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Cytotoxic Activity of the Extracts from *Curcuma zedoaria*

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ABSTRACT. The effect of the hexane extract of *Curcuma zedoaria* roots and its solvent fractions were investigated on the proliferation of SiHa, SNU-1 and HepG2 cell lines. Among those fractions, final fraction H2-3-1 and H2-3-3 showed cytotoxic effect on SiHa and HepG2 cell lines. The hallmark of apoptosis, DNA fragmentation, also appeared in the final fractions H2-3-1 and H2-3-3 after 24h treatment in SiHa cell line. Furthermore, those fractions were shown to be able to induce cell death in [^{3}H]thymidine incorporation test. These two fractions, H2-3-1 and H2-3-3 were determined as (-)-α-curcumene and β-tumerone respectively by NMR and mass spectrum. From these results, it is speculated that te hexane extract of *Curcuma zedoaria* is necessary for further studies as a potent inhibitor of the growth of cancer cells.

Keywords: *Curcuma zedoaria* roots, Hexane fraction, SiHa cells, Cytotoxicity, DNA fragmentation, Apoptosis inducer.

INTRODUCTION

Curcuma zedoria (Zingiberaceae) is a perennial plant that is mainly distributed in Asian countries. *Curcuma zedoaria* (*C. zedoria*) has been clinically used for the treatment of stomache, blood stagnation and promoting menstration as an oriental medicine (Han, 1998; Matsuda *et al.*, 1998; Yoshioka *et al.*, 1998). The methanolic extract exhibited a significant prolongation of hexobarbital (HB)-induced hypnosis (Shin *et al.*, 1989). Previous studies on *C. zedoaria* showed the isolation of various curcuminoids and terpenoids such as germacrone, curdione and zedoarol β-elemen (Yoshinori *et al.*, 1986; Tradimed, 1996) The essential of *C. zedoaria* exhibits antimicrobial activity against Staphylococcus aureus and Vibrio comma (Rao *et al.*, 1970). In cancer, polysaccharides and protein-bound polysaccharides of *C. zedoaria* showed inhibition of sarcoma-180 (Moon *et al.*, 1985), and the water fraction of *C. zedoria* increased NKCA activity (Kang *et al.*, 1987). The water extract of *C. zedoria* showed antimutagenic activity against benzopyrene-induced mutations in the Salmonella/microsomal system (Lee *et al.*, 1988).

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In our continuing search for anticancer agents, we found that the hexane extract of *C. zedoaria* showed inhibitory activity against SiHa (Cervix cancer cell line) and HepG-2 (hepatic cancer cell line), and showed apoptosis effect on SiHa cells. We herein report the bioassay directed fractionation of the hexane extract of *C. zedoaria.*

MATERIALS AND METHODS

Plant Materials

The Rhizome of *C. zedoaria* was purchased from a pharmaceutical company (Youngin Co) in Pusan.

Chemical Materials

Curcumin (Arldrich), Silica Gel 60 (Merck), Silica Gel 60 F254 (Merck), Dichloromethane (Merch), Ethyl acetate (J.T.Baker), Methyl Alcohol (CARLO ERBA), Hexane (KANTO Co.), Vilber lourmat (UV lamp, Marne La Vallee, France), RPMI (Hyclone), DMEM (Hyclone), 3-[4,5-dimethylthiazole-2yl]-2,5-diphenyltetrazolium bromide (Sigma), agarose (Sigma), 1-kb ladder (Bio-rad), [^{3}H] thymidine (Amersham), SiHa, HepG 2 and SNU cell lines were provided from the Korean cell bank.

