Agric. Chem. Biotechnol. **43**(3), 156-160(2000)

Induction of DNA Breakage by the Hot-water Extracts of Fructus Chaenomelis (*Chaenomeles sinensis* Koehne)

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Received July 24, 2000

The possible mechanism of the DNA strand breaking activity of the hot-water extract of Fructus Chaenomelis (dried fruit of *Chaenomeles sinensis*) in a closed circular duplex replica form DNA (RFI DNA) was studied through agarose gel electrophresis under various conditions. Induction of DNA strand scission by the hot-water extract of *C. sinensis* occurred in dose and time-dependent manners. Cu^{2+} was indispensable for the induction of DNA strand breakage. Exogeneous chelating agents inhibited the DNA breaking activity, conforming the catalytic action of Cu^{2+} on generation of free radicals responsible for oxidative damage. Antioxidant enzymes and some radical scavengers were used to investigate the major radical species triggering the DNA strand scission, demonstrating that a highest inhibitory activity was found in the presence of catalase, while less in the presence of tiron (a scavenger for superoxide radical), 2-aminoethyl-isothiuroniumbromide-HBr, cysteamine (scavengers for hydroxyl radical), and 1,4-diazabicyclo [2,2,2] octane (a scavenger for singlet oxygen) in decreasing order. The findings implied that oxygen radical species generated in presence of transition divalent cation during the oxidation of some compounds contained in the hot-water extract of *C. sinensis* is mainly responsible for inducing genotoxicity.

Key words: Chaenomeles sinensis, *DNA strand scission, antioxidant enzymes, radical scavengers.*

The antimutagenic activity of hot-water extracts of 130 medicinal plants have previously been reported, indicating the results that 11 medicinal plants including Fructus Chaenomelis (*Chaenomeles sinensis* Koehne), Herba Menthae (*Mentha arvensis* var. piperascens Malinv.), Roasted Fructus Crataegi (*Crataegus pinnatifida* Bunge), Radix Linderae (*Lindera strychifolia* Sieb. et Zucc. Villar), Radix Scutellariae (*Scutellaria baicalensis* Georgi), Fructus Ligustri (*Ligustrum lucidum* Ait.), Semen Platycladi (*Platycladus orientalis* L. Planco), Herba Lycopi (*Lycopus lucidus* Turcz.), Rhizoma Coptidis (*Coptis japonica* Nakai), Fructus Chaenomelis (*Crataegus pinnatifida* Bunge) and Rhizoma Alismatis (*Alisma canaliculatum* All. Br.) had higher antimutagenic activities than other 119 medicinal plants.[1] Further experiment on the hot-water extracts of 11 medicinal plants showed that Fructus Chaenomelis (*Chaenomeles sinensis* Koehne), in the presence of Cu^{2+} induced DNA breakage most effectively,[2] suggesting the involvement of some radical species generated in the presence of Cu^{2+} during the oxidation of the cellular components.

In fact, cellular compounds such as unsaturated fatty acids and mono- or polymeric sugars have been well-documented to be involved in the generation of ROS and other free radicals as by-products via the oxidation-reduction process during aerobic cellular metabolism. Previous studies also showed potent roles of these radicals in causing tumorigenesis, aging, and atheroscleosis through chronic accumulation of genetic leison,[3-7] as well as cell injury and cell death through acute oxidative damage.[8-10]

Fructus Chaenomelis (*Chaenomeles sinensis* Koehne) is not only a popularly prescribed oriental medicinal plant, but also is consumed massively as a beverage foodstuff such as tea. Therefore, it is important to elucidate the precise mechanism of the DNA damaging action exerted by the hot-water extract of *C. sinensis* to protect consumers against any potential toxic effects caused by its daily intake. In this paper, we attempted to define the main causes of DNA strand breakage induced by the hot-water extract of *C. sinensis* from biochemical aspect.

