



## A novel and potent biological antioxidant, Kinobeon A, from cell culture of safflower

Tsutomu Kanehira<sup>a</sup>, Susumu Takekoshi<sup>b,\*</sup>, Hidetaka Nagata<sup>b</sup>, Kentaro Matsuzaki<sup>a</sup>,  
Yasuhiro Kambayashi<sup>c</sup>, Robert Y. Osamura<sup>b</sup>, Takao Homma<sup>a</sup>

<sup>a</sup>Department of Applied Chemistry, Tokai University Graduate School of Engineering, Hiratsuka, Kanagawa 259-1292, Japan

<sup>b</sup>Department of Pathology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan

<sup>c</sup>Department of Environmental and Preventive Medicine, Graduate School of Medical Science, Kanazawa University,  
13-1 Takara-machi, Kanazawa 920-8649, Japan

Received 26 February 2003; accepted 27 June 2003

### Abstract

Kinobeon A was originally isolated from cultured cells of safflower (*Carthamus tinctorius* L., Compositae). It had never previously been directly isolated from safflower or other plants, animals or microorganisms. In this report, we demonstrate the anti-oxidative effects of kinobeon A and compare the results with those two known natural antioxidants, lignan (nordihydroguaiaretic acid) and quercetin. The NADPH-induced microsomal lipid peroxidation system was employed to assess anti-oxidative effects of kinobeon A. Addition of kinobeon A to the system significantly decreased the formation of thiobarbituric acid reactive substances (TBARS) in a dose-dependent manner with effects similar to those of lignan and quercetin. Formation of TBARS was completely inhibited at 10  $\mu$ M of kinobeon A. Employing the xanthine/xanthine oxidase/nitroblue tetrazolium system and the  $\text{KO}_2$ /XTT system, the superoxide anion scavenging activity of kinobeon A was greater than that of lignan or quercetin.  $\text{IC}_{50}$  values calculated for kinobeon A in these two systems were 1  $\mu$ M and 0.8  $\mu$ M, respectively. Kinobeon A exerted cytoprotective effects following oxidative treatments with hydrogen peroxide, cumene hydroperoxide, menadione and xanthine oxidase (XOD). Addition of kinobeon A to the systems markedly enhanced survival ratios of Madin-Darby bovine kidney cells, while their survival significantly decreased with the oxidative treatment alone. Kinobeon A exhibited stronger effect on the cell viability than lignan or quercetin when menadion or XOD were used as inducing reagents of oxidative stress. The present study demonstrates for the first time that kinobeon A prevents oxidative stresses and could be a useful cytoprotective reagent.

© 2003 Elsevier Inc. All rights reserved.

**Keywords:** Antioxidant; Kinobeon A; Lignan; Oxidative stress; Safflower

\* Corresponding author. Tel.: +81-463-93-1121x2570; fax: +81-463-91-1370.

E-mail address: [takekos@is.icc.u-tokai.ac.jp](mailto:takekos@is.icc.u-tokai.ac.jp) (S. Takekoshi).

## Introduction

Reactive oxygen species (ROS) generated during normal cell metabolism are usually inactivated by host antioxidant defense mechanisms. These include both anti-oxidative enzymes such as superoxide dismutase, glutathione peroxidase and catalase, and non-enzymatic factors including several low molecular weight compounds such as anti-oxidative vitamins and some other anti-oxidative micro-nutrients obtained from the diet. However, oxidative stress can occur when these defense mechanisms become compromised. Oxidative stress has been implicated in a variety of pathological and chronic degenerative processes including the development of cancer, atherosclerosis, inflammation, aging and neurodegenerative disorders (Zhou and Zheng, 1991). Therefore, supplements of dietary anti-oxidative compounds can enhance the antioxidant capacity of the body and can effectively act to prevent oxidative stress-related diseases.

Recently, natural antioxidants such as flavonoids, lignans and related phenolic compounds have given rise to much attention. Plants that are rich in these compounds are ideal sources of natural antioxidants. Safflower (*Carthamus tinctorius* L., Compositae) has long been used as a source of edible fat, food colorants and Chinese medicine. There are some reports about anti-oxidative compounds from safflower describing their activity in scavenging free radical species such as superoxide anion ( $O_2^-$ ) (Kanehira et al., 2000) and  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical (Zhang et al., 1996, 1997), indicating the significance of safflower as a source material of antioxidants. Kinobeon A was originally isolated from cultured cells of safflower. It had never previously been directly isolated from safflower or other plants, animals or microorganisms (Wakayama et al., 1994). Kinobeon A possesses a unique chemical structure as shown in Fig. 1. As reported previously (Wakayama et al., 1994), the compound was not produced by cells grown in the optimum growth medium, while addition of certain compounds (cellulose powder, D-amino acids) resulted in kinobeon A formation. Although biosynthetic pathways of the compound still remain unclear, the physical and/or chemical modification in cultured media might cause stresses to the plant cells and this modification may induce self-defense mechanisms against cell damage, including that of the anti-oxidative system. Pharmacological effects of kinobeon A have not been investigated. Based on its structure, it may function as an antioxidant.

