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RESEARCH ARTICLE

Podophyllin, but not the constituents quercetin or kaempferol, induced genotoxicity *in vitro* and *in vivo* through ROS production

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Abstract
The genotoxic potential of podophyllin (PD) was investigated in this study. PD increased bacterial revertants and abnormal chromosomal structures in a concentration-dependent manner, both with and without metabolic activating enzymes, and increased the incidence of micronuclei in imprinted control region mouse reticulocytes. Results from three studied constituents of PD, such as podophyllotoxin, kampferol, and quercetin, suggested that the mutagenic effect of PD was not due to the presence of podophyllotoxin, kampferol, and quercetin and might be related to other components and the formation of reactive oxygen species. The detailed mutagenic mechanisms need further investigation, and the medicinal use of PD needs to be cautioned against.

Keywords: *Podophyllin; Genotoxicity; Ames test; Chromosome aberration; Micronucleus; Reactive oxygen species*

Introduction

Herbal medicines are gradually becoming accepted by people as legitimate pharmaceuticals and are generally thought of as dietary supplements in most parts of the world. Many people believe that these so-called herbal medicines are natural and harmless. These misconceptions have encouraged people to use herbal medicines without precaution. Chinese herbal nephropathy (CHN) was first reported in 1993 as patients ingested Chinese herbal medicines to lose weight (Vanherweghem et al., 1993). Upon closer examination, the true cause of the CHN was the misuse of *Stephaniae* Radix in place of *Aristolochiae* species. Aristolochiae species contain aristolochic acid (AA), was shown to be a strong rodent carcinogen (Arlt et al., 2002), and was listed as a known carcinogen by the International Agency for Research on Cancer (IARC, 2002). Because of the serious toxicity induced by AA, the plants of the *Aristolochiae* species were withdrawn from the market in some countries. Due to the above incidences, the use of herbal medicine for the public health needs to be evaluated, especially as increasing amounts of herbal product are distributed through the world's markets.

Podophyllin (PD) is an alcoholic plant extract obtained from the dried rhizomes and roots of the common plants of *Podophyllum emodi* (Indian *Podophyllum*) and *Podophyllum peltatum* (*Mayapple or Mandrake)*. The plants of *Podophyllum* were used as traditional medicines for the treatment of cathartics and skin disorders, and also as antihelmintics and antifungals (Rivera and Tyring, 2004). Those are usually combined with henbane or belladonna to prevent side-effects. It is often used as pills or tinctures. The dried ripened fructus of

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Podophyllum were formulated as pills to cure stasis, dysmenorrhea, dystocia, and stillbirth in the Chinese Pharmacopoeia (2005). In addition to the aforementioned *Podophyllum* species, *Podophyllum pleianthum*, a folk medicine distributed in Taiwan, was traditionally used to treat snake bites. Because of their diverse biological actions, their widespread use without caution has resulted in severe poisoning or fatal cases (West et al., 1982). The common reaction was neuropathy, which lasted for several years. PD is an irritant to the eyes and mucus membranes. It can result in severe systematic toxicity after ingestion or topical application, which is usually reversible but has been fatal (Reynold and Prasad, 1996). Symptoms of toxicity include nausea, vomiting, abdominal pain, and diarrhea. Though it has a number of adverse effects, it is still included in pharmacopeias (e.g., the 2006 United States Pharmacopoeia; USP), but its clinical use is limited to applying on warts.

Podophyllotoxin (Figure 1), the major constituent of PD, was separated and identified in 1880. Subsequently, a series of aryltetralin-type ligands were isolated, which included α-peltatine, β–peltatine, podophylltoxone, and 6-methoxypodophyllotoxin, etc. (Xiao et al., 2002). In addition to these ligands, flavonoids such as quercetin and kampferol (Figure 1) also exist in PD.