Materials and Methods

Materials. Fructus Chaenomelis (*C. sinensis* Koehne) was kindly provided by an oriental clinic in Uiwang, Kyunggi-do. Double stranded replicative form I DNA (RFI DNA) of bacteriophage Φ×174 was purchased from Life

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Abbreviations: AET, 2-aminoethyl-isothiuroniumbromide-HBr; DABCO, 1,4-diazabicyclo[2,2,2]octane; DETAPAC, diethylenediaminepentaacetic acid; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglyco-bis[β-aminoethylether] N,N,N',N'-tetraacetic acid; RF, replica form; ROS, reactive oxygen species; SOD, superoxide dismutase.

Technologies (Gaithersburg, MD, USA). Superoxide dismutase (EC 1.15.1.1 from human erythrocytes), catalase (EC 1.11.1.6 from bovine liver), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used without further purification.

Preparation of the hot-water extract of *C. sinensis*. Extract was prepared according to the method described in our previous work.[1,2] In brief, dried fruit of *C. sinensis* (12 g) was extracted with boiled water (120 m*l*) for 3 h, and the filtrate was concentrated to yield 8 g using a rotary evaporator.

Reaction conditions for DNA breakage. The reactions were performed by the method established by Ueda *et al.*[11] with a slight modification. The standard reaction mixture (20 μ*l*) contained 200 ng of Φ×174 RFI DNA, 1.25 μg of the hot-water extract, and 50 μM of $CuCl_2$ in 50 mM Tris-Cl buffer (pH 7.1), unless otherwise noted. Complete conversion of RFI DNA into RFII and RFIII DNAs was made by adding sodium borohydride and mitomycin C (final concentrations, 0.5 and 0.4 mM, respectively) to the standard reaction. The reactions were carried out for 1 h at 37°C and stopped by adding 2 μ*l* of 0.1 M EDTA solution containing 50% glycerol and 1% (w/v) bromophenol blue. Φ×174 duplex DNAs in a final volume of 22 μ*l* were analyzed through agarose gel electrophoresis.

Agarose gel electrophoresis. The samples were layered onto 1.0% agarose gel. Electrophoresis was conducted at 4 V/cm for 90 min in TAE buffer (40 mM Tris-acetate, pH 8.2, containing 1 mM EDTA and 1 μg/m*l* ethidium bromide). All experiments were carried out with 1 μg/m*l* ethidium bromide in the agarose gel to separate the RFI and RFII DNAs efficiently. RFI DNA (supercoiled, covalently closed, circular duplex DNA) migrated faster than RFIII DNA (linear duplex DNA), which, in turn, migrated faster than RFII DNA (nicked, open circular duplex DNA) under the condition described above. After electrophoresis, the DNA bands were visualized directly with an ultraviolet transilluminator (253 nm).[12]

Quantitative analysis of duplex DNA on the gel. Absorbance intensities of forms I, II and III DNA molecules on the gel were recorded as a function of distance along the gel through direct scanning using the CCD camera integrated-gel imaging and document system (Vilber Lourmat, France). Automated calculation of the integral absorbance intensity of the areas under the DNA bands using the integrated analyst software resulted in quantitation of the relative amount of forms I, II and III Φ×174 RF DNAs (Bio-1D, Vilber Lourmat, France).

Results and Discussion

Induction of DNA strand scission by the hot-water extract. We have already found that the hot-water extract of Fructus Chaenomelis (*C. sinensis*) induced severe strand scission on Φ×174 RFI DNA in the presence of Cu^{2+}.[2]

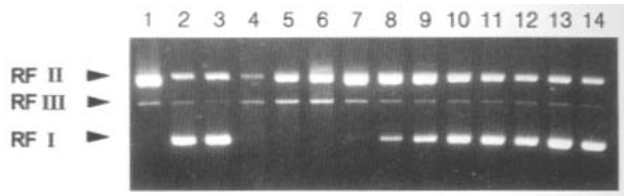

Fig. 1. Dose-dependent effect of the hot-water extract of *C. sinensis* on the induction of Φ×174 duplex DNA strand breakage in the presence of $CuCl_2$ (50 μM). 1; with $CuCl_2$, sodium borohydride, and mitomycin C: 2; without $CuCl_2$: 3; with $CuCl_2$: 4; 5 μg: 5; 2.5 μg: 6; 1.25 μg: 7; 0.625 μg: 8; 0.313 μg: 9; 0.156 μg: 10; 0.078 μg: 11; 0.039 μg: 12; 0.019 μg: 13; 0.010 μg: 14; 0.005 μg. Control reactions shown in lanes 1 and 3 were performed without adding the hot-water extract. The amount of the extract used in the reaction shown in lane 2 was the same as that in lane 4.

