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Effects of Natural Products on the Induction of NAD(P)H: Quinone Reductase in Hepa 1c1c7 Cells for the Development of Cancer Chemopreventive Agents

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Abstract – NAD(P)H:quinone reductase (QR) is one of the protective phase II enzymes against toxicity that accomplishes the capacity of detoxification by modulating the effects of mutagens and carcinogens. The detoxification mechanism is that quinone reductase promotes the 2-electron reduction of quinones to hydroquinones which are less reactive. This study is to search new inducers of quinone reductase from natural products, which can be used as cancer chemopreventive agents. Plant extracts were evaluated by using quinone reductase generating system with Hepa 1c1c7 murine hepatoma cell lines for enzyme inducing properties and crystal violet staining method for the measurement of cytotoxicity provoked. We have tested approximately 106 kinds of natural products after partition into n-hexane, ethyl acetate and aqueous layers from 100% methanol extracts of natural products. The ethyl acetate fractions of *Vitex rotundifolia* (fruits, 2FC: 12.7 μg/ml), *Cnidium officinale* (aerial parts, 2FC: 10.5 μg/ml), *Chrysanthemum sinese* (flowers, 2FC: 17.4 μg/ml) and the hexane fractions of *Angelica gigas* (roots, 2FC: 13.2 μg/ml), *Smilax china* (roots, 2FC: 11.9 μg/ml), *Sophora flavescens* (roots, 2FC: 16.3 μg/ml) revealed the significant induction of quinone reductase in a murine hepatic Hepa 1c1c7 cell culture system.

Key words – NAD(P)H : quinone reductase, Cancer chemoprevention, Hepa 1c1c7 cell, Phase II enzyme.

Introduction

NAD(P)H : quinone reductase (EC 1.6.99.2) is previously termed as DT-diaphorase (Bayney *et al.*, 1989; Shaw *et al.*, 1991) and is a flavoprotein of the cytosol (Bayney *et al.*, 1987; Ysern and Prochaska, 1989), which has a unique capacity to promote the 2-electron reduction of quinones to hydroquinones (Ysern and Prochaska, 1989; Iyanagi and Yamazaki, 1970; Chen *et al.*, 1992; Prestera *et al.*, 1993). According to the reports, quinones are metabolized by one-electron transfer by phase I enzymes such as cytochrome P-450 to semiquinone intermediates and these intermediates interact rapidly with oxygen to produce superoxide radicals (Bayney *et al.*, 1989), which is believed to be a major mechanism responsible for the toxic effect produced by quinones (Schlager and Powis, 1990). It was reported that quinone reductase blocks the toxic effects of quinone compounds by reducing to hydroquinones (Iyanagi and Yamazaki, 1970; Huang *et al.*, 1979), which are less reactive than semiquinone radicals and more easily diminished from the cells via conjugation (Lind, 1985). Quinone reductase acts as an intracellular detoxification system against mutagens, carcinogens and other toxic compounds (Prochaska *et al.*, 1985; Prochaska and Talalay, 1988).

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Quinone reductase inducing activity prevents the production of mutagenic quinone metabolites and decreases the covalent conjugation of oxygenated metabolites to microsomal proteins (Petersen *et al.*, 1989; Prochaska, *et al.*, 1985) and this enzyme maintains the ability of the cells to survive the stress of oxidative metabolites (Ernster, 1967; Forrest *et al.*, 1990; Talalay and Benson, 1982; Ysern and Prochaska, 1989; Iyanagi and Yamazaki, 1970; Merk *et al.*, 1991; Dicker and Cederbaum, 1993). Detoxification enzymes are belong to phase II enzymes (Prestera *et al.*, 1993) that are broadly distributed in mammalian cells and organs and inducing activities of these enzymes exert ability of protecting against toxic agent (Bayney *et al.*, 1987; Prestera *et al.*, 1993). Elevation of the activity of detoxification enzyme like quinone reductase is carried out by administration of several chemical compounds (Wattenberg, 1985; Prestera *et al.*, 1993), as exemplified by β-naphthoflavone, coumarin, disulfiram, indole 3-acetonitrile and indole 3-carbinol (Wattenberg, 1974; Wattenberg, 1975; Wattenberg *et al.*, 1977; Wattenberg and Leong, 1968; Wattenberg and Leong, 1970; Wattenberg and Loub, 1978; Wattenberg and Lam, 1979). It has been reported that an increase in the activities of phase II enzymes such as quinone reductase in organs was observed by the oral feeding of a polyphenolic fraction isolated from green tea in drinking water to mice. These results can be implicated in relation to the cancer chemopreventive effects of green tea against the induction of tumors in various target organs (Khan *et al.*, 1992). We have focused on the development of novel quinone reductase inducing compounds from natural products that might be potent and nontoxic, which can be used as important cancer chemopreventive agents.