Quercetin, a well-known flavonoid and minor constituent of PD, is found in most herbs, vegetables, fruits, and in beverages such as teas and wines. Studies have shown that flavonoids have multiple biological activities, such as acting as an antioxidant (Rice-Evans, 2001; Fujisawa and Kadoma, 2006), anti-inflammatory, antiviral, platelet-aggregation inhibitor (Landolfi, et al., 1984; Hertog and Hollman, 1996), antiaging agent (Rice-Evans, 2001), and for preventing cardiovascular disease (Cao et al., 1998; Rice-Evans, 2001; Carollo et al., 2007). However, in the 1970s, quercetin was reported as mutagenic in the Ames test (Bjeldanes and Chang, 1977; Nagao et al., 1981), chromosome aberration test (Silva et al., 1996, 1997), and micronucleus test (Sahu et al., 1981; Caria et al., 1995). Kampferol also was shown to have mutagenic activity in the Ames and chromosome aberration tests (Nagao et al., 1981; Silva et al., 1996, 1997).

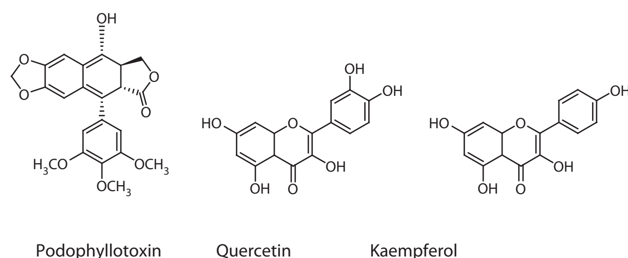

Figure 1. Structures of podophyllotoxin, quercetin and kampferol.

The aim of this study was to clarify PD's genotoxic potential and investigate the genotoxic mechanisms ascribed to the constituents, podophyllotoxin, kampferol, and quercetin. Two *in vitro* short-term mutagenicity bioassays, the Ames Salmonella assay and the chromosome aberration test with mammalian cells, were conducted. In addition, a micronucleus test was used to evaluate genotoxicity *in vivo*. Our results showed that PD was strongly mutagenic *in vitro* and *in vivo*, and the mutagenic effect was not from quercetin and kampferol.

Materials and methods

Materials

PD, podophyllotoxin, quercetin, kampferol, Aroclor 1254, D(+)-glucose, sodium chloride, acridine orange, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (β–NADP), colcemid, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), 4-nitro-*O*-phenylenediamine (4-NOP), 2-aminoanthracene (2-AA), benzo[a]pyrene (BaP), mitomycin C (MMC), and sodium azide were all obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Giemsa stain solution was purchased from Mute Chemical Co., Ltd. (Tokyo, Japan). Fetal calf serum, penicillin/streptomycin/glutamine, F-12K medium, and trypsin were obtained from Gibco BRL (Grand Island, New York, USA). PD constituent analysis was kindly provided by Dr. Cheng Hui-Wen, Ph. D. Results from the liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) examined the constituents of PD and showed that the relative amounts of the constituents were 31.9, 3.2, and 1.8% of podophyllotoxin, kampferol, and quercetin, respectively (Lin et al., 2007).

Experimental animals

For the Ames test, Wistar rats (200 g) were used for the preparation of the liver microsomal (S9) fraction. For the micronucleus test, male imprinting control region (ICR) mice, aged 8–9 weeks and weighing 30–40 g, were employed. All animals were purchased from the Animal Center of the College of Medicine, National Taiwan University. The animals were allowed at least a 1-week acclimation period in their animal housing at 25°C and a 12-h light-dark cycle. All animal treatments were approved by the Institution Animal Care and Use Committee (IACUC) of the College of Medicine, National Taiwan University, which follows the Animal Welfare Protection Act of the Department of Agriculture, Executive Yuan (Taipei, Taiwan).

