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Constituents of the Essential Oil of the *Cinnamomum cassia* Stem Bark and the Biological Properties

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GC-MS analysis on the essential oil (CC-oil) of *Cinnamomum cassia* stem bark led to the identification of cinnamaldehyde (CNA, **1**), 2-hydroxycinnamaldehyde (2-CNA), coumarin (**2**), and cinnamyl acetate. The major volatile flavor in CC-oil was found to be 2-CNA. Coumarin was first isolated from this plant by phytochemical isolation and spectroscopic analysis. CNA and CC-oil showed potent cytotoxicity, which was effectively prevented by N-acetyl-L-cysteine (NAC) treatment. Intraperitoneal administration with CNA considerably decreased malondialdehyde (MDA) formation and glutathione S-transferase activity in rats. These results suggest that CC-oil and CNA can regulate the triggering of hepatic drug-metabolizing enzymes by the formation of a glutathione-conjugate.

Key words: *Cinnamomum cassia,* Lauraceae, cinnamaldehyde, essential oil, cytotoxicity, glutathione *S*-transferase

INTRODUCTION

The stem Bark of *Cinnamomum cassia* has been traditionally used in both Eastern and Western countries for treating dyspepsia, gastritis, blood circulation disturbances and inflammatory diseases (Tang *et al.*, 1992). *C. cassia* has a very unique and strong flavor, and the most characteristic substance is known to be cinnamaldehyde (CNA). However, a previous study by the authors showed a large 2-cinnamaldehyde peak by gas chromato-graphy (GC) on the essential oil of *C. cassia* CC-oil). Therefore, it was assumed that CC-oil is a source of 2-CNA rather than of CNA. Several biological activities such as peripheral vasodilatory (Tang *et al.*, 1992), antitumor (Lee C. W. *et al.*, 1999), antifungal (Bang, K. H. *et al.*, 2000) and cytotoxic (Kwon *et al.*, 1998) and mutagenic activity (Dillon *et al.*, 1998) were mainly focused on CNA. Lee *et al.* (1999) have suggested that 2-hydroxycinnamaldehyde (2-CNA) may be an important antitumor agent. With the exception of that report, the availability of CC-oil has not been evaluated as a source of 2-CNA.

In order to elucidate the phytochemical contents, *in vitro* cytotoxicity and *in vivo* hepatic function, various active compounds were isolated and the effect of these compounds on the lipid peroxidation and the activity of hepatic drug-metabolizing enzymes was investigated. Therefore, the establishment of the *in vivo* toxicity and pharmacological effect of CNA may lead to a better use of CC-oil.

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MATERIALS AND METHODS

Instrument

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were recorded on a Hitachi 260-01 spectrometer on KBr Electron Impact-Mass spectra (EI-MS, ionization voltage 70 eV) were taken on a Finnigan Mat TSQ-700.

Plant material and steam-distillation

Commercially available plant material was purchased from commercially available sources and identified as *C. cassia* by Dr. G.T. Kim (Department of Forestry, Sangji University, Wonju, Korea). This plant material (1.5 kg)

was extracted by steam distillation for 4 h. The distilled liquid was extracted with diethyl ether and dehydrated with anhydrous sodium sulfate followed by evaporation on a rotatory evaporator at 40°C. The residual oil (9 g) was obtained and used for chemical analysis, further purification, and biological assays.

Isolation of compounds 1 and 2

The extracted material was subjected to silica gel column chromatography (Art No. 7734, Merck, Germany, 3 × 21 cm, 60 g) and eluted with *n*-hexane-ethyl acetate (10:1). The eluate was collected in 10 ml fractions. After the elution volume reached 400 ml, the solvent was switched to *n*-hexane-ethyl acetate (10:2). A total of 96 fractions were collected and checked by spraying a vanillin-sulfuric acid reagent. Fractions showing a similar TLC pattern were combined and dried *in vacuo*. As a result, five fractions (fraction 1-5) were obtained. Fraction 2 of the retention volume 290-390 ml was further purified by preparative TLC and yielded pure volatile compound **1** (colorless oil, 1.2 g). Fraction 4 over the retention volume of 640-760 ml was recrystallized in *n*-hexane and yielded compound **2** (colorless needles, 20 mg), which could be observed under UV.