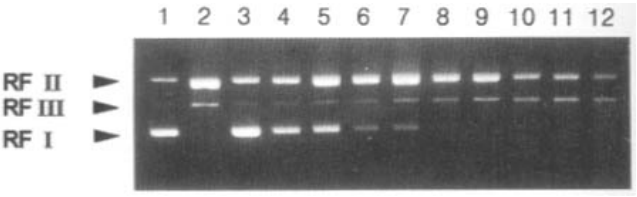

Fig. 2. Incubation time-dependent effect of the hot-water extract of *C. sinensis* (5 μg) on the induction of Φ×174 duplex DNA strand breakage in the presence of $CuCl_2$ (50 μM). 1; without $CuCl_2$: 2; with $CuCl_2$, sodium borohydride, and mitomycin C: 3; without incubation: 4; 5 min: 5; 10 min: 6; 20 min: 7; 30 min: 8; 1 hr: 9; 2 hr: 10; 5 hr: 11; 10 hr: 12; 24 hr. The control reaction shown in lane 2 alone was performed without adding the hot-water extract.

Therefore, we first examined the dosage effect of the hot-water extract on the induction of RFI DNA strand scission. Figure 1 shows that induction was proportional to the sample amount, *i.e.*, the amount of RFII DNA generated by single strand scission on RFI DNA increased in proportion to the sample amount added in the standard reaction. The conversion to RFIII DNA reached its maximum after the peak of the conversion to RFII DNA with a little delay, and both duplex DNAs decreased upon further addition of the sample (Fig. 1, lanes 5 to 8). Figure 2 shows the effect of incubation time with the sample on the induction of DNA strand scission. RFI DNA was incubated in the presence of 50 μM $CuCl_2$. The conversion of RFI DNA to RFII DNA was proportional to the incubation time (0 to 30 min), *i.e.*, the amount of RFII DNA increased with decreasing amount of RFI DNA. The intensity of RFIII DNA appeared somewhat clear following the complete conversion of supercoiled RFI DNA to open circular RFII form (Fig. 2, lane 8), and the maximum amount of linear RFIII form DNA was yielded by 2 h incubation. The amount of RFIII DNA increased slightly with decreasing amount of RFII DNA (1 to 5 h). When incubation was more than 2 h, duplex DNAs were degraded into smaller fragments. The difference in the kinetics of the topological conversion between the duplex

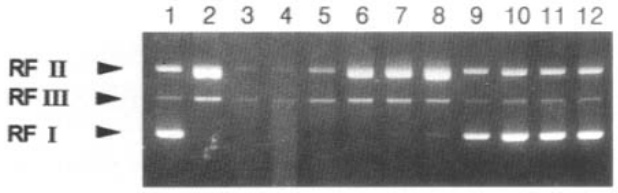

Fig. 3. Effect of $CuCl_2$ concentration on the induction of Φx174 RFI DNA strand breakage by the hot-water extract of *C. sinensis* (5 μg). 1; without $CuCl_2$: 2; with $CuCl_2$ (50 μM), sodium borohydride, and mitomycin C: 3; with 50 μM $CuCl_2$: 4; with 100 μM $CuCl_2$: 5; with 45 μM $CuCl_2$: 6; with 40 μM $CuCl_2$: 7; with 38 μM $CuCl_2$: 8; with 36 μM $CuCl_2$: 9; with 30 μM $CuCl_2$: 10; with 20 μM $CuCl_2$: 11; with 10 μM $CuCl_2$: 12; with 5 μM $CuCl_2$. The control reaction shown in lane 2 alone was performed without adding the hot-water extract.