Rat-liver microsome (S9) preparation

Rat-liver microsomes (S9) used for metabolic activation were prepared, as described previously (Matsuoka et al., 1979; Maron and Ames (1983). To obtain the liver microsomal fraction, each of the Wistar rats was injected intraperitoneally (i.p.) with Aroclor 1254 (30 mg/kg body weight), and 4 days later, the rats were sacrificed by cervical dislocation. The livers were homogenated, diluted 1:4 with 0.15 M of KCl, and centrifuged for 10 min at 900 × *g*. The supernatant was pooled and diluted (to a protein concentration of 30 mg/mL), frozen in small aliquots, and stored at –70 to –80°C until use. The final preparation of the S9 mixture was made in accordance with the protocol of Ames et al. (1975). The composition and final concentrations of the S9 reaction mix used for the CHO-K1 cell chromosome aberration test were as follows: G6P, 4.4 mM; β–NADP, 0.84 mM; KCl, 30 mM; $NaHCO_3$, 0.032%; and S9 fraction, 10%.

Ames salmonella/microsome test

The method we used followed that of Maron and Ames (1983) and Organization for Economic Cooperation and Development guidelines (OECD, 1997). The *Salmonella typhimurium* bacteria and histidine auxotrophic strains, TA98, TA100, and TA102, were obtained from MOLTOX (Molecular Toxicology, Annapolis, Maryland, USA) and grown for 14 h at 35 ± 2°C with continuous shaking. Bacteria were grown to a density of 1–2 × 10^9 cells/mL with a OD600 absorbance of 0.2–0.3. Top agar, containing 2 mL of heated agar, 0.1 mL of test chemical, 0.1 mL of bacteria, and 0.5 mL of S9 solution, were mixed and added to three different minimal glucose agar plates. All plates were incubated at 37°C for 48 h, and the number of bacteria colonies was determined. The entire experiment was replicated again on a different day, with a total of six plates for each concentration of PD or test chemicals with and without S9. Each tester strain was routinely checked to confirm its features for optimal response to known mutagenic chemicals with the following agents: 4-NOP (0.5 μg/plate), MMC (0.5 μg/plate), and 2-AA (5 μg/plate). A test compound was judged to be mutagenic in the plate test if it produced, in at least one concentration and one strain, a response equal to three times (or more) of the control incidence with a positive dose-response relationship (de Serres and Shelby, 1979; Suter et al., 2002). The only exception was strain TA102, which has a relatively high spontaneous revertant number, whereby an increase by a factor of 1.5 above the control level was taken as an indication of a mutagenic effect.

Chromosomal aberration test

The test was conducted by using the method of Cheng et al. (2004, 2005) and Matsuoka et al. (1979), with some modification. Chinese hamster ovary epithelial cells (CHO-K1, ATCC: CCL-61) were plated into 6-cm dishes at 5 x 10^5 cells/plate; after overnight incubation, cells were treated with DMSO (solvent), MMC (1 μg/mL), and various concentrations of test chemicals for 3 h with or without S9, followed by 0.1 μg/mL colcemid for 3 h. After trypsinization, cells were treated with hypotonic solution (0.03 M of potassium chloride and 0.01 of M sodium citrate) at 37°C for 10 min, fixed in Carnoy's solution (methanol:acetic acid, 3:1), and spread on glass slides by the air-drying method. Specimens were stained with a 3% Giemsa solution in 0.07-M phosphate buffer (pH 6.8) for 30 min. For determination of both of the chromosome aberrations, 100 metaphases per experimental group were scored. Structural chromosome aberrations observed in each experimental group were classified into seven types as follows: chromosome-type gap (G), chromosome-type break (B), chromosome-type ring (R), chromosome-type dicentric (D), chromatid-type gap (g), chromatid-type break (b), and chromatid-type exchange (e).