Compound **1**: colorless oil, UV λ_{max}, DMSO): 290 nm; IR (KBr, cm^{-1}) ν_{max}: 1621 (aromatic C=C), 1684 (α,β-unsaturated aldehyde); ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 6.72 (1H, dd, *J*=7.7 and 15.9 Hz, H-8), 7.44 (1H, d, *J*=15.9 Hz, H-7), 7.45 (2H, d-like, H-2,6), 7.54 (3H, m, H-3,4,5), 9.70 (1H, d, *J*=7.7 Hz); ^{13}C-NMR (50 MHz,, $CDCl_3$): 193.5 (C-9), 152.6 (C-8), 133.8 (C-1), 131.1 (C-3), 128.9 (C-2,6), 128.7 (C-3,5), 128.3 (C-7); MS (70 eV) *m/z* (%): 132.1 (M^+, 69), 131.1 ([M-H]$^+$, 100), 103.1 ([M-CO]$^+$, 44), 77 ([phenyl]$^+$, 24).

Compound **2**: colorless needle from *n*-hexane; mp 68-70° ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 7.72 (1H, d, *J*=9.5 Hz, H-3), 7.51 (2H, dd, *J*=7.0, 1.8 Hz, H-5,8), 7.32 (2H, t, *J*=7.0 Hz, H-6,7), 6.44 (1H, d, *J*= 9.5 Hz, H-4); ^{13}C-NMR (50 MHz, $CDCl_3$): 160.8 (C-2), 154.0 (C-9), 143.4 (C-4), 131.8 (C-3), 127.8 (C-5), 124.3 (C-8), 118.8 (C-3), 116.9[a] (C-6), 116.6[a] (C-10); MS (70 eV) m/z (%): 146 (M^+, 100), 118 ([M-CO]$^+$, 98), 90 (47), 63 (20). The values with a superscript (a) may be interchangeable.

GC-Analysis

Column {DB-1 (length 30 meters, i.d. 0.25 mm, film thickness 0.25 mm, J&W Scientific, USA)}, Column temp. program {init. temp. 50°C (3 min), temp. increase velocity (8°C/min), final temp. 250°C (10 min)}; solvent cut (3 min); temp. program {injector (250°C), transfer line (250 °C), ion source (150°C), manifold (70°C)}, detector {Electron Impact-Quadrupole 1 (EI energy 70 eV); carrier gas {He (99.99%), flow rate (1.5 ml/min)}.

Peak 1 (cinnamaldehyde)-RT 13.97 min, Mass spectrum of peak 1 was identical to that of compound **1**.

Peak 2 (2-hydroxycinnamaldehyde)-RT 16.87 min; MS *m/z* (%): 148 (M^+, 3), 147 ([M-H]$^+$, 100), 115 (49).

Peak 3 (coumarin)-RT 17.07 min, Mass spectrum of peak 3 was identical to that of compound **2**.

Peak 4 (cinnamy acetate): RT 17.19 min, MS *m/z* (%): 176 (M+, 25), 134 ([M-HCHO]$^+$, 44), 133 ([M-CH_3CO]$^+$, 115 ([ph-CH=C=CH]$^+$, 100), 105 (26), 92 (24), 77 ([C_6H_5]$^+$, 15), 43 (53).

MTT assay

The *in vitro* tests against 3LL cells (human lung carcinoma) was carried out according to the method described previously (Denizot *et al.*, 1996). The cells (1×10^4) were seeded in each well containing 100 μl of the RPMI medium supplemented with 10% FBS in a 96-well microtiter plate and incubated overnight. The test samples, CC-oil, CNA, and compound **2**, were dissolved in dimethylsulfoxide (DMSO) and added in serial dilution (the final DMSO concentrations in all assays did not exceed 0.01 %). Twenty-four hours after seeding, 100 μl of the new media or test samples were added, and the plates were then incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved with 50 μl DMSO. The optical density was measured at 540 nm.

Animal

The experimental animals were purchased from Korean Experimental Animal Co. and were adapted to constant conditions (temperature: 20 ± 2°C, humidity: 40-60%, light/dark cycle: 12 h) for more than two weeks. For the animal experiments, Sprague-Dawley male rats weighing 100-120 g were used. The animals were fasted 24 h prior to the experiments. Considering the variation of enzyme activity during one day, the animals were sacrificed at a set time (10:00 A.M.-12:00 A.M.).