Experimental

Chemicals – β-Naphthoflavone was purchased from Aldrich Chem. Co., USA. Bovine serum albumin (BSA), Tween-20, flavin adenine dinucleotide (FAD), glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), glucose-6-phosphate dehydrogenase (G-6-P-D), menadione, Nonidet P-40 (NP-40), dicoumarol, crystal violet and sodium dodecyl sulfate (SDS) were from Sigma Chem. Co., USA.

Plant materials – Herbal medicines were purchased from a herb market in Seoul and voucher specimens have been deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Each of the dried plants were sliced, and then extracted 3 times with 100% MeOH at room temperature. The MeOH extracts were concentrated under reduced pressure below 40°C, and then the concentrated MeOH extracts were partitioned into n-hexane, ethyl acetate (EtOAc) and then water.

Cells and cell culture – Hepa 1c1c7 cells were purchased from American Type Culture Collection, USA and α-minimal essential medium (without ribonucleosides or deoxyribonucleosides) was purchased from Sigma Chem. Co., USA. Hepa 1c1c7 cells were cultured in α-minimal essential medium supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere (5% CO_2).

Quinone reductase assay – The following method was based on the previous report with modification (Prochaska and Santamaria, 1988). Cultured Hepa 1c1c7 cells were plated at a density of 0.5×10^4 cells per well in a 96-well microtiter plate and incubated for 48 hr at 37°C in a humidified atmosphere (5% CO_2) with the test samples (0.5% DMSO, final). Cultured cells were washed with phosphate-buffered saline (PBS, pH 7.4) and lysed with 25 μl of lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 15 mM $MgCl_2$ and 0.5% NP-40, pH 8.0) for 10 min at 37°C. 200 μl of reaction mixture (12.5 mM Tris-HCl, pH 7.4, 0.135 mg BSA, 0.01% Tween-20, 0.05 mM FAD, 1 mM G-6-P, 0.03 mM NADP, 0.06 mg

MTT, 0.4 U G-6-P-D, 0.05 mM menadione and distilled water) was added into 96 well plates and incu- bated for 30 min at 37°C. 25 μl of a stopping solution (0.3 mM dicoumarol dissolved in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4) was added into each well and the plates were shaked for 5 min at room temperature. The absorbance was measured with a microplate reader at a 610 nm ($THERMO_{max}$, Molecular Device Co., USA).

Cytotoxicity measurement – Plant extract were evaluated for cytotoxicity by measuring cell survival at the end of the each experiment using the crystal violet staining method (Holobaugh and McChesney, 1990). Cultured Hepa 1c1c7 cells were washed with PBS (pH 7.4) and stained with 30 μl per well of 0.2% crystal violet dissolved in 2% ethanol for 10 min at 37°C, and then washed with PBS (pH 7.4). 200 μl of solution (0.5% SDS in 50% ethanol) was dispensed into each well and incubated for 1 hr at 37°C. Absorbance was measured with a microplate reader at a wavelength of 610 nm ($THERMO_{max}$, Molecular Device Co., USA).

Determination of specific activity – Specific activity was determined by the followed formula (Prochaska and Santamaria, 1988).

$$\text{specific activity} = \frac{\text{absorbance change of MTT/min}}{\text{absorbance of crystal violet}} \times 3{,}345\ \text{nmol/mg}$$

where 3,345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT.