Micronucleus assay

The micronucleus assay from peripheral blood cells was performed, as described (Cheng et al., 2004; Hayashi et al., 1990). Male ICR mice, weighing 30–40 g, were employed and divided into five groups. The PD suspension was prepared in distilled water and administered by gavage, using a stomach tube. MMC (1 mg/kg) was administrated by i.p. injection to serve as a positive control. Then, 24, 48, and 72 h after the treatment, 3–4 μL of peripheral blood was collected from the tail vein and transferred onto a slide prestained with acridine orange (1 mg/mL) and then covered with a coverglass. The number of micronucleated cells were counted in 1,000 reticulocytes per animal. Slides were analyzed by using a Zeiss Axiophot 2 fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY, U.S.A.) with a combination of a blue excitation (e.g., 488 nm) and a yellow-to-orange barrier filter (e.g. 515 nm long pass), with an X100 objective lens.

Analysis of ROS production by flow cytometry

Intracellular reactive oxygen species (ROS) generation was measured by a flow cytometer with an oxidation-sensitive DCFH-DA fluoroprobe (Rothe and Valet,

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1990). CHO-K1 cells, (2 x 10^6 /mL) were treated with various concentrations of PD (25, 50, and 100 µg/mL) for 2 h, then trypsinized and stained with 20 µg/mL of DCFH-DA for 30 min at 37°C in the dark. Cells were then collected after phosphate-buffered saline (PBS) washing for fluorescence measurements. The level of intracellular ROS was determined with a FACS Calibur™ flow cytometer (Becton Dickinson, San Jose, California, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. For each treatment, 10^4 cells were counted and the experiment was performed in triplicate.

Statistical analysis

All experiments were performed at least in triplicate, and data are presented as the mean ± standard error of the mean. Statistical analysis, including the Student's *t*-test, were performed, using SigmaPlot (Systat Software Inc., Point Richmond, California, USA), and $p < 0.05$ was considered as significantly different.

Results

PD-induced bacterial revertants in ames test

The data for the PD-induced histidine revertants in the three strains of *Salmonella typhimurium* were listed in Table 1. Our data showed that PD increased colony formation in strains TA98, TA100, and TA102 in a concentration-dependent manner (Table 1A). The increase at a PD concentration of 1,000 µg/plate in TA98, TA100, and TA102 was 12.0 2.7 and 1.9-fold, respectively. In the presence of S9, the increases were significantly enhanced, reaching 18.6-, 5.2-, and 2.5-fold, respectively (Table 1B). The induction of the TA98 strain to greater than three times relative to control values either with or without S9 was regarded as a positive mutagenic effect (FDA, 2004).

Three major constitutive compounds of PD were simultaneously examined in the Ames Salmonella/microsome test in order to clarify the mutagenic effects. Table 2 showed the number of revertants induced by different compounds at the same concentration. Quercetin significantly increased colony formation in strains TA98, TA100, and TA102 with or without S9. The increases induced by quercetin (200 µg/plate) ranged from 32.7- to 45.8-fold over the negative control in the TA98 strain (either with or without S9), and the mutagenicity was attenuated in the presence of S9. Kampferol increased colony formation in all strains with S9, and TA102 without S9. Podophyllotoxin did not show a mutagenic effect in the Ames test. Based on the chemical analysis (Lin et al., 2007), the percent composition of quercetin and kampferol in PD were 1.8 and 3.2%, respectively. The amount of quercetin and kampferol in 200 µg of PD were 3.6 and 6.4 µg. This amount also induced revertants in the TA98 strain both with or without S9 (Table 2).

Induction of chromosome aberration by PD in CHO-K1 cells

PD-induced abnormal chromosome structures were examined with CHO-K1 cells. In Table 3, MMC (1 µg/mL) and BaP (5 µg/mL), were used as the positive controls, and they significantly induced abnormal chromosome structures in the absence (Table 3A) or presence (Table 3B) of S9. PD (25, 50, and 100 µg/mL) increased the abnormal structure of chromosomes at 3 h, 24 h

Table 1. Induction of His⁺ revertants in three strains of *Salmonella typhimurium* by podophyllin (PD) with and without metabolic activation (by S9).