Isolation of enzyme sources

After being anesthetized with CO_2 gas, animals were decapitated from the abdomen aorta. The liver was washed by fluxing in saline and the liver tissue was homogenized in a four-fold quantity of ice-cooled 0.1M sodium phosphate buffer (pH 7.4). This suspension was centrifuged in the cool state (600 × g, 10 min). The supernatant was again subjected to centrifugation (10,000 × g, 20 min) and the precipitate was taken. This was subsequently suspended with an equivalent volume of 0.1 M sodium phosphate buffer (pH 7.4) and further subjected to centrifugation (105,000 × g, 30 min). The resultant mitochondrial fraction was used to measure the catalase

activity as an enzyme source. The supernatant obtained from centrifugation of the final one (105,000 × g, 60 min) was used as an enzyme source of the cytosolic fraction to measure the activities of xanthine oxidase and aldehyde oxidase. The resulting precipitate was centrifuged (105,000 × g, 60 min) in 0.1 M sodium phosphate buffer (pH 7.4) and the obtained microsomal fraction was used for the enzyme sources to measure the cytochrome P_{450}, aminopyrine N-demethylase, and aniline hydroxylase activities.

Measurement of malondialdehyde

The animals were sacrificed by decapitating the blood from the abdominal aorta under slight anesthesia with CO_2 gas. The liver was thoroughly perfused with ice-cold normal saline through the portal vein until uniformly pale and weighed. The thiobarbituric acid (TBA) reactive substance in the liver was measured as a marker of lipid peroxidation by the method reported by Ohkawa *et al.* (1979). An aliquot (0.4 ml) of the 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of an 8.1% SDS, 1.5 ml of a 20% acetate buffer (pH 3.5) and 1.5 ml of a 0.8% TBA solution. The mixture was heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol:pyridine (15:1) was added for extraction, and the absorbance of the *n*-butanol:pyridine layer at 532 nm was measured to identify the TBA reactive substance.

Determination of aminopyrine *N*-demethylase activity

The aminopyrine *N*-demethylase activity was determined according to the Nash's method (1953). briefly, the solution (2 ml) containing 2 mM aminopyrine, 0.5 mM NADPH, 10 mM $MgCl_2$, 150 mM KCl and 1 mM semicarbazide in 0.1 M Na^+/K^+ phosphate buffer (pH 7.5) was reacted for 15 min. The reaction was subsequently quenched by adding 15% $ZnSO_4$ and saturated $Ba(OH)_2$. This reactant was centrifuged and the supernatant was colored with Nash reagent. Finally, the activity of aminopyrine N-demethylase was calculated from the absorbance at the 415 nm wavelength.

Determination of aniline hydroxylase activity

The aniline hydroxylase activity was determined according to the method reported by Bidlack and Lowery (1982). Briefly, 1 mM aniline, 0.5 mM NADPH and the enzyme sources were added to the solution containing 50 mM Tris. HCl buffer (pH 7.4), 10 mM $MgCl_2$ and 150 mM KCl to a final volume adjusted to 2.0 ml. This solution was reacted at 37°C for 20 min and the reaction was terminated by adding 20% trichloroacetic acid. The reaction mixture was then centrifuged, and 10% Na_2CO_3 and 0.2N NaOH/2% phenol were added to the obtained supernatant. This solution was reacted at 37°C for 30 min and the absorbance was measured at 640 nm. The enzyme activity was calculated from a standard calibration curve. The unit of enzyme activity was expressed as the amount (nmole) of *p*-aminophenol in 1 mg protein produced per minute.

Determination of xanthine oxidase activity

The xanthine oxidase activity was determined according to the the method reported by Stirpe and Della (1969). In summary, a mixture of 3.0 ml of 0.1 M potassium phosphate buffer (pH 7.5) and 0.1 ml of 60 mM sodium xanthine (substrate) was reacted at 37°C. Subsequently, the protein from the mixture was removed by adding 20 % trichloroacetic acid. The absorbance of the obtained supernatant was measured at 292 nm. The activity level was calculated using a standard calibration curve. The unit of enzyme activity was expressed as nmoles uric acid in 1 mg protein producing per minute.