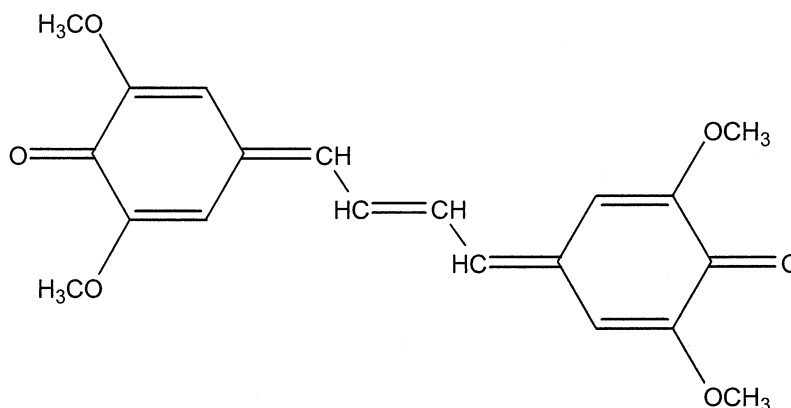


Fig. 1. Structure of kinobeon A.

Multiple pharmacological effects of lignans have been reported, including anti-tumor (Schultze-Mosgau et al., 1998; Ito et al., 2001), anti-viral (Kernan et al., 1997; Hwu et al., 1998; Chen et al., 1998) and anti-inflammatory (Diaz-Lanza et al., 2001) activities. Lignans also possess free radical-scavenging abilities, and thus anti-radical property is directed toward hydroxy radical (HO $\cdot$ ) and O $_2^-$ , which are reactive oxygen species implicated in the initiation of lipid peroxidation (Harper et al., 1999; Ng et al., 2000).

The aims of this study were to do a comprehensive investigation of the anti-oxidative activities of kinobeaon A, comparing them with two known natural antioxidants, lignan (nordihydroguaiaretic acid) and quercetin; and further, to assess their possible protective effects on oxidative stress in cell cultures.

## Methods

### *Kinobeaon A*

Kinobeaon A was derived from our laboratory source, as described in the previous report (Wakayama et al., 1994). Cells of safflower (*Carthamus tinctorius* L.) was derived from two month-young leaves on a cell-growth medium (825 mg/l of NH $_4$ NO $_3$ , 950 mg/l of KNO $_3$ , 85 mg/l of KH $_2$ PO $_4$ , 220 mg/ml of CaCl $_2$ ·2H $_2$ O, 185 mg/ml of MgSO $_4$ ·7H $_2$ O, 22.3 mg/ml of MnSO $_4$ ·4H $_2$ O, 6.2 mg/l of H $_3$ BO $_3$ , 8.6 mg/l of ZnSO $_4$ ·7H $_2$ O, 0.83 mg/l of KI, 0.25 mg/l of NaMoO $_4$ ·2H $_2$ O, 0.025 mg/l of CuSO $_4$ ·5H $_2$ O, 0.025 mg/l of CoCl $_2$ ·6H $_2$ O, 27.8 mg/l of FeSO $_4$ ·7H $_2$ O, 37.3 mg/l of Na $_2$ EDTA, 100 mg/l of *myo*-inositol, 0.1 mg/l of thiamine-HCl, 0.5 mg/l of pyridoxine-HCl, 0.5 mg/l of nicotinic acid, 2.0 mg/l of glycine, 0.1 mg/l of 30 g/l of sucrose, 0.186 mg/l of  $\alpha$ -naphthaleneacetic acid, 0.225 mg/l of *N*-6-benzyladenine and 2g/l of gellan gum). The cells (3.5 g) were placed in a 300 ml-flask containing 50 ml of the cell-growth medium (without gellan gum), and then cultured on the rotary shaker (75 rpm) in darkness at 25 °C for 4 days. The cells (3.5g) were then transferred into 75 ml/flask of the kinobeaon A-production medium which was modified the cell-growth medium by adding 165.19 mg/l of D-phenylalanine and 40 g/l of cellulose powder to the medium and by eliminating CaCl $_2$ ·2H $_2$ O and MgSO $_4$ ·7H $_2$ O from the medium, and incubated on the shaker for 4 days. During this incubation, kinobeaon A was produced by the cells and adsorbed on cellulose powder in the medium. The product (kinobeaon A) adsorbed on the cellulose powder was extracted with 3.2 litre of acetone/methanol (1:1, v/v). After filtrating through sintered glass and concentrating the eluate with a rotary evaporator under reduced pressure to a volume of 50 ml, the concentrate was passed through a TOYOPEARL HW-40F (Tosoh, Tokyo, Japan) column ( $\phi$ 2.5 cm  $\times$  50 cm) using acetone/methanol as an eluent, and then passed through a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column ( $\phi$ 1.6 cm  $\times$  60 cm) using methanol as an eluent. The resulting fraction containing kinobeaon A was concentrated to dryness with a rotary evaporator under reduced pressure to give a red amorphous residue. The residue was recrystallized from methanol at room temperature. 200 mg of kinobeaon A was obtained from 83 g of cultured cells. Purity of the compound was estimated to be >99% from physicochemical analysis with HPLC, UV/VIS spectra and  $^1$ H NMR spectra (data not shown).

### *Other chemicals*

Quercetin, nordihydroguaiaretic acid, menadione, xanthine and cumene hydroperoxide (Cu-OOH) were purchased from Sigma Chemical (MO, USA). Hydrogen peroxide (H $_2$ O $_2$ ) was from Mitsubishi Gas Chemical (Tokyo, Japan). Xanthine oxidase (XOD) was from Roche Diagnostics (Mannheim, Ger-

many). Dimethyl sulfoxide (DMSO) and malondialdehyde were from Merck (Darmstadt, Germany).  $\text{Fe}^{3+}$  (3 mM)-ADP (50 mM) complex, a stock solution, was diluted to adequate concentration with water before use.