Strains	Negative control[a]	Positive control[c,d]	His⁺/plate PD (µg/plate) 1	20	200	1,000
(A) Without S9						
TA 98	26 ± 5	238 ± 16***	17 ± 4**(0.7)[b]	19 ± 5*(0.7)	103 ± 15***(4.0)	313 ± 23***(12.0)
TA 100	80 ± 6	799 ± 12***	73 ± 15 (0.9)	62 ± 13*(0.8)	139 ± 4***(1.7)	213 ± 5***(2.7)
TA 102	149 ± 3	1259 ± 39***	119 ± 22*(0.8)	146 ± 19 (1.0)	245 ± 4*** (1.6)	290 ± 10***(1.9)
(B) With S9						
TA 98	32 ± 6	3396 ± 363***	26 ± 8 (0.8)	42 ± 10 (1.3)	201 ± 35*** (6.3)	594 ± 103***(18.6)
TA 100	77 ± 8	4273 ± 89***	66 ± 8*(0.9)	88 ± 9*(1.1)	222 ± 45***(2.9)	398 ± 2***(5.2)
TA 102	198 ± 19	2863 ± 120***	124 ± 13**(0.6)	245 ± 6*(1.2)	433 ± 30***(2.2)	487 ± 11***(2.5)

Values are presented as the mean ± standard error ($N \geq 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the negative control.
[a]The 2-µL dimethyl sulfoxide plate was used as the negative control.
[b]Multiples of increase relative to the negative control.
[c]Positive control in the –S9 plate: TA 98, 4-NOP 2 µg/plate; TA 100, sodium azide 5 µg/plate; TA102, MMC 0.5 µg /plate.
[d]Positive control in the +S9 plate: TA 98, TA 100, and TA102, 2-AA: 5 µg/plate.

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Table 2. Induction of His+ revertants in three strains of *Salmonella typhimurium* by podophyllin (PD) and PD components with and without metabolic activation (by S9).

	His+/plate						
	Negative	PD	Podophyllotoxin	Quercetin		Kampferol	
Strains	control[a]	(200 µg/plate)	(200 µg/plate)	(200 µg/plate)	(3.6 µg/plate)	(200 µg/plate)	(6.4 µg/plate)
(A) Without S9							
TA 98	34 ± 4	297 ± 20***(8.7)[b]	33 ± 3(1.0)	1557 ± 103***(45.8)	164 ± 15*** (4.8)	34 ± 5 (1.0)	28 ± 3 (0.8)
TA 100	109 ± 8	248 ± 30 ***(2.3)	87 ± 11(0.8)	471 ± 88***(4.3)	195 ± 13** (1.8)	112 ± 15 (1.0)	156 ± 16* (1.4)
TA 102	279 ± 31	430 ± 58**(1.5)	307 ± 41(1.1)	642 ± 115*(2.3)	420 ± 20 (1.5)	458 ± 36***(1.6)	377 ± 38 (1.4)
(B) With S9							
TA 98	41 ± 4	428 ± 23***(10.4)	25 ± 7(0.6)	1341 ± 57***(32.7)	273 ± 24***(6.7)	218 ± 29***(5.3)	218 ± 29***(5.3)
TA 100	111 ± 6	324 ± 27***(2.9)	78 ± 8(0.7)	555 ± 47***(5.0)	139 ± 5***(1.3)	244 ± 19***(2.2)	104 ± 10 (1.0)
TA 102	336 ± 29	565 ± 52**(1.7)	246 ± 38(0.7)	987 ± 49**(2.9)	598 ± 33**(1.8)	503 ± 31**(1.5)	379 ± 56* (1.1)

Values are presented as the mean ± standard error ($N \geq 6$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the negative control.
[a]The 2-µL dimethyl sulfoxide plate was used as the negative control.
[b] Multiples of increase relative to the negative control.

Table 3A. Chromosome aberrations of CHO-K1 cells treated with podophyllin (PD) and PD components with and without metabolic activation (by S9).