Determination of the aldehyde oxidase activity

The aldehyde oxidase activity was determined according to the method reported by Rajagopalan (1968). A mixture of 0.1 M potassium phosphate buffer (pH 7.5), N-methylnicotinamide (substrate) and the enzyme solution was reacted. The absorbance of the produced 2-pyridone was measured at 300 nm. The enzyme activity was calculated using a standard calibration curve. The unit of enzyme activity was expressed as nmoles of 2-pyridone in 1 mg protein producing per minute.

Determination of the glutathione *S*-transferase activity

The glutathione *S*-transferase activity was determined according to the method reported by Habig (1974). In summary, 75 µl of 40 mM reduced glutathione was added to 0.1M potassium phosphate buffer (pH 6.5) in the test tube followed by adding 100 µl of the enzyme solution. In the blank, the reaction was terminated by 20% trichloroacetic acid. In each of the test- and blank solutions, the mixture was reacted at 25°C for 5 min and followed by being reacted again with 25 µl of 2,4-dinitrochlorobenzene (substrate). The reaction was quenched with 20 % trichloroacetic acid and centrifuged. The absorbance of each supernatant obtained after centrifuging each test- and blank reactant was measured at 340 nm wavelength. The activity (nmole) was calculated using a molar absorption coefficient (9.6 $mM^{-1}cm^{-1}$).

Statistics

The data is reported as a mean ± S.D.. Statistical significance was analyzed by the Duncan's multiple range test.

RESULTS AND DISCUSSION

The essential oil of *C. cassia* (CC-oil) was obtained from the plant material by steam distillation with a yield of

0.7% (w/w). Column chromatographic separation of the essential oil afforded five fractions (fraction 1-5). Preparative TLC purification of fraction 2 yielded compound **1** (colorless oil) and recrystallization of fraction 4 afforded compound **2** (colorless needle). In the ^{1}H-NMR spectrum of compound **1**, the aldehydic proton was splitted as doublet (J=7.7 Hz) at δ 9.70 and the two olefinic protons (δ 7.44 and δ 6.72) of H-7 and H-8 were found as doublet (J=15.9 Hz) and doublet of doublets (J=7.7 and 15.9 Hz), respectively. The coupling constant of 15.9 Hz indicated that the double bond has a *trans*-geometry. Two and three protons signals were observed at δ 7.45 (d-like) and δ 7.54 (m), respectively, which was assigned to aromatic protons. ^{13}C-NMR data supported that compound **1** is CNA as shown in the experimental section. In the ^{1}H-NMR spectrum of Compound **2**, two *ortho*-coupled protons {d 7.72 (1H, d, J=9.5 Hz) and d 6.44 (1H, d, J= 9.5 Hz)} and the two *di*-ortho-coupled protons {d 7.51 (1H, dd, J=7.0, 1.8 Hz)} and d 7.32 (1H, t, J=7.0 Hz) were observed. Nine peaks in the ^{13}C-NMR spectrum indicated that compound **2** is coumarin. The molecular ion (*m/z* 132) in mass spectrum of compound **2** demonstrated that it is a non-substituted coumarin. GC-MS analysis showed four major peaks in the chormatogram (Fig. 1). Peaks 1-4 were identified as CNA, 2-CNA, coumarin, and CNA. The mass spectra of CNA and 2-CNA showed the characteristic base peaks $[M-H]^+$ at m/z 131 and m/z 147, respectively.

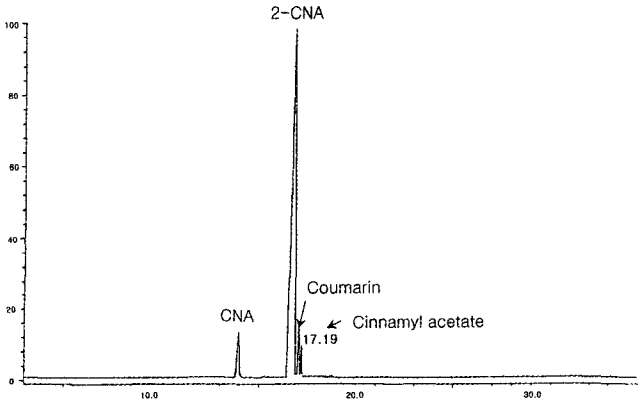
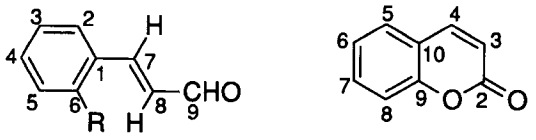