#### *Antioxidant activity in rat liver microsome*

Male Wistar rats (age: 12 weeks) were killed by decapitation, and the livers were quickly excised and rinsed with ice-cold 0.15 M KCl. Rat livers (600 g) were quickly thawed, rinsed with ice-chilled 0.15 M KCl, and then homogenized with 4 volumes of 0.15 M KCl using a Teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at  $10,000 \times g$  for 15 min, and the sediment was discarded. The supernatant was further centrifuged at  $78,000 \times g$  for 90 min to precipitate microsomes. Microsomal pellets were suspended in 700 ml of 0.15 M KCl solution containing 10 mM EDTA (pH 7.0) by gently homogenizing. The suspension was then centrifuged at  $78,000 \times g$  for 60 min. The pellets were suspended in 700 ml of 0.1 M potassium phosphate buffer (pH 7.5) and precipitated in the same manner as above. The final pellets were suspended in 50 ml of  $\text{H}_2\text{O}$  to give a protein concentration of 40 to 50 mg/ml. The assay reaction mixture contained microsomes (2 mg/ml),  $12 \mu\text{M Fe}^{3+}$ -ADP, 5 mM  $\text{MgSO}_4$ , 5 mM glucose-6-phosphate, and 20  $\mu\text{g/ml}$  of glucose-6-phosphate dehydrogenase. After preincubation of the reaction mixture without NADPH at 37 °C for 2 min, the reaction was started by adding 0.5 mM NADPH. Thiobarbituric acid-reacting substances (TBARS) were determined as previously described (Ohkawa et al., 1979), and expressed as nmol/ml malondialdehyde equivalent. Tetramethoxypropane was used as a standard.

#### *Cell culture*

Madin-Darby bovine kidney (MDBK) cells were obtained from RIKEN cell bank, and were cultured under 5%  $\text{CO}_2$ /air at 37 °C in Ham's F-12 (Gibco BRL, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Immuno-Biological Laboratories, Gunma, Japan).

#### *Cell viability*

Test compounds (kinobeaon A, lignan and quercetin) did not show cytotoxicity against MDBK cells in the range used in this experiments.  $\text{LD}_{50}$  value of kinobeaon A was 100  $\mu\text{M}$ . The DMSO concentration was 0.1% in all incubations. MDBK cells ( $1 \times 10^3$  cells/ml) were seeded in each well of 96-well plates and were allowed to attach to the bottom face of the plates under 5%  $\text{CO}_2$ /air at 37 °C overnight. After changing the medium, each test compound was added to the medium within the range of 0–20  $\mu\text{M}$ , and the cells were incubated under 5%  $\text{CO}_2$ /air at 37 °C for 4 days. The cells were then treated in absence of test compounds with 200  $\mu\text{M H}_2\text{O}_2$ , 50  $\mu\text{M Cu-OOH}$ , 40  $\mu\text{M}$  menadione or 13 mU/ml XOD in a medium containing 10% FBS under 5%  $\text{CO}_2$ /air at 37 °C for 2 days. Cell viability was determined using the cell counting kit-8 technique (DOJINDO Laboratories, Kumamoto, Japan). Briefly, 10  $\mu\text{l}$  of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay solution was added to each well and incubated at 37 °C for 3 h. The absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm using a microplate reader Spectra Max 250 (Molecular Devices, Sunnyvale, CA, USA). Absorbance (A450-A655) correlates with the number of living cells.

### *O<sub>2</sub><sup>-</sup> scavenging activity using xanthine/XOD/NBT system*

The assay was carried out according to the method of [Beauchamp and Fridovich \(1971\)](#). The reaction mixture contained 0–2.5  $\mu\text{M}$  kinobeaon A, lignan or quercetin, 41.5 mM  $\text{NaHCO}_3\text{-Na}_2\text{CO}_3$  buffer (pH 10.2), 0.1 mM xanthine, 0.1 mM EDTA, 0.005% bovine serum albumin and 25  $\mu\text{M}$  nitroblue tetrazolium (NBT). After preincubation at 25 °C for 10 min, 0.21 U/ml XOD was added to the mixture, and the mixture was incubated at 25 °C for 20 min. The buffer solution was added in place of test compound solution as a positive control, and was used in place of XOD solution as a negative control. The reaction was stopped by adding 0.2 mM  $\text{CuCl}_2$ . The activity was estimated by measuring the absorbance at 560 nm using spectrophotometer, MPS-2000 (Shimadzu, Kyoto, Japan). The  $\text{O}_2^-$  scavenging activity was expressed as % of control.  $\text{IC}_{50}$  values were calculated from the residual activity (%) in a series of dilutions of the test compounds, with the concentration that inhibits 50% reported as the  $\text{IC}_{50}$ .