(A) Without S9			Number of aberrant cells/100 cells[a]						
Treatment (µg/mL)		Aberrant cells (%)	G	B	D	R	g	b	e
3 h	Negative[a] control	1.0 ± 1.0	0 ± 0	0 ± 0	1.0 ± 1.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	MMC[b]	5.7 ± 0.9***	2.0 ± 0.6	0 ± 0	0.3 ± 0.3	0 ± 0	0 ± 0	3.0 ± 0.6	0.3 ± 0.3
	PD (µg/ml)[b]								
	25	7.3 ± 0.3**	0.7 ± 0.3	1.0 ± 0.6	1.0 ± 0.0	0.7 ± 0.7	2.3 ± 0.9	1.7 ± 0.9	0 ± 0
	50	8.0 ± 3.6	0.7 ± 0.7	0.3 ± 0.3	1.0 ± 0.0	0.3 ± 0.3	3.7 ± 2.2	2.0 ± 1.0	0 ± 0
	100	12.0 ± 1.0**	1.3 ± 1.3	0 ± 0	1.3 ± 0.3	0 ± 0	4.7 ± 1.5	4.0 ± 0.6	0.7 ± 0.7
	Q[c] 1.8	1.3 ± 0.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.7 ± 0.3	0.7 ± 0.3	0 ± 0
	K 3.2	1.7 ± 1.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.7 ± 0.3	1.0 ± 1.0	0 ± 0
24 h	Negative[a] control	1.0 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0.7 ± 0.3	0.3 ± 0.3	0 ± 0	0 ± 0
	MMC[b]	11.7 ± 1.2***	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.7 ± 0.3	6.0 ± 0.6	2.0 ± 0.6	2.0 ± 0.6
	PD (µg/ml)								
	25	7.7 ± 0.3***	0.3 ± 0.3	0 ± 0	2.3 ± 1.3	0 ± 0	1.0 ± 0.6	3.0 ± 1.2	1.0 ± 0.6
	50	9.0 ± 2.9*	0.3 ± 0.3	0 ± 0	2.0 ± 0.0	0 ± 0	1.0 ± 0.6	2.7 ± 1.2	3.0 ± 1.7
	100	14.0 ± 4.6*	2.3 ± 2.3	0.7 ± 0.3	2.7 ± 0.9	0 ± 0	2.3 ± 0.9	4.3 ± 0.9	1.7 ± 0.9
	Q 1.8	2.7 ± 0.9	0 ± 0	0 ± 0	0.3 ± 0.3	0.3 ± 0.3	0.7 ± 0.3	1.3 ± 0.3	0 ± 0
	K 3.2	2.7 ± 0.3	0 ± 0	0.3 ± 0.3	0.7 ± 0.3	0 ± 0	0.3 ± 0.3	1.0 ± 0.6	0.3 ± 0.3

without S9, and at 3 h with S9 in a concentration-dependent manner (Table 3A and 3B). Gap and break defects were the main abnormal chromosome structures in PD-induced aberrations. Quercetin and kampferol, in concentrations found in 100 µg/mL of PD (1.8 and 3.2 µg/mL, respectively), did not induce abnormal structures in chromosomes in CHO-K1 cells.

In vivo *induction of micronuclei by PD*

The *in vivo* micronucleus induction in mice by PD is shown in Table 4. The positive control MMC significantly induced micronucleus formation. PD demonstrated a concentration dependent (from 17.5 to 140 mg/kg) increase in micronucleus formation at 24, 48, and 72 h. The highest induction was 2.81 ± 0.30 of micronucleated reticulocytes at 48 h, which was a 3.51-fold increase relative to the control value of 0.80 ± 0.11. Quercetin (2.52 mg/mL) and kampferol (4.48 mg/mL) were added according to their corresponding concentrations in PD (140 mg/mL) and had no effect on micronucleus formation up to 72 h.