Fig. 1. Gas chromatogram of the essential oil of *C. cassia*



CNA : R=H
2-CNA : R=OH

Coumarin

Fig. 2. Structure of cinnamaldehyde (**1**, CNA), 2-hydroxycinnamaldehyde (2-CNA) and coumarin (**2**)

As shown in Fig. 3, both CC-oil and CNA produced considerable cytotoxicity on 3LL Lewis carcinoma cells. Coumarin (**2**) and cinnamylacetate exhibited only mild cytotoxicity. This result implicated that of the *C. cassia* essential oil, the cytotoxic effect as well as the isolated CNA, and that the former contain other strong cytotoxic constituents. To determine the events involved in CC-oil and CNA-induced cytotoxicity, the NAC of a known antioxidant was examined for their capacity to interfere with the cytotoxic process. The results showed that both the CC-oil and CNA-induced cell death could be suppressed by NAC, a well known scavenger of reactive oxygen intermediates (ROI) (Fig. 3). The experiments on the CNA-induced cytotoxicity suggested that the electrophilicity of CNA induces strong cytotoxicity in 3LL-Lewis cells together with a notion that the cellular sulfhydryl may be mediated. Furthermore, oxidative injury may play a pivotal role in both the CC-oil and CNA-elicited cytotoxicity. Considering the chemical structure of CNA and NAC, it was suggested that Michael addition of the sulfhydryl compound to CNA plays a pivotal role in preventing the cytotoxicity. However, the flavor substance, 2-CNA, with stronger activity can be predicted as, which is more abundant in the plant. The hydroxy group of 2-CNA may enhance the electrophilicity because the ring-activating group, OH, is bonded at the meta-position of the alkenyl chain. The mild cytotoxicity of coumarin was not blocked by NAC. It appears that the four compounds that were shown on the GC chromatogram may be correlated biosynthetically. However, unrelated compounds amongst those aromatic compounds were not found on the GC chromatogram. It is well known that the cyclization of 2-hydroxyphenylpropanoid should form coumarin. In addition, the reduction of CNA can produce cinnamyl alcohol. The formation of OH, NH and SH are susceptible to the structure of an α,β-unsaturated carbonyl.

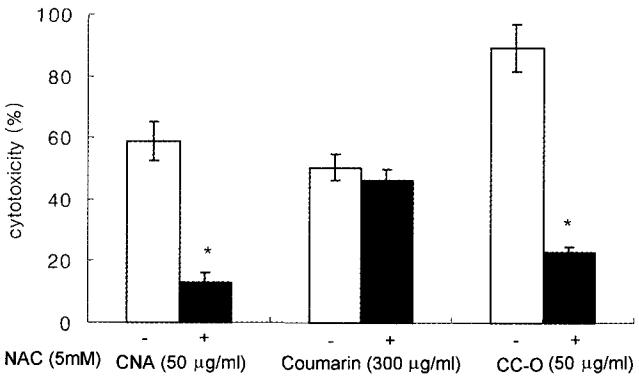


Fig. 3. Effect of N-aetyl-L-cysteine on the CNA and CC-oil-induced cytotoxicity on 3LL-Lewis cells. Each column represents the mean ± S.D. from at least three independent experiments performed in triplicates. The asterisk (*) indicates the means that are significantly different ($p<0.01$) from the control.