DNAs (RFI to RFII vs. RFII to RFIII) was considered partly due to the resolution afforded by the fractionation methodology; agarose gel electrophoretic analysis is extremely sensitive to the detection of random strand breakage on supercoiled DNA (RFI), as RFI DNA is easily converted to open circular DNA (RFII) by strand scission regardless of the site and polarity, whereas RFII DNA converted to linear forms (RFIII) in a delayed manner because accumulation of additional DNA breakages with proper strand polarity is prerequisite for the further topological conversion of duplex DNA. Moreover, RFIII DNAs with slight changes in molecular weight caused by random DNA breakage in both strands are indistinguishable in the present assay system, which may explain why the amount of RFIII DNA did not decrease in a dose- or a time-dependent manner.

Effects of metal ions and chelating agents on DNA strand scission. We investigated the effects of transition metal ions and chelating agents on the DNA strand breaking activity of the hot-water extract of *C. sinensis*. Using the optimal condition described above, Φx174 RFI DNA was incubated with 1.25 μg of the samples in the presence of 50 μM of each metal ions. The highest DNA strand breaking activity was found in the presence of Cu^{2+}, and the DNA strand breakage depended on the Cu^{2+} concentration being higher than 36 μM (Fig. 3, lanes 5 to 9). Fe^{2+} was found to be much less effective than Cu^{2+}, and other transition metal ions (Mg^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+}) showed almost no effect at the concentration used in standard reaction (Table 1). Catalytic effect of Cu^{2+} on DNA strand scission reaction was proved through the blocking of the reaction by exogeneous chelating agents (Table 2). The chelating agents (100 μM of each) effectively blocked DNA strand scission, although EGTA, and DETAPAC were slightly more inhibitory than EDTA. In spite of the different chelating ability of each agent, the degree of inhibition depended on the concentration of the chelating agents (data not shown). These findings demonstrated that DNA strand scission by the hot-water extract of *C. sinensis* was catalyzed by Cu^{2+}.

Table 1. Effects of metal ions on strand scission in Φx174 RF DNA.

Metals (50 μM)	extract (1.25 μg)	Relative amount of DNA(%)			
		RF I	RF II	RF III	Others
Control (-$CuCl_2$)	-	80.6	19.3	0	0.1
Control (+$CuCl_2$)	+	0	89.6	10.3	0.1
$CoCl_2$	+	80.0	19.0	0.9	0.1
$FeSO_4$	+	31.1	66.2	2.6	0.1
$MgCl_2$	+	78.3	19.6	2.0	0.1
$MnCl_2$	+	76.4	21.9	1.6	0.1
$ZnCl_2$	+	77.7	20.6	1.6	0.1

All reactions were performed under the standard reaction condition.

Table 2. Effects of chelating agents on strand scission in Φx174 RFI DNA in the presence of 50 μM $CuCl_2$.

Chelating agents	extract (1.25 μg)	Relative amount of DNA(%)			
		RF I	RF II	RF III	Others
Control (-$CuCl_2$)	-	81.4	17.2	1.3	0.1
Control (+$CuCl_2$)	+	0	92.0	7.9	0.1
100 μM EDTA	+	77.3	19.3	3.2	0.2
100 μM EGTA	+	80.1	17.5	2.2	0.2
100 μM DETAPAC	+	79.4	19.1	1.4	0.1

All reactions were performed under the standard reaction condition.

Effects of antioxidant enzymes and radical scavengers on DNA strand scission. The results described suggest the involvement of free radical species including ROS and other peroxide compounds generated during the Cu^{2+}-mediated oxidation of the compounds contained in the extract of *C. sinensis* in the DNA strand scission. Some mono- and polysaccharide derivatives, aminohexoses, autoxidized lipids, and phenyl compounds have been known to damage viral and cellular DNAs with reactive radical species and other peroxide compounds (*e.g.*, superoxide anion, hydroxyl radical, hydrogen peroxide, lipid peroxides, and non-radical products such as carbonyl compounds).[13-16] Therefore, it is important to find the major reactive oxygen(s) in charge of breaking the DNA. We thus investigated the inhibitory effects of cellular antioxidation-related enzymes and various radical scavenging agents on the DNA strand scission induced by *C. sinensis*. Catalase, which removes hydrogen peroxide, completely inhibited DNA strand scission, whereas SOD, which removes superoxide anion, exhibited less inhibition than the catalase (Table 3). However, inhibitory activity of SOD was found to be additively augmented by simultaneous treatment with catalase. 1,2-Dihydroxybenzene-3,5-disulfonic acid (tiron), a scavenger for superoxide anion, strongly inhibited the DNA strand damage caused by the extract of *C. sinensis*.[17] AET, a hydroxyl radical scavenger, also showed almost the same level of inhibition as that of tiron (Table 4).[18] DABCO, a scavenger for singlet oxygen, showed intermediate