ROS induced by PD in CHO cells

CHO-K1 cells loaded with the fluorescence probe, DCFH-DA, were used to measure the generation of ROS with flow cytometry. PD increased the DCF

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Table 3B. Chromosome aberrations of CHO-K1 cells treated with podophyllin (PD) and PD components with and without metabolic activation (by S9).

(B) With S9			Number of aberrant cells/100 cells						
Treatment		Aberrant cells (%)	G	B	D	R	g	b	e
3 h	Negative control[a]	0.3 ± 0.3	0.3 ± 0.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	BaP[b]	15.5 ± 1.2***	0.7 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	5.3 ± 0.9	2.3 ± 0.3	2.3 ± 0.3	4.3 ± 0.9
	PD (μg/ml)[b]								
	25	9.0 ± 1.9*	1.5 ± 0.8	0 ± 0	3.2 ± 0.8	0.5 ± 0.2	2.2 ± 0.9	1.7 ± 0.6	0 ± 0
	50	9.7 ± 1.7**	0.2 ± 0.2	0.3 ± 0.2	0.8 ± 0.3	0.2 ± 0.2	2.7 ± 0.9	5.2 ± 1.3	0.3 ± 0.3
	100	14.0 ± 2.7**	1.0 ± 1.0	0 ± 0	2.3 ± 0.3	0.3 ± 0.3	5.3 ± 2.3	4.7 ± 0.9	0.3 ±0.3
	Q 1.8	1.0 ± 0.0	0 ± 0	0.3 ± 0.3	0.3 ± 0.3	0 ± 0	0 ± 0	0.3 ± 0.3	0 ± 0
	K 3.2	2.3 ± 0.3	0 ± 0	0 ± 0	0.3 ± 0.3	0.3 ± 0.3	0 ± 0	1.7 ±0.7	0 ± 0

[a]Dimethyl sulfoxide (DMSO) was taken as the negative control.
[b]PD was dissolved in DMSO, and the solvent control (DMSO) did not exceed 0.1%. MMC at 1 μg/mL and benzo(a)pyrene (BaP) at 5 μg/mL were used as the positive control without and with S9 medium. Quercetin at 1.8 μg/mL and kampferol at 3.2 μg/mL were added according to their contents existing in 100 μg/mL PD.
[c]Q, quercetin; K, kampferol.
[d]Values are presented as the mean ± standard error ($n = 3$); *p < 0.05; **p < 0.01; ***p < 0.001 vs. the negative control. G, chromosome gap; B, chromosome break; D, dicentric; R, ring; g, chromatid gap; b, chromatid break; e, exchange.

Table 4A. Micronucleus formation in peripheral blood cells of mice treated with podophyllin (PD) and PD components *in vivo*.

Treatment group	Number of micronucleate cells/1,000 nucleated cells — Time (h)		
	24	48	72
Control	0.57 ± 0.15	0.80 ± 0.11	0.87 ± 0.19
MMC (1 mg/kg)	2.93 ± 0.22***	3.26 ± 0.22***	2.62 ± 0.17**
PD (17.5 mg/kg)	1.48 ± 0.20**	1.16 ± 0.12	2.24 ± 0.28
PD (35 mg/kg)	1.30 ± 0.16**	2.11 ± 0.30*	2.60 ± 0.27*
PD (70 mg/kg)	1.67 ± 0.28**	2.18 ± 0.07***	2.90 ± 0.27**
PD (140 mg/kg)	2.54 ± 0.21***	2.81 ± 0.30***	2.98 ± 0.72

Table 4B. Micronucleus formation in peripheral blood cells of mice treated with podophyllin (PD) and PD components *in vivo*.