Table I. Effect of cinnamaldehyde (CNA) isolated from *C. cassia* essential oil (CC-oil) on hepatic drug-metabolizing enzymes in rats

Group Dose	Normal	Cinnamaldehyde (mg/kg)	
	0	25	50
MDA[2)]	25.3 ± 0.51[1)a]	20.4 ± 0.29[b]	18.6 ± 0.18[c]
Glutathione S-transferase[3)]	206.8 ± 9.43[a]	119.4 ± 11.01[a]	118.7 ± 8.96[a]
Glutathione[4)]	2.19 ± 0.18[a]	2.31 ± 0.20[a]	2.27 ± 0.24[a]
Epoxide hydrolase[5)]	12.3 ± 0.31[a]	23.6 ± 0.24[b]	28.2 ± 0.19[c]
Aminopyrine demethylase[6)]	2.86 ± 0.19[a]	1.73 ± 0.13[b]	1.06 ± 0.15[c]
Aniline hydroxylase[7)]	0.58 ± 0.06[a]	0.36 ± 0.07[b]	0.29 ± 0.09[b]
Aldehyde oxidase[8)]	1.38 ± 0.11[a]	1.44 ± 0.10[a]	1.38 ± 0.17[a]
Xanthine oxidase[9)]	2.34 ± 0.90[a]	2.47 ± 0.12[a]	2.50 ± 0.17[a]

Rats were intraperitoneally injected daily for seven days and animals were decapitated 24 h after the last injection.
[1)]Vlaues are mean ± S.D. for six experiments. Values with same superscript letter are not significantly different in each row ($p<0.05$); Unit: [2)]nmol/g of tissue [3)]1,2-dinitro-4-nitrobenzene nmol/mg protein/min [4)]μmol/g of tissue [5)]nmol/g of tissue [6)]HCHO nmol/mg protein/min [7)]p-aminophenol nmol/mg protein/min [8)]2-pyridone nmol/mg protein/min [9)]uric acid nmol/mg protein/min

Therefore, the occurrence of cinnamyl acetate may be associated with this fact. In addition, the weak cytotoxicity of coumarin and its lower inhibition by NAC can be attributed to the absence of the aldehydic function in that molecule.

Cytotoxic screening of natural substances frequently allows the prediction of several beneficial biological activities in addition to cancer. Therefore, it was a great concerning as to whether the CC-oil and CNA can provide considerable *in vivo* toxicity or not. By administering CNA with a 25 and 50 mg/kg dose for 7 days, MDA production due to hepatic lipid peroxidation and the activities of glutathione S-transferase were significantly decreased (Table I). It appears that the excess substrate inhibited the enzyme activity by an enzyme-catalyzed reaction. Many natural cytotoxic natural products have been reported to induce antioxidant enzymes. Hepatic microsomal enzymes, aldehyde oxidase and xanthine oxidase, both capable of generating hydroxyl radicals, were not significantly influenced by CNA administration, whereas the activities of hepatic microsomal aminopyrine demethylase and aniline hydroxylase, which are associated with phase I responsible for the xenobiotics, were considerably decreased. Generally, many mutagenic substances induce microsomal cytochrome P_{450} enzymes and form epoxide intermediates with strong electrophilicity as can be seen in carcinogens such as benzopyrene and aflatoxins. Therefore, the lowering of the two enzyme activities provided beneficial information regarding the treatment of diseases in adults. The increase of epoxide hydrolase activity suggested that epoxidation of the aromatic ring in CNA should not be a central route to the metabolism. Furthermore, the absence of a significant decrease in glutathione was in agreement with the absence of a MDA increase.

Therefore, from the finding of a blockade of cytotoxicity with NAC and the inhibition of glutathione *S*-transferase activity in rats, it was evident that both CNA and CC-oil were conjugated with sulfhydryl biomolecules in both 3LL cells and in rats. This covalent bonding may be the earliest stage for almost all the known biological activities of CNA. Based on the highly associated anti-inflammatory and anti-lipid peroxidative system in the body, investigations on inducible nitric oxide synthase, cyclooxigenase and tumor necrosis factor formations are necessary. The assumptions in this study depend on the traditional use of *C. cassia* and the known biological activities of CNA. It was demonstrated that antioxidant defense mechanism in higher organisms and the cytotoxicity mechanism in unicellular organisms share a similar process for detoxification and cytotoxicity, respectively. Furthermore, CNA stimulation of cells in the rat may induce hepatic drug-metabolizing enzymes and show a lower MDA value than normal rats. This suggests that it could be a promising anti-lipid peroxidative agent. In addition, CC-oil can also be effective in the diseases associated with aging since the oil contains greater amount of 2-CNA than of CNA.

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