Results and Discussion

It has been reported that quinone reductase plays an important role in the protection of cells against cell necrosis and carcinogenesis induced by quinones and their metabolic precursors, which are ubiquitous in nature (Prochaska *et al.*, 1985; Prochaska and Talalay, 1988). A lot of evidence suggested that phase II enzyme such as quinone reductase is induced coordinately with other phase II enzymes by various substances that protect against carcinogenic effects in animal models (Talalay and Benson, 1982). Therefore, development of inducing agents of phase II enzymes such as quinone reductase from natural products can be a new strategy for the control of cancer. In this study, induction of quinone reductase was measured by the quinone reductase generating system that produce blue color which is dependent on the reduction of MTT, as quinone reductase promotes the reduction of menadione to menadiol that reduces MTT (Prochaska and Santamaria, 1988; Dicker and Cederbaum, 1993). Dicoumarol, which has been reported as a strong inhibitor that interdicts quinone reductase inducing activity (Merk *et al.*, 1991), was used as

Table 1. Effects of plant extracts on the induction of quinone reductase in Hepa 1c1c7 cells

Scientific name/Family	Plant parts	Fold concentration (μg/ml)					
		Aqueous		Ethyl acetate		Hexane	
		1.5FC	2FC	1.5FC	2FC	1.5FC	2FC
Acanthopanax senticosus/Araliaceae	Rb	>20	>20	>20	>20	>20	>20
Aconitum koreanum/Ranunculaceae	Rt	>20	>20	>20	>20	>20	>20
Adenophora trachelioides/Compositae	Rt	>20	>20	>20	>20	>20	>20
Akebia quinata/Lardizabalaceae	Tu	>20	>20	>20	>20	>20	>20
Albizzia julibrissin/Leguminosae	Bk	>20	>20	>20	>20	>20	>20
Amomum cardamomum/Zingiberaceae	Sd	>20	>20	>20	>20	>20	>20
Anemarrhena asphodeloides/Liliaceae	Rt	>20	>20	>20	>20	>20	>20
Angelica gigas/Umbelliferae	Rt	>20	>20	>20	>20	5.6	13.2
Angelica tenuissima/Umbelliferae	Rt	>20	>20	>20	>20	>20	>20
Aralia continentalis/Araliaceae	Rt	>20	>20	>20	>20	>20	>20