Extraction and Isolation

The air dried plant (3 kg) were extracted by 99% MeOH (15 l) three times at 60°C for 24 hr. The methan-

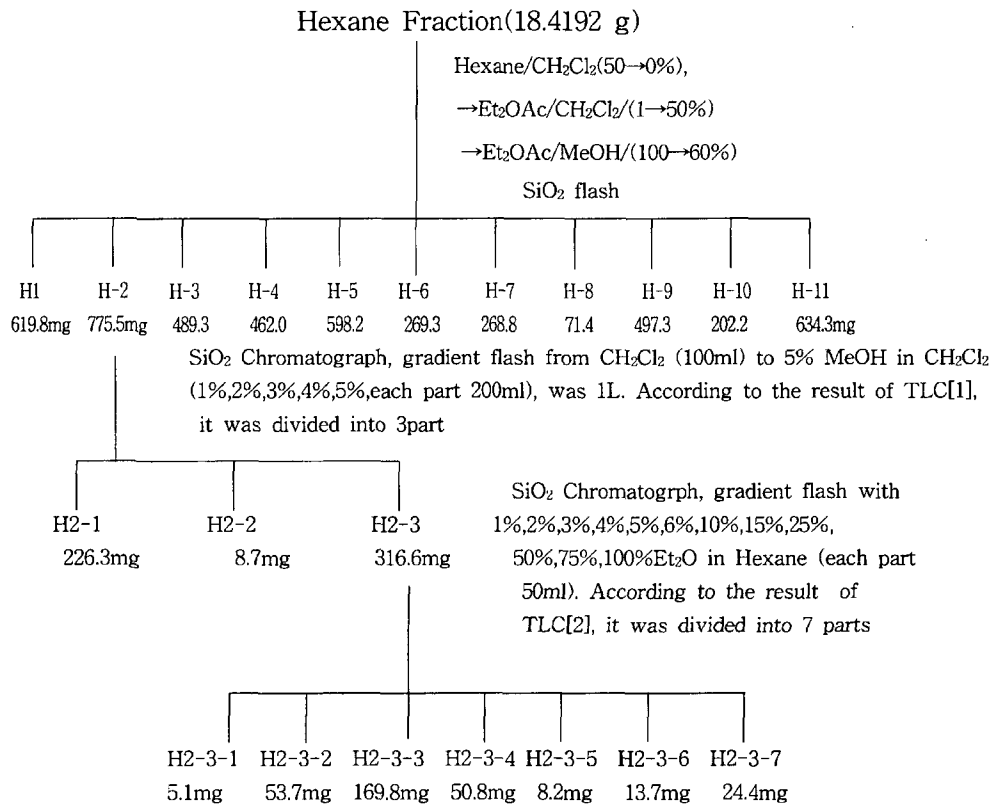


Fig. 1. Extraction scheme of *C. zedoaria*.

olic extracts (50.471 g) were combined with water, and then extracted with hexane. Separation of the hexane fractions (18.419 g) were proceeded according to the following scheme (Fig. 1).

Cytotoxicity Test

Basic theory was according to Mossman's method (Mossmann *et al.*, 1983). 10^5 cell/100ul of each cell were spread on 96 well plate with media. After incubation for 24 hrs, spin down slightly and take out media and put new media. Put 10 ul of each drug in triplet and incubate 48 hrs and put 12 ul of 3-[4,5-dimethylthiazole-2yl]-2,5-diphenyltetrazolium bromide (MTT) 5 mg/ml PBS vigorously and swirl slightly. Incubate for 4 hrs with no light. After spinning down for 3 min, suction the media and put 100 ul sol (DMSO : ethanol=1 : 1) on each well. Wrap the plate and shake for 5 min. Read Elisa (540 nm) and calculate IC_{50} value.

DNA Fragmentation Analysis

The 5×10^6 cells that were treated for 24 hr and centrifuged at 5,000 rpm for 3 min, then were washed twice with ice-PBS. After removing suspension, suspend cells were suspended with 300 ul digestion buffer (10 mM Tris-HCL, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K). After incubation for 12~18 h at 50°C in a tightly capped tube, the DNA was extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1, Sigma, USA). After precipitation, the pellet was resuspended in a 30 ul TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through a 1.5% agarose gel. After staining the agarose gel with ethidium bromide (0.1 ug/ml) for 15 min, the gel was destained with D.D.W for 10 min. The DNA bands were visualizd by UV light.