### *Superoxide anion scavenging activity using KO<sub>2</sub>/XTT system*

A non-enzymatic  $\text{O}_2^-$  formation system, which generates  $\text{O}_2^-$  directly from potassium superoxide ( $\text{KO}_2$ ), was employed according to the previous report ([McPherson et al, 2002](#)). Stock solutions (100 mM) of  $\text{KO}_2$  were prepared fresh in anhydrous DMSO containing 500 mM crown ether dicyclohexyl-18-crown-6. Test compounds (0–3  $\mu\text{M}$  of kinobeaon A, lignan or quercetin) were incubated in 50 mM  $\text{Na}_2\text{CO}_3$  (pH 9.4). Following a 5 min preincubation period at 25 °C,  $\text{KO}_2$  (4.2 mM final) was added from stock solutions and the reactions were allowed to proceed for an additional 3 min. The ability of the  $\text{KO}_2$  solutions to release superoxide anion upon addition to aqueous solution was monitored by the reduction of 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene- sulfonic acid hydrate (XTT) measured as the increase of in absorbance at 470 nm. Control incubations received  $\text{KO}_2$  that had been prepared in 0.1 M HCl to promote dismutation to hydrogen peroxide. DMSO and 18-crown-6 were added to all control incubations. The  $\text{O}_2^-$  scavenging activity was expressed as % of control.  $\text{IC}_{50}$  values were calculated from the residual activity (%) in a series of dilutions of the test compounds.

### *Statistical analysis*

Values of cell viability are shown as mean  $\pm$  standard error. Statistical analysis was performed with ANOVA. Statistical significances were defined as  $P < 0.001$  (\*\*\*),  $P < 0.01$  (\*\*),  $P < 0.05$  (\*).

## **Results**

### *Effects of kinobeaon A on lipid peroxidation*

The effect of kinobeaon A, lignan (nordihydroguaiaretic acid) and quercetin on the peroxidation of lipids was examined using the NADPH-dependent microsomal lipid peroxidation system. The reaction was carried out within the range of 0–25  $\mu\text{M}$  kinobeaon A, lignan or quercetin. The value of TBARS increased with time, while it was depressed effectively as the concentration of kinobeaon A increased ([Fig. 2A](#)). The formation of TBARS was completely inhibited by 10  $\mu\text{M}$  of kinobeaon A. Similar

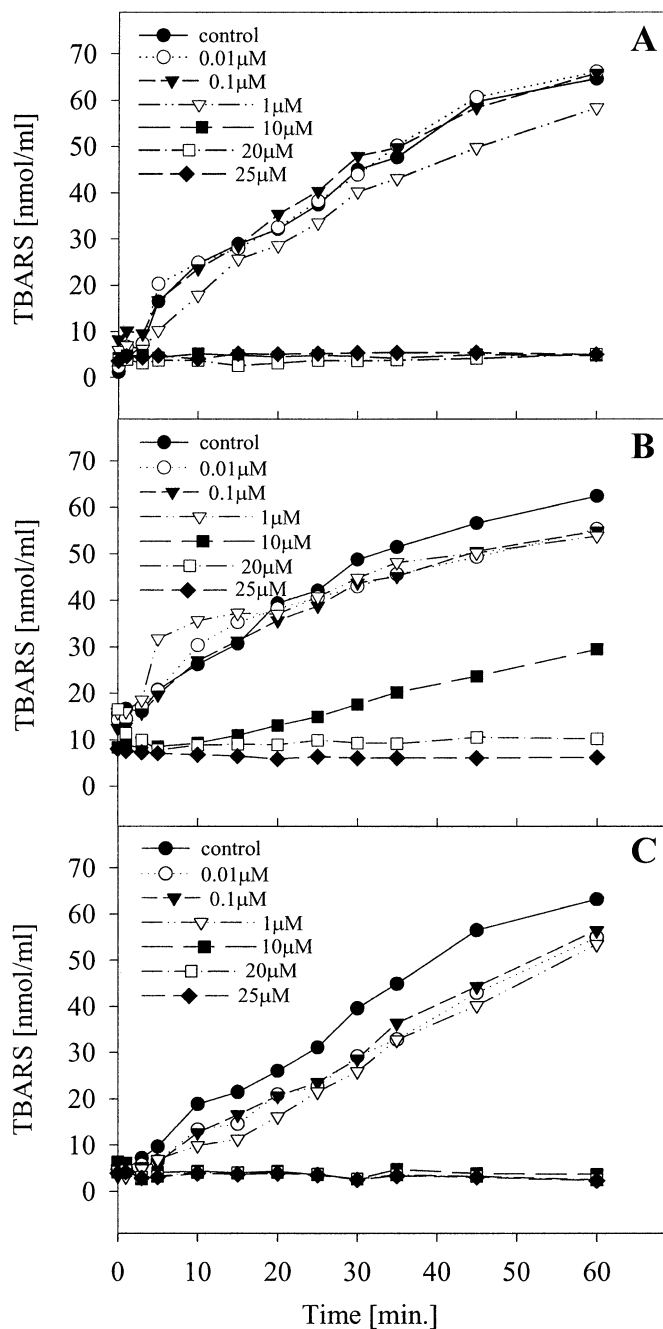


Fig. 2. Effects of kinobeeon A, lignan and quercetin on the NADPH-dependent microsomal lipid peroxidation. Kinobeeon A, lignan (nordihydroguaiaretic acid) or quercetin within the range of 0–25  $\mu\text{M}$  were incubated in the NADPH-dependent microsomal lipid peroxidation system for 0–60 min. A, kinobeeon A; B, lignan; C, quercetin. Kinobeeon A decreased TBARS value in a concentration-dependent manner. 10  $\mu\text{M}$  of kinobeeon A completely inhibited the lipid peroxidation. This experiment was done in triplicate, and a representative figure is displayed.

inhibitory curves were obtained when using lignan (Fig. 2B) and quercetin (Fig. 2C). The formation of TBARS was completely inhibited by 20  $\mu\text{M}$  of lignan and 10  $\mu\text{M}$  of quercetin.