Table 1. Contiuned

Scientific name/Family	Plant parts	Fold concentration (μg/ml)					
		Aqueous		Ethyl acetate		Hexane	
		1.5FC	2FC	1.5FC	2FC	1.5FC	2FC
Artemisia argyi/Compositae	Lf	>20	>20	>20	>20	>20	>20
Artemisia capillaris/Compositae	Lf, St	>20	>20	>20	>20	>20	>20
Asparagus cochinchinensis/Liliaceae	Bk	>20	>20	>20	>20	>20	>20
Benicasa cerifera/Cucurbitaeae	Fr	>20	>20	>20	>20	>20	>20
Boswellia carteriz/Bruseraceae	Rr	>20	>20	>20	>20	>20	>20
Bupleurum falcatum/Umbelliferae	Rt	>20	>20	>20	>20	>20	>20
Cassia tora/Leguminosae	Sd	>20	>20	>20	>20	>20	>20
Chaenomeles sinensis/Rosaceae	Fr	>20	>20	>20	>20	>20	>20
Chrysanthemum sibiricum/Compositae	Lf, St	>20	>20	>20	>20	>20	>20
Chrysanthemum sinese/Compositae	Fl	>20	>20	9.4	17.4	>20	>20
Cibotium barometz/Polypodiaceae	Ap	>20	>20	>20	>20	>20	>20
Cichorium intybus/Compositae	Rt	>20	>20	>20	>20	>20	>20
Cimicifuga heracleifolia/Ranunculaceae	Rt	>20	>20	>20	>20	>20	>20
Citrus unshiu/Rutaceae	Fb	>20	>20	>20	>20	>20	>20
Cnidium officinale/Umbelliferae	Ap	>20	>20	2.5	10.5	>20	>20
Cocculus trilobus/Menispermaceae	Rt	>20	>20	>20	>20	>20	>20
Codonopsis pilosula/Campanulaceae	Fr	>20	>20	>20	>20	>20	>20
Coptis chinensis/Ranunculaceae	Rt	>20	>20	>20	>20	>20	>20
Cornus officinalis/Cornaceae	Fr	>20	>20	>20	>20	>20	>20
Corydalis ternata/Papaveraceae	Rt	>20	>20	>20	>20	>20	>20
Crataegus pinnatifida/Rosaceae	Fr	>20	>20	>20	>20	>20	>20
Cuscuta australia/Convolvulaceae	Sd	>20	>20	>20	>20	>20	>20
Cynanchum wilfori/Asclepiadaceae	Wp	>20	>20	>20	>20	>20	>20
Cyperus rotundus/Cyperaceae	Rt	>20	>20	>20	>20	>20	>20
Dianthus chinensis/Caryophyllaceae	Wp	>20	>20	>20	>20	>20	>20
Dioscorea batatas/Dioscoreaceae	Rb	>20	>20	>20	>20	>20	>20
Dryopteris crassirhizoma/Polypodiaceae	Rt	>20	>20	>20	>20	>20	>20
Epimedium koreanum/Berberidaceae	Lf,St	>20	>20	>20	>20	>20	>20
Equiseturn hyemale/Equisetaceae	Wp	>20	>20	>20	>20	>20	>20
Eucommia ulmoides/Eucommiaceae	Bk	>20	>20	>20	>20	>20	>20
Foeniculum vulgare/Umbelliferae	Fr	>20	>20	>20	>20	>20	>20
Gardenia jasminoides/Rubiaceae	Fr	>20	>20	>20	>20	>20	>20
Gentiana scabra/Gentianaceae	Rt	>20	>20	>20	>20	>20	>20
Glycine max/Leguminosae	Sd	>20	>20	>20	>20	>20	>20
Glycyrrhiza uralensis/Leguminosae	Rt	>20	>20	>20	>20	>20	>20
Hylomecon vernale/Papaveraceae	Wp	>20	>20	>20	>20	>20	>20
Juglans sinensis/Juglandaceae	Sd	>20	>20	>20	>20	>20	>20
Kalopanax pictum/Araliaceae	Bk	>20	>20	>20	>20	>20	>20
Leonurus sibiricus/Labiatae	Lf,St	>20	>20	>20	>20	>20	>20
Ligusticum delavayi/Umbelliferae	Rt	>20	>20	>20	>20	>20	>20
Lonicera confusa/Caprifoliaceae	Fl	>20	>20	>20	>20	>20	>20
Lonicera japonica/Caprifoliaceae	Fl	>20	>20	>20	>20	>20	>20
Lycium chinense/Solanaceae	Fr	>20	>20	>20	>20	>20	>20
Machilus thunbergii/Lauraceae	Bk	>20	>20	>20	>20	>20	>20
Malva verticillata/Malvaceae	Sd	>20	>20	>20	>20	>20	>20
Mentha arvensis/Labiatae	Ap	>20	>20	>20	>20	>20	>20
Morus alba/Moraceae	Rt	>20	>20	>20	>20	>20	>20
Nelumbo nucifera/Nymphaeaceae	Fr	>20	>20	>20	>20	>20	>20
Nepeta japonica/Labiatae	Ap	>20	>20	>20	>20	>20	>20
Paeonia albiflora/Ranunculaceae	Rt	>20	>20	>20	>20	>20	>20
Paeonia obovata/Ranunculaceae	Rt	>20	>20	>20	>20	>20	>20

