetylshikonin analogues showed a good antitumor activity in mice bearing S-180 cells in peritoneal cavity (Table 2). The phenylacetylshikonin derivatives showing higher T/C values, compared to phenylacetylshikonin (T/C, 173%), are as follows; 3,4,5-trimethoxy-(175%), 4-biphenyl-(176%), 4-ethoxy-(186%), 2,5-dimethoxy-(186%), α-acetoxy-(195%) and 4-(N,N-dimethylamino) phenylacetylshikonin (192%). Antitumor effects of phenylacetylshikonin analogues could not be corelated to their cytotoxicity. α-Methoxyphenylacetylshikonin with ED_{50} values of 0.098 and 0.033 μg/ml in L1210 and K562 cells, respectively, exerted a toxic effect at a dose of 7.75 mg/kg, while the administration of 2.18 mg/kg showed a good antitumor activity (T/C, 157%). In earlier study, it was confirmed that introduction of an acetyl group at C-1' of shikonin potentiated both of antitumor and inhibitory effects (Kim and Ahn, 1990). In a similar manner, the presence of an α-acetoxy group in phenylacetyl moiety enhanced the T/C value remarkably; 195% for α-acetoxyphenylacetylshikonin. From these observations, it is assumed that introduction of acetyl group might facilitate the binding to an active site of acceptor or accelerate the redox cycling through the enhanced electrophilicity (Ahn *et. al.* 1996). 4-(N,N-dimethylamino)phenylacetylshikonin which was synthesized for the purpose of improving the solubility gave a T/C value (T/C 192%) much higher than unsubstituted phenylacetylshikonin (T/C= 173%). This might be due to its cationic property or enhanced solubility in physiological fluid.

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REFERENCES CITED

Ahn, B. Z., Baik, K. U., Kweon, G. R., Lim, K. and Hwang, B. D., Acylshikonins; synthesis and inhibition of DNA topoisomerase-I. *J. Med. Chem.*,

38, 1044-1047 (1995).

Ahn, B. Z., Song, G. Y., Baik, G. U. and Sok, D. E., Cytotoxicity of acylshikonin analogues against L 1210 cells and antitumor activity in Sarcoma tumors. *Cancer. Res.*, (submited to 1996).

Ahn, B. Z. and Sok, D. E., Michael acceptors as a tool for anticancer drug design. Review in *Current Pharmaceutical Design.* Bentham Science Publishers (in press 1996).

Hayashi, M., Pharmacological studies on crude plant drugs, shikon and tooki (II). Shikonin and acetylshikonin. *Nippon Yakurigaku Zasshi.*, 73, 177-191 (1977a).

Hayashi, M., Pharmacological studies on crude plant drugs, shikon and tooki (II). Shikonin and acetylshikonin. *Nippon Yakurigaku Zasshi.*, 73, 193-203 (1977b).

Kim, H. and Ahn, B. Z., Antitumor effect of acetylshikonin and synthetic naphthazarin derivatives on L1210 and S-180 cells. *Yakhak Hoeji.*, 34, 262-266 (1990).

Kyogoku, K., Terayama, H., Tachi, Y., Suzuki, T. and komatsu, M., Comparison of contents, constituents, and antibacterial effects of fat soluble fraction between nanshikon and koshikon. *Shoyakugaku Zasshi.*, 27, 31-36 (1973).

National Cancer Institute USA; Cell culture technical procedures, 1972. Papageorgiou, V. P., Wound healing properties of naphthoquinone pigment from *Alkana tinctoria. Experientia.*, 34, 1499-1501 (1978).

Papageorgiou, V. P., Winkler, A. M., Sagredos, A. N. M. and Digenis, G. A., Studies on the relationship of structure to antimicrobial properties of naphthoquinones and other constituents of *Alkana tinctoria. Planta Medica.*, 35, 56-60 (1979).

Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., scudiero, D. S. A., Monks, A. and Boyd, M. R., Comparison of *in vitro* anticancer drug screening data generated with a tetrazolium assay versus a protein assay against a divise panel of human cell lines. *J. Natl. Cancer Inst.*, 2, 1113-1118 (1990).

Sankawa, U., Ebizuka, Y., Miyazaki, T., Isomura, Y., Otsuka, H., Shibata, S., Inomato, M. and Fukuoka, F., Antitumor activity of shikonin and its deriatives. *Chem. Pharm. Bull.* 25, 932-2935 (1977).

Sankawa, U., Otsuka, H., Kataoka, Y., Hoshi, Y. A. and Kuretani, K., Antitumor activity of shikonin and their deriatives II. *Chem. Pharm. Bull.*, 29, 116-122 (1981).

Shukla, Y. N., Tandon, J. S., Bhakuzi, D. S. and Dhar, M. M., Naphthoquinones of *Arnebia nobilis. Phytochemistry.*, 10, 1905-1915 (1971).

Skehan, P., Storeng, R., Scudiero, D., Monk, A., McMahon, J., Vistica, D., Warren, J. T., Kenney, S. and Boyd, M. R., New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.*, 82, 1107-1112 (1990).

Thayer, P. S., Himnelfarb, P. and Watt, G. L., Cytotoxicity assay with L1210 cell *in vitro* and KB cells *in vitro. Cancer. Chemother. Rep.*, 2, 1-25 (1971).

Tikkanen, L., Matsushima, T., Natori, S. and Yoshihira, K., Mutagenicity of natural naphthoquinones and benzoquinones in the *Salmonella* microsome test. *Mutation Research.*, 124, 25-34 (1983).