### Superoxide anion scavenging activity

The  $\text{O}_2^-$  scavenging activity of kinobeon A is shown in Fig. 3A and 3B, comparing it with lignan and quercetin. In both the xanthine/XOD/NBT system and the  $\text{KO}_2$ /XTT system, kinobeon A dose-dependently increased the  $\text{O}_2^-$  scavenging activity.  $\text{IC}_{50}$  values were as follows: kinobeon A, 1.0  $\mu\text{M}$ ;

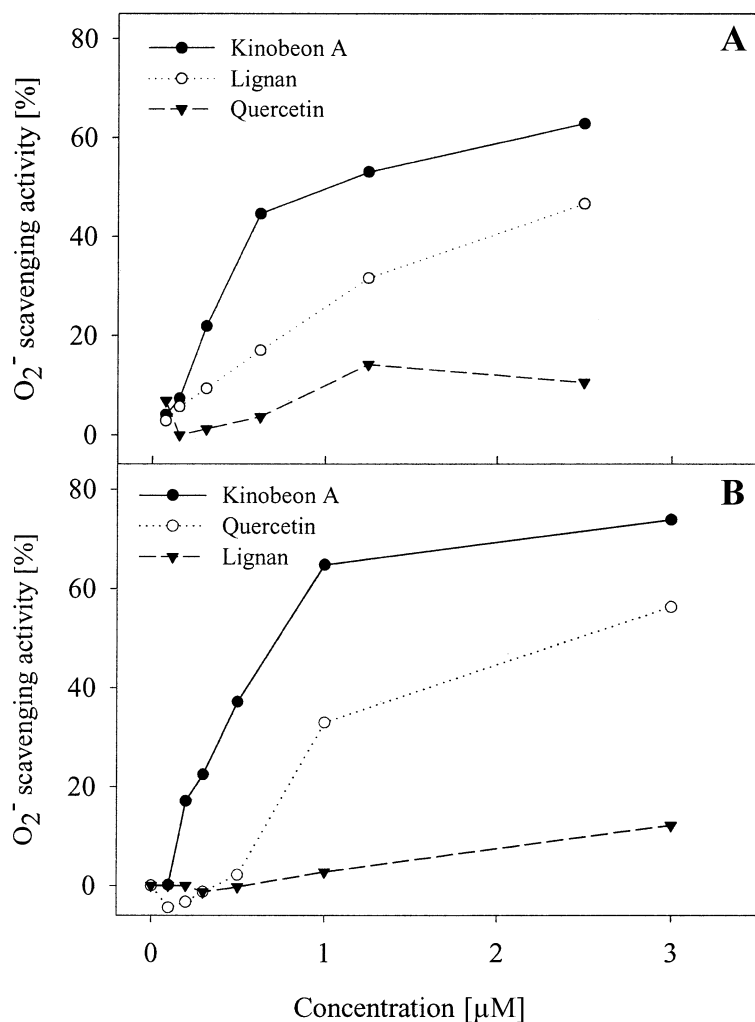


Fig. 3. Effects of kinobeon A, lignan and quercetin on superoxide anion scavenging activity. The xanthine/XOD/NBT system (A) and the  $\text{KO}_2$ /XTT system (B) were used for the formation of superoxide anion ( $\text{O}_2^-$ ). Kinobeon A scavenged  $\text{O}_2^-$  in a concentration-dependent manner.  $\text{IC}_{50}$  values calculated for kinobeon A in these two systems were 1  $\mu\text{M}$  and 0.8  $\mu\text{M}$ , respectively. The concentration of kinobeon A, at which  $\text{O}_2^-$  was scavenged 50%, was lower than that of lignan (nordihydroguaiaretic acid) and quercetin. This experiment was done in triplicate, and a representative figure is displayed.



lignan, 3.0  $\mu\text{M}$ ; quercetin, 30.0 (in the xanthine/XOD/NBT system); kinobeon A, 0.8  $\mu\text{M}$ ; lignan, 12.0  $\mu\text{M}$ ; quercetin, 2.5  $\mu\text{M}$  (in the  $\text{KO}_2$ /XTT system). Kinobeon A had the highest activity among these three compounds.

#### Cytotoxicity due to oxidative stress and protection by kinobeon A

Four oxidative systems,  $\text{H}_2\text{O}_2$ , Cu-OOH, menadione and XOD, were employed to investigate the cytoprotective effects of kinobeon A in MDBK cells compared with those of lignan and quercetin. Treatment of MDBK cells with the four oxidative systems significantly decreased the cell viability (Fig. 4A–4D). Treatment of the cells with 1–20  $\mu\text{M}$  of kinobeon A prior to  $\text{H}_2\text{O}_2$ , Cu-OOH, menadione and XOD exposure resulted in a remarkable increase in the cell viability in a concentration-dependent manner, compared to the results without pretreatment with kinobeon A. Vehicle (DMSO) did not affect