Table 3. Effects of catalase and superoxide dismutase (SOD) on strand scission in Φx174 RFI DNA in the presence of 50 μM $CuCl_2$[b].

Enzymes	Concentration (unit/ml)	Relative amount of DNA(%)			
		RF I	RF II	RF III	Others
Control (-$CuCl_2$)	-	79.7	20.3	0	0
Control (+$CuCl_2$)	-	0	85.7	14.1	0.2
Heat inactivated catalase[a]	144	10.4	89.5	0	0.1
Catalase	144	81.0	18.9	0	0.1
Catalase	64.7	56.8	41.2	1.8	0.2
Heat inactivated SOD[a]	66	0	95.6	4.3	0.1
SOD	132	13.0	45.6	41.2	0.2
SOD	66	18.9	61.2	19.7	0.2
Catalase(64.7 units/ml)+SOD	132	19.8	40.5	39.6	0.1
Catalase(64.7 units/ml)+SOD	66	30.5	45.3	24.0	0.2

[a]The enzyme was inactivated by boiling for 15 min.
[b]1.25 μg of the extract was added to all reaction.
All reactions were performed under the standard reaction condition.

Table 4. Effects of radical scavengers on strand scission in Φx174 RFI DNA in the presence of 50 μM $CuCl_2$.

Scavengers	extract (1.25 μg)	Relative amount of DNA(%)			
		RF I	RF II	RF III	Others
Control (-$CuCl_2$)	-	74.5	25.4	0	0.1
Control (+$CuCl_2$)	+	0	87.7	12.2	0.1
10 mM Tiron	+	85.1	13.8	0.9	0.2
100 mM Potassium iodide	+	19.4	76.7	3.7	0.2
100 mM DABCO	+	20.5	75.9	3.4	0.2
10 mM AET	+	45.6	51.1	3.1	0.2
100 mM Cysteamine	+	23.5	74.4	0	0.1
100 mM D-Mannitol	+	3.1	89.9	6.9	0.1
1 M Isopropanol	+	3.0	85.4	11.5	0.1
100 mM DMSO	+	3.4	88.5	7.9	0.2
1 mM Ethanol	+	3.5	87.7	8.7	0.1

All reactions were performed under the standard reaction condition.

inhibitory activity,[19] which was also found in the presence of 2-mercaptoethylamine-HCl (cysteamine) and less in the presence of potassium iodide, both known as the hydroxy radical scavengers.[20,21] On the other hand, we found low levels of inhibition on DNA strand breakage by other hydroxyl radical scavengers such as sodium benzoate, ethanol, DMSO, D-mannitol, and isopropanol,[22-24] an evidence that oxygen radicals (in particular, superoxide and hydroxyl radicals), probably generated during Cu^{2+}-mediated autoxidation of some cellular components extracted by hot water, play a critical role in damaging the Φx174 duplex DNA. The discrepancies of scavenging effect on a radical species appeared to be caused by chemical heterogeneity of the compounds in the sample used in this study.

The ethanolic extract of *C. sinensis* has recently been reported to show a potent inhibition of tyrosinase,[25] an indication of the possibility of application in cosmetics. However, for commercial application, intrinsic cytotoxic or genotoxic activities should be eradicated or detoxificated to protect normal cells from possible necrosis or neoplastic transformation. Therefore, purification and chemical identification of the compounds, together with functional characterization of each purified compound both *in vitro* and *in vivo* are crucial. Further studies on the development of a novel process in food industry as well as in cosmetic industry are needed, eventually leading to a mass consumption of *C. sinensis* as a bio-functional resource.

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