[^{3}H]thymidine Incorporation Measurement

To measure proliferation, cell were seeded in 96-well plates in 200 μl of complete medium with or without extracted fraction at a density of 5×10^6 cells/ml of SiHa cell. After 1 day of incubation, cells in each well were pulsed for 18 h with 4 μCi of [^{3}H]thymidine (specific activity, 27 Ci/mmol) (Amersham, Braunschweig, Germany) and collected with cell harvester (Skatron, Tranby, Norway); incorporated radioactivity was measured in a liquid scintillation counter (Beckman Instrumens, Inc, Fullerton, CA, USA).

RESULTS AND DISCUSSION

The hexane fractions of the rhizoma of *C. zedoaria*

Table 1. IC_{50} values of *C. zedoaria* fractions on SiHa, SNU-1 and HepG2 cell lines

Substance	IC_{50}value (μg/ml)		
	SiHa	SNU-1	HepG 2
H1	74.6	121.4	87.6
H2	34.2	39.5	41.6
H3	74.9	121.0	88.7
H4	72.5	122.0	69.1
H5	67.8	156.0	72.6
H6	80.2	95.0	78.6
H7	122.7	128.2	121.1
H8	100.7	120.2	108.6
H9	98.5	105.8	99.7
H10	146.7	106.4	156.7
H11	105.8	108.9	150.8
H2-1	210.0	97.0	155
H2-2	143.0	102.0	243
H2-3	36.0	60.0	43.0
H2-3-1	38.5	98.9	40.5
H2-3-2	80.2	62.7	122.1
H2-3-3	41.6	100.3	32.1
H2-3-4	122.3	101.1	181.2
H2-3-5	98.0	82.1	96.1
H2-3-6	130.6	69.1	122.1
H2-3-7	190.2	88.1	200.2

were evaporated *in vacuo*, suspended in H_2O and partitioned with CH_2Cl_2. The CH_2Cl_2 fraction was subject to column chromatography over silica gel eluting with gradient solvent system as shown in Fig. 1. These extraction system was processed according to the cytotoxic effect of the fractions (Table 1).

Until the 3rd fractionation, effective fraction (H2-3) showed almost equivalent cytotoxic effect on SiHa, SNU-1 and HepG2 cell lines. Finally, H2-3-1 and H2-3-3 fractions showed lowest IC_{50} values on SiHa and HepG2 cell lines. Therefore, we continued to investigate whether or not the cytotoxic effect is caused by apoptosis in SiHa cells. In order to determine the apoptotic effect of these final fractions, we examined the apoptotic responses, as judged by the appearance of a DNA ladder in gel electrophoresis (Steller, 1995; Im *et al.*, 1999). A characteristic pattern of nucleosomal DNA fragmentation was detected 24 hr after exposure to 50 ug/ml and 100 ug/ml of H2-3-1 and H2-3-3 fractions (Fig. 2).

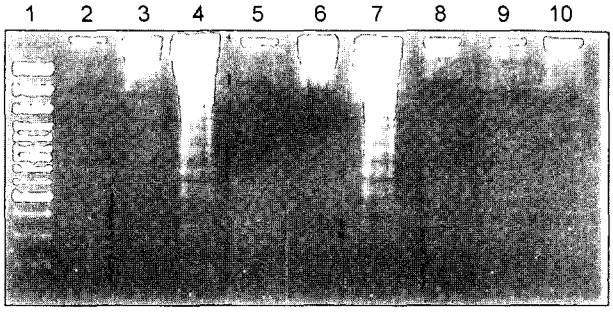


Fig. 2. Effects of *C. zedoaria* frs on DNA fragmentation in SiHa cell line. 1, marker; 2, H2-3-1 10 ug/ml; 3, H2-3-1 50 ug/ml; 4, H2-3-1 100 ug/ml; 5, H2-3-3 10 ug/ml; 6, H2-3-3 50 ug/ml; 7, H2-3-3 100 ug/ml; 8, H2-3-5 10 ug/ml; 9, H2-3-5 50 ug/ml; 10, H2-3-5 100 ug/ml treated. Cells were incubated for 24 hrs.