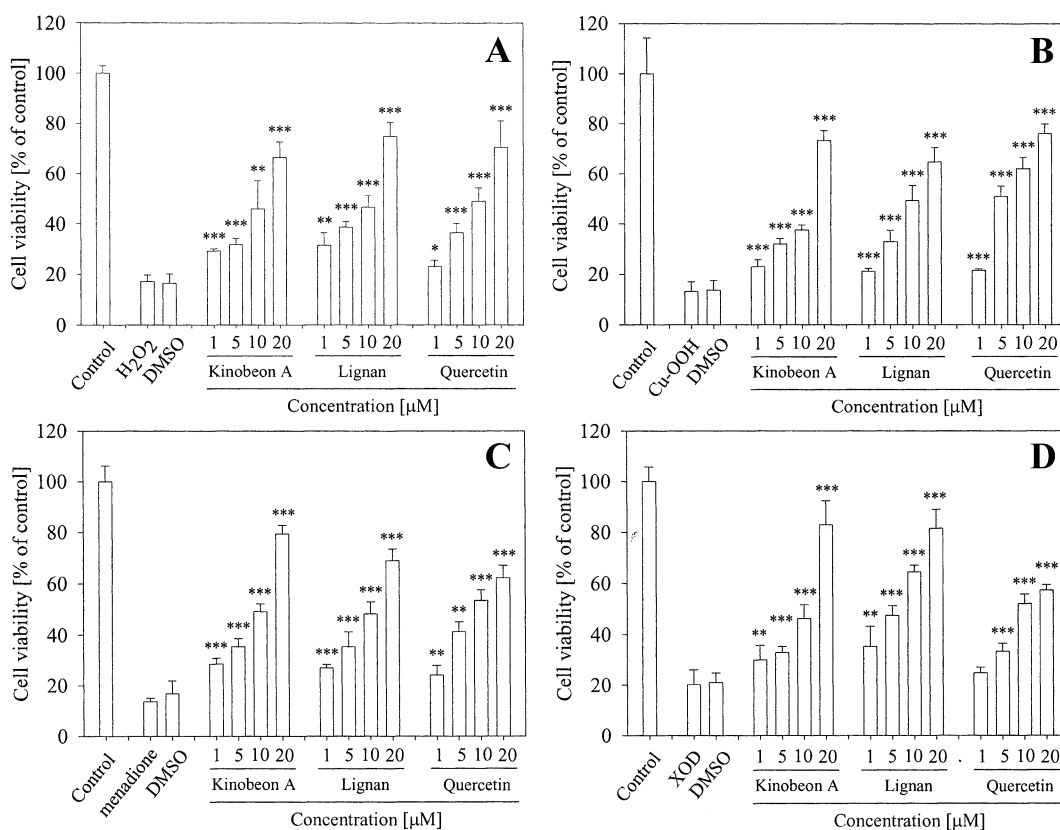


Fig. 4. Effects of kinobeon A, lignan and quercetin on oxidative stress-induced cytotoxicity in MDBK cells. Cytoprotective effects of kinobeon A in MDBK cells following treatment with  $\text{H}_2\text{O}_2$ , Cu-OOH, menadione and xanthine oxidase (XOD) were investigated. Cell survival decreased by these four oxidative agents was restored by adding kinobeon A, lignan and quercetin in a dose-dependent manner. At 20  $\mu\text{M}$ , the tested compounds restored cell survival to 66–83% of non-oxidation control. A,  $\text{H}_2\text{O}_2$ ; B, Cu-OOH; C, menadione; D, XOD. DMSO refers to oxidative agent plus DMSO. Values of cell viability are represented as mean (% of control)  $\pm$  standard error of 5 independent tests. \*\*\* $P$ <0.001; \*\* $P$ <0.01; \* $P$ <0.05 compared with values obtained without pretreatment with kinobeon A, lignan and quercetin.



the cell viability, which decreased with all of the oxidative stresses. Lignan and quercetin showed dose-dependent cytoprotective effects against all four oxidative stresses similar to that of kinobeeon A. Kinobeeon A at a concentration of 20  $\mu\text{M}$  increased the value of cell survival, which decreased to 14–20% due to oxidative stresses, to 66–83% of the control. At 20  $\mu\text{M}$  of kinobeeon A, the relative viability values of cells treated with lignan and quercetin to the values treated with kinobeeon A under each oxidative stress were as follows:  $\text{H}_2\text{O}_2$  (kinobeeon A: lignan: quercetin = 1.00: 1.12: 1.06), Cu-OOH (1.00: 0.88: 1.04), menadione (1.00: 0.87: 0.78), and XOD (1.00: 0.98: 0.69). Kinobeeon A exhibited stronger effect on the cell viability than lignan and quercetin, when menadione and XOD were used as inducing reagents of oxidative stress. Similar cytoprotective actions of kinobeeon A were observed in human hepatoma cell line, HepG2 against oxidative stresses (data not shown).

## Discussion

In this study, the antioxidant activity of kinobeeon A was compared with that of two representative natural antioxidants, lignan (nordihydroguaiaretic acid) and quercetin.