Treatment group	Number of micronucleated cells/1,000 nucleated cells — Time (h)		
	24	48	72
Control	0.50 ± 0.09	0.53 ± 0.21	0.76 ± 0.09
MMC (5 mg/kg)	3.88 ± 0.48***	5.44 ± 0.70***	5.92 ± 0.63***
Quercetin (2.52 mg/kg)	0.43 ± 0.12	0.24 ± 0.02**	0.28 ± 0.06*
Kampferol (4.48 mg/kg)	0.42 ± 0.12	0.58 ± 0.12	0.39 ± 0.09*

Data are expressed as the mean ± standard error from the six independent experiments. Quercetin at 2.52 mg/mL and kampferol at 4.48 mg/mL were added according to their contents existing in 140 mg/mL of PD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the control.

fluorescence intensity in a concentration-dependent manner (Figure 2), but quercetin and kampferol did not. The increased fluorescence intensity induced by PD (100 μg/mL) was 79.7 ± 5.7% relative to the control value of 39.3 ± 4.3%. H_2O_2 was used as the positive control.

Discussion

In this study, PD acted as a direct mutagen in bacteria and caused chromosome aberrations in CHO-K1 cells. PD increased the number of revertants in *Salmonella* strains TA 98, TA100, and TA102 with or without S9. In the presence of S9, the mutagenic effect of PD was significantly enhanced, especially in the TA98 strain. Part of the PD-induced mutagenicity might be through metabolic activation. PD also increased the number of abnormally structured chromosomes both with and without S9. Although many reports examine the toxicity of PD, comprehensive reports on the mutagenic effects of PD are few (Ferguson and Pearson, 1992).

Petersen and Weisman (1995) estimated the amounts of quercetin and kampferol in 20% PD were about 10% and recommended using pure podophyllotoxin or a lower percentage PD formulation to

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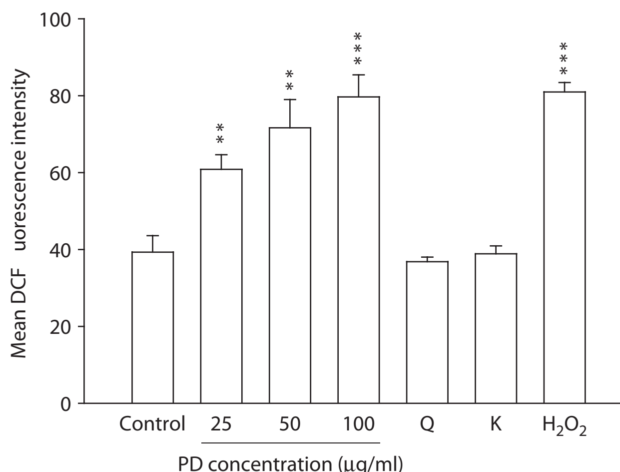

Figure 2. Effects of podophyllin (PD) on reactive oxygen species (ROS) generation in CHO-K1 cells. PD dose dependently induced DCF fluorescence intensity. Cells were incubated with various concentrations of PD (25, 50, and 100 μg/mL) for 2 h, then trypsinized and stained with 2',7'-dichloroflyorescin diacetate (DCFH-DA) at 37°C for 30 min. Dimethyl sulfoxide and H_2O_2 (200 μM) were used as the negative and positive controls, respectively. Quercetin (Q 1.8 μg/mL) and kampferol (K 3.2 μg/ml) were used as the paralleled test. Data are expressed as the mean ± standard error from six independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the negative control.

prevent toxicity. Although PD formulations may contain variable amounts of the active ligand podophyllotoxin, the dry weight of this constituent is almost never identified.

Due to the uncertainty of the exact constituents in PD, we characterized the constituents in PD based on their mutagenic effects. As a resin mixture, the percentages of podophyllotoxin, quercetin, and kampferol in PD were detected by LC/MS/MS as 31.9, 1.8, and 3.2%, respectively (Lin et al., 2007). Podophyllotoxin, the main component in PD, did not show a mutagenic effect in the Ames test. Podophyllotoxin is a white crystalline substance isolated from PD. It has several biological activities similar to PD, including an antiviral activity against type I herpes simplex and measles (Rivera and Tyring, 2004) and has shown antitumor activity (Canel, et al., 2000).

Quercetin is a widely distributed flavonoid, mainly found