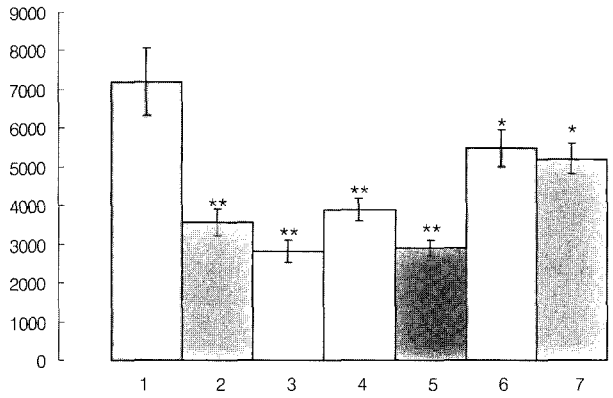
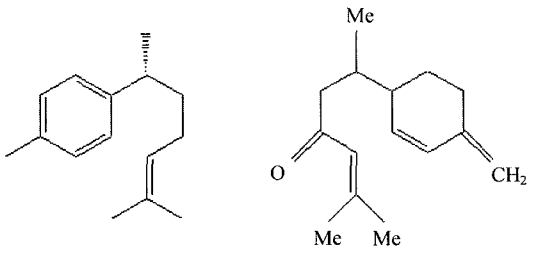


Fig. 3. [^{3}H]Thmidine incorporation into in SiHa cell, nontreated or treated as follows. 1, control; 2, H2-3-1 50 ug/ml; 3, H2-3-1 100 ug/ml; 4, H2-3-3 50 ug/ml; 5, H2-3-3 100 ug/ml; 6, H2-3-5 50 ug/ml; 7, H2-3-5 100 ug/ml. Cells were collected by using a cell harvester, and radioactivity was measured in a scintillation counter. The results are mean±SD. Significantly different from control value (*p<0.05, *p<0.01).

We also examined the apoptotic response as judged by the induction of cell death by [^{3}H]thymidine incorporation test (Ewa *et al.*, 1997). A characteristic pattern of cell death induction was detected 24 hr after exposure to 50 ug/ml and 100 ug/ml of H2-3-1, 3, 5 fractions (Fig. 3). H2-3-1 and H2-3-3 fractions would also be able to induce cell death, since [^{3}H]thymidine incorporations into 50 ug/ml and 100 ug/ml of those fractions were much lower than in control SiHa cells (Fig. 3).

We analyzed these two fractions by ^{13}C-NMR and ^{1}H-NMR and mass spectrum. They are shown to be one major compound in each fraction. (-)-α-Curcumene and β-tumerone maybe involved in those active fractions H2-3-1 and H2-3-3 respectively, from the result of GC mass fragmentation pattern (Fig. 4). The identification of β-tumerone in *C. zedoaria* was shown by the literature (Hong *et al.*, 2001). A well known compound, curcumin from *C. zedoaria* that showed cytotoxic activity in the OVCAR-3 cell line (Syur *et al.*, 1998) was not found in hexane fraction of *C. zedoaria*.

In conclusion, we demonstrated that *C. zedoaria* has cytotoxic components and they can also induce apoptosis. These cytotoxic components maybe (-)-α-Cur-



(-) -α -Curcumene β -Turmerone

Fig. 4. The putative structure of fraction H2-3-1 is (-)-α-Curcumene and H2-3-5 is β-tumerone. This is a analysed result of GC mass fraction pattern peakmatching data base.

cumene and β-tumerone. These findings suggest that the potential use of single component, (-)-α-Curcumene and β-tumerone in SiHa cell deserves further exploration.

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