Kinobeeon A was shown to have more significant inhibitory activity against lipid peroxidation in rat liver microsomes (Fig. 2A–2C) and shown to have more effective  $\text{O}_2^-$  scavenging activity in both the xanthine/ XOD/NBT and the  $\text{KO}_2$ /XTT systems than those of lignan or quercetin (Fig. 3A and 3B). The present study proved for the first time that kinobeeon A had antioxidative activities. It is a well-documented fact that some anti-oxidative compounds isolated from safflower oil cake possess scavenging abilities against reactive oxygen species, such as  $\text{O}_2^-$  and against  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radicals (Zhang et al., 1996, 1997). While anti-oxidative compounds from plant cell cultures are rather rare (Wang et al., 1999), kinobeeon A may be produced from safflower cells by modifying medium components (Wakayama et al., 1994) and/or physical stresses (such as rotation of culture bottles to achieve cell suspension). Environmental changes including those induced by chemical and/or physical conditions in culture media seem to elicit self-defense systems of the plant cells to prevent its own damage. An induced antioxidant activity might be one of the self-defense systems for safflower cells. Kinobeeon A possesses four methoxy groups ( $-\text{OCH}_3$ ), highly conjugated double bonds and a quinone-like structure. Lu et al. reported that methoxy groups may contribute to free radical scavenging activity in the structures of lignans (Lu and Liu, 1991). There are also some reports describing that the degree of methylation of hydroxyl groups in the structure of a lignan (nordihydroguaiaretic acid) significantly altered its antiviral activities (Hwu et al., 1998; Chen et al., 1998). The four  $-\text{OCH}_3$  groups in the structure of kinobeeon A may be the functional groups primarily responsible for the effective inhibition of lipid peroxidation and/or the scavenging free radicals. We have examined  $\text{O}_2^-$  scavenging capacity of kinobeeon A using different  $\text{O}_2^-$  generation systems. Kinobeeon A markedly scavenged  $\text{O}_2^-$  in both the xanthine/XOD/NBT system (enzymatic reaction) and  $\text{KO}_2$ /XTT system (non-enzymatic reaction). This result indicates that kinobeeon A can directly scavenge  $\text{O}_2^-$ .

Treatment with kinobeeon A prior to  $\text{H}_2\text{O}_2$ , Cu-OOH, menadione and XOD exposure resulted in a significant increase in survival of MDBK and HepG2 cells, compared to the results in absence of treatment with kinobeeon A (Figs. 4A–4D). The recovery ratios of the cell viability by kinobeeon A against oxidative treatments were stronger than those of lignan or quercetin when menadione and XOD were used as inducing reagents of oxidative stress. This was the first documented evidence that kinobeeon A had cytoprotective activities. Many studies have documented that a typical anti-oxidative lignan,

nordihydroguaiaretic acid, may scavenge free radicals such as hydroxyl ( $\text{HO}^\cdot$ ), *tert*-butyl alkoxy ( $t\text{-BO}^\cdot$ ) and *tert*-butyl peroxy ( $t\text{-BOO}^\cdot$ ) radicals, and may suppress  $\text{H}_2\text{O}_2$ - and *t*-butylhydroperoxide-induced cytotoxicity (Harper et al., 1999; Ng et al., 2000). The cytoprotective effects of flavonoids have also been explained through the direct radical scavenging action and the synergistic interaction with the cellular anti-oxidative enzyme, glutathione peroxidase (Nagata et al., 1999). Based on the results in this paper, cytoprotective effects of kinobeaon A could be explained by its ability to scavenge radicals, such as alkoxy, lipid peroxy and  $\text{O}_2^-$  radicals relating to various biological effects of lignans, flavonoids and other phenolic compounds as follows: (a) direct radical and lipid peroxide scavenging actions (Harper et al., 1999; Ng et al., 2000) and synergistic interaction with other antioxidants (Nagata et al., 1999); (b) antioxidant properties preventing oxidative attack on membrane lipids by sparing vitamin E or by regenerating vitamin E as does vitamin C (Gey and Puska, 1989); (c) inhibition of lipoxygenases (Agarwal et al., 1991; Capdevila et al., 1988) which are known to be stimulated by lipid peroxides and could be involved in the oxidative stress and (d) inhibition of cellular enzymes involved in signal transduction (Nikaido et al., 1981; Kumada et al., 1978). Although oxidation products of kinobeaon A in the oxidation systems used in this study are not identified, we observed that certain peak(s) was increased with time during oxidation reaction, indicating that relatively stable oxidation products might be occurred (unpublished data). To reveal the mode of action on the anti-oxidative activity of kinobeaon A, further study of structure-activity relationships will be required. The elucidation of the mechanism of the kinobeaon A production in the safflower cell culture system may also help clarify the mechanism of radical scavenging and cytoprotective activity by kinobeaon A.

In conclusion, kinobeaon A is able not only to act as an  $\text{O}_2^-$  scavenger and/or to inhibit lipid peroxidation but also to prevent cytotoxicity induced with oxidative stress at the cellular level. Our current results suggest that a small dose of kinobeaon A could be a potent drug to prevent oxidative stress related diseases.

## Acknowledgements

Authors thank Dr. Erwin J. Landon for critical reading of the manuscript. Dr. Takekoshi has been supported by grants-in-aid for Scientific Research from the Ministries of Education, Science and Culture, and Health and Welfare of Japan, and funds from Tokai University School of Medicine, Project Research.

## References

- Agarwal, R., Wang, Z.Y., Bik, D.P., Mukhtar, H., 1991. Nordihydroguaiaretic acid, an inhibitor of lipoxygenase, also inhibits cytochrome P-450-mediated monooxygenase activity in rat epidermal and hepatic microsomes. *Drug Metabolism and Disposition: the Biological Fate of Chemicals* 19 (3), 620–624.
- Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44 (1), 276–287.
- Capdevila, J., Gil, L., Orellana, M., Marnett, L.J., Mason, J.I., Yadagiri, P., Falck, J.R., 1988. Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism. *Archives of Biochemistry and Biophysics* 261 (2), 257–263.
- Chen, H., Teng, L., Li, J.-N., Park, R., Mold, D.E., Gnanre, J., Hwu, J.R., Tseng, W.N., Huang, R.C.C., 1998. Antiviral activities of methylated nordihydroguaiaretic acids. 2. Targeting Herpes Simplex virus replication by the mutation insensitive transcription inhibitor tetra-O-methyl-NDGA. *Journal of Medicinal Chemistry* 41 (16), 3001–3007.