Table 1. Contiuned

Scientific name/Family	Plant parts	Fold concentration (μg/ml)					
		Aqueous		Ethyl acetate		Hexane	
		1.5FC	2FC	1.5FC	2FC	1.5FC	2FC
Panax ginseng/Araliaceae	Rt	>20	>20	>20	>20	>20	>20
Perilla sikokiana/Labiatae	Sd	>20	>20	>20	>20	>20	>20
Peucedanum japonicum/Umbelliferae	Rt	>20	>20	>20	>20	>20	>20
Pharbitis nil/Convolvulaceae	Sd	>20	>20	>20	>20	>20	>20
Phyllostachys nigra/Gramineae	St	>20	>20	>20	>20	>20	>20
Phytolacca esculenta/Phytolaccaceae	Rt	>20	>20	>20	>20	>20	>20
Pinellia ternata/Araceae	Tu	>20	>20	>20	>20	>20	>20
Platycodon grandiflorum/Campanulaceae	Rt	>20	>20	>20	>20	>20	>20
Pleuropterus cilinervis/Polygonaceae	Rt	>20	>20	>20	>20	>20	>20
Polygonum cuspidata/Polygonaceae	Rt	>20	>20	>20	>20	>20	>20
Poria cocos/Polyporaceae	Fr	>20	>20	>20	>20	>20	>20
Prunella vulgaris/Labiatae	Fl	>20	>20	>20	>20	>20	>20
Prunus armeniaca/Hamamelidaceal	Sd	>20	>20	>20	>20	>20	>20
Prunus persica/Hamamelidaceal	Sd	>20	>20	>20	>20	>20	>20
Pueraria thunbergiana/Leguminosae	Fl,Rt	>20	>20	>20	>20	>20	>20
Raphanus sativus/Cruciferae	Sd	>20	>20	>20	>20	>20	>20
Rehmania glutinosa/Scrophulariaceae	Rt	>20	>20	>20	>20	>20	>20
Rheum palmatum/Polygonaceae	Rt	>20	>20	>20	>20	>20	>20
Rhus javanica/Anacardiaceae	Lf	>20	>20	>20	>20	>20	>20
Rubus coreanus/Hamamelidaceae	Fr	>20	>20	>20	>20	>20	>20
Salvia miltiorrhiza/Labiatae	Rt	>20	>20	>20	>20	>20	>20
Sambucus williamsii/Caprifoliaceae	Ap	>20	>20	>20	>20	>20	>20
Sanguisorba officinalis/Rosaceae	Rt	>20	>20	>20	>20	>20	>20
Santalum album/Santalaceae	St	>20	>20	>20	>20	>20	>20
Saussurea lappa/Compositae	Rt	>20	>20	>20	>20	>20	>20
Schizandra chinensis/Magnoliaceae	Fr	>20	>20	>20	>20	>20	>20
Scopolia japonica/Solanaceae	Rt	>20	>20	>20	>20	>20	>20
Scrophularia ningpoensis/Scrophulariaceae	Rt	>20	>20	>20	>20	>20	>20
Scutellaria baicalensis/Labiatae	Rt	>20	>20	>20	>20	>20	>20
Siegesbeckia pubescens/Compositae	Wp	>20	>20	>20	>20	>20	>20
Sinapis alba/Cruciferae	Sd	>20	>20	>20	>20	>20	>20
Smilax china/Liliaceae	Rt	>20	>20	>20	>20	2.2	11.9
Sophora flavescens/Leguminosae	Fl	>20	>20	>20	>20	>20	>20
Sophora flavescens/Leguminosae	Rt	>20	>20	>20	>20	4.7	16.3
Sparganium stoloniferum/Sparganiaceae	Rt	>20	>20	>20	>20	>20	>20
Taraxacum platycarpum/Compositae	Rt	>20	>20	>20	>20	>20	>20
Torilis japonica/Umblliferae	Fr	>20	>20	>20	>20	>20	>20
Torreya nucifera/Taxaceae	Fr	>20	>20	>20	>20	>20	>20
Trichosanthes kirilowii/Cucurbifaceae	Sd	>20	>20	>20	>20	>20	>20
Triticum aestivum/Gramineae	Sd	>20	>20	>20	>20	>20	>20
Tussilago farfara/Compositae	Fl	>20	>20	>20	>20	>20	>20
Typha orientalis/Typhaceae	Fl	>20	>20	>20	>20	>20	>20
Vitex rotundifolia/Verbenaceae	Fr	>20	>20	0.97	12.7	>20	>20
Zanthoxylum bungeanum/Rutaceae	Bk	>20	>20	>20	>20	>20	>20
Zingiber officinale/Zingiberaceae	Rt	>20	>20	>20	>20	>20	>20

Abbreviations are aerial parts(Ap), bark(Bk), flowers(Fl), fruits(Fr), fruits bark(Fb), leaf(Lf), root bark(Rb), roots(Rt), ruber resin(Rr), seeds(Sd), stem(St), tuber(Tu) and whole plants(Wp). 0.5×10^4 cells/0.2 ml/well was used and 30 min incubation after 48 hr treatment was performed for enzyme assay. 2FC and 1.5FC are concentration required to increase 2 and 1.5 fold the specific activity of quinone reductase as compared to negative control group (DMSO).

a stopping solution. In order to investigate the induction effects on quinone reductase by some natural products, we have evaluated one hundred six kinds of natural products corresponding to 47 different families by measuring the reduction rate of MTT produced by quinone reductase in Hepa 1c1c7 cells. Table 1 shows data for concentration required to increase 2 and 1.5 fold the specific activity of quinone reductase (2FC and 1.5FC, respectively) by natural products as compared to negative control group (DMSO). The induction effect by β-naphthoflavone showed the maximum value of quinone reductase inducing activity at a density of 0.5×10^4 cells/0.2 ml/well at 30 min incubation time after 48 hrs of sample treatment (Fig. 1 and Table 2). Based on this result, 30 min incubation time was used hereafter. We identified 6 fractions that elevate