- Diaz-Lanza, A.M., Abad-Martinez, M.J., Fernandez-Matellano, L., Recuero-Carretero, C., Villaescusa-Castillo, L., Silvan-Sen, A.M., Bermejo-Benito, P., 2001. Lignan and phenylpropanoid glycosides from *Phillyrea latifolia* and their in vitro anti-inflammatory activity. *Planta Medica* 67 (3), 219–223.
- Gey, K.F., Puska, P., 1989. Plasma vitamins E and A inversely correlated to mortality from ischemic heart disease in cross-cultural epidemiology. *Annals of the New York Academy of Sciences* 570, 268–282.
- Harper, A., Kerr, D.J., Gescher, A., Chipman, J.K., 1999. Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. *Free Radical Research* 31 (2), 149–160.
- Hwu, J.R., Tseng, W.N., Gnabre, J., Giza, P., Huang, R.C.C., 1998. Antiviral activities of methylated nordihydroguaiaretic acids. 1. Synthesis, structure identification, and inhibition of tat-regulated HIV transactivation. *Journal of Medicinal Chemistry* 41 (16), 2994–3000.
- Ito, C., Itoigawa, M., Ogata, M., Mou, X.Y., Tokuda, H., Nishino, H., Furukawa, H., 2001. Lignans as anti-tumor-promoter from the seeds of *Hernandia ovigera*. *Planta Medica* 67 (2), 166–168.
- Kanehira, T., Homma, T., Saito, K., 2000. On restricting activity of carthamin and safflower yellow B against superoxide anion formation in an experimental model system. *Roumanian Biotechnological Letters* 5 (5), 361–364.
- Kernan, M.R., Sendl, A., Chen, J.L., Jolad, S.D., Blanc, P., Murphy, J.T., Stoddart, C.A., Nanakorn, W., Balick, M.J., Rozhon, E.J., 1997. Two new lignans with activity against influenza virus from the medicinal plant *Rhinacanthus nasutus*. *Journal of Natural Products* 60 (6), 635–637.
- Kumada, Y., Naganawa, H., Takeuchi, T., Umezawa, H., 1978. Biochemical activities of the derivatives of dehydrodicaffeic acid dilactone. *The Journal of Antibiotics* 31 (2), 105–111.
- Lu, H., Liu, G.T., 1991. Effect of dibenzo[a,c]cyclooctene lignans isolated from *Fructus schizandrae* on lipid peroxidation and anti-oxidative enzyme activity. *Chemico-Biological Interactions* 78 (1), 77–84.
- McPherson, D.B., Kilker, R.P., Foley, T.D., 2002. Superoxide activates constitutive nitric oxide synthase in a brain particulate fraction. *Biochemical and Biophysical Research Communications* 296, 413–418.
- Nagata, H., Takekoshi, S., Takagi, T., Honma, T., Watanabe, K., 1999. Antioxidative action of flavonoids, quercetin and catechin, mediated by the activation of glutathione peroxidase. *The Tokai Journal of Experimental and Clinical Medicine* 24 (1), 1–11.
- Ng, T.B., Liu, F., Wang, Z.T., 2000. Antioxidative activity of natural products from plants. *Life Sciences* 66 (8), 709–723.
- Nikaido, T., Ohmoto, T., Noguchi, H., Kinoshita, T., Saitoh, H., Sankawa, U., 1981. Inhibitors of cyclic AMP phosphodiesterase in medicinal plants. *Planta Medica* 43 (1), 18–23.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95 (2), 351–358.
- Schultze-Mosgau, M.H., Dale, I.L., Gant, T.W., Chipman, J.K., Kerr, D.J., Gescher, A., 1998. Regulation of c-fos transcription by chemopreventive isoflavonoids and lignans in MDA-MB-468 breast cancer cells. *European Journal of Cancer* 34 (9), 1425–1431.
- Wakayama, S., Kusaka, K., Kanehira, T., Yamada, Y., Kawazu, K., Kobayashi, A., 1994. Kinobeaon A, A novel red pigment produced in Safflower tissue culture systems. *Zeitschrift für Naturforschung* 49c, 1–5.
- Wang, P., Ou, S., Peilin, C., 1999. Optimization of conditions for safflower cell culture and accumulation of cellulosic product tocopherols. *Chinese Journal of Biotechnology* 15 (4), 231–237.
- Zhang, H.L., Nagatsu, A., Sakakibara, J., 1996. Novel antioxidants from safflower (*Carthamus tinctorius* L.) oil cake. *Chemical and Pharmaceutical Bulletin* 44 (4), 874–876.
- Zhang, H.L., Nagatsu, A., Watanabe, T., Sakakibara, J., Okuyama, H., 1997. Antioxidative compounds isolated from safflower (*Carthamus tinctorius* L.) oil cake. *Chemical and Pharmaceutical Bulletin* 45 (12), 1910–1914.
- Zhou, Y.C., Zheng, R.L., 1991. Phenolic compounds and an analog as superoxide anion scavengers and antioxidants. *Biochemical Pharmacology* 42 (6), 1177–1179.