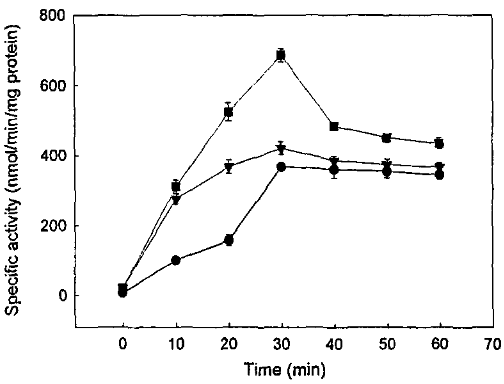

Fig. 1. Time course effect of quinone reductase induced by β-naphthoflavone in Hepa 1c1c7 cells. ● 0.01 μg/ml, ▼ 0.5 μg/ml, ■ 5 μg/ml.

quinone reductase activity. These are ethyl acetate fractions of *Vitex rotundifolia* (fruits, 2FC: 12.7 μg/ml), *Cnidium officinale* (aerial parts, 2FC: 10.5 μg/ml), *Chrysanthemum sinese* (flowers, 2FC: 17.4 μg/ml) and the hexane fractions of *Angelica gigas* (roots, 2FC: 13.2 μg/ml), *Smilax china* (roots, 2FC: 11.9 μg/ml), *Sophora flavescens* (roots, 2FC: 16.3 μg/ml). The EtOAc fraction of *Cnidium officinale* and the hexane fraction of *Smilax china* exhibited the most potent inducing effect and equally about 0.03 times induction effect as compared to that of β-naphthoflavone(0.36 μg/ml), which was used as a positive control in this assay. We have utilized crystal violet staining for measurement of cytotoxicity induced by natural products in Hepa 1c1c7 cells. The EtOAc fraction of *Cnidium officinale* and the hexane fraction of *Smilax china* revealed a negligible cytotoxicity (Fig. 2). It has been reported that glucosinolates found in cruciferous vegetables (Tawfiq *et al.*, 1995), rosemary derived from the leaves of *Rosmarinus officinalis* (Offord *et al.* 1995) and sulforaphane in SAGA broccoli (*Brassica oleracea*) (Zhang *et al.*, 1992) reveal the potential activity to increase the detoxification effect of an important human carcinogen by the induction of phase II enzymes such as quinone reductase. Therefore, active fractions are to be fractionated with column chromatography to find active principles in this system for the development of cancer chemopreventive agents from natural products.

Table 2. Dose-dependent effect of β-naphthoflavone for the induction of quinone reductase in Hepa 1c1c7 cells

	Ratio of specific activity (treated/control)		
Cell No. / Conc. (μg/ml)	0.2×10^4 cells/0.2 ml/well	0.5×10^4 cells/0.2 ml/well	1×10^4 cells/0.2 ml/well
5	6.09±0.05	7.21±1.12	3.94±0.91
2.5	3.72±0.19	5.24±0.15	3.29±1.11
1	3.15±0.47	5.19±0.24	2.22±0.89
0.5	2.57±0.09	4.20±0.18	2.21±0.75
0.25	1.89±0.05	3.47±0.23	1.87±0.05
0.1	1.72±0.03	1.75±0.11	1.41±0.09
0.05	0.67±0.09	1.30±0.07	1.08±0.23
0.01	0.57±0.08	1.17±0.12	1.07±0.14

30 min incubation after 48 hr treatment was performed for enzyme assay.

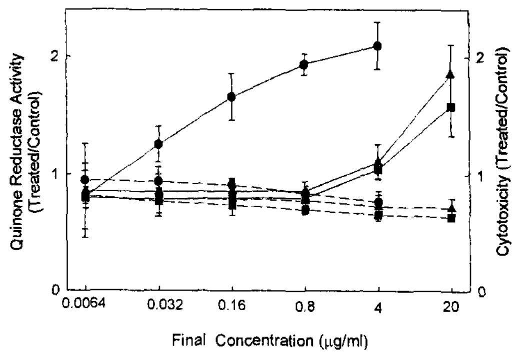

Fig. 2. Induction of quinone reductase activity (——) and cytotoxicity (⋯⋯) by β-naphthoflavone (●), the EtOAc fraction of the aerial parts of Cnidium officinale (▲) and the hexane fraction of the roots of Smilax china (■) in Hepa 1c-1c7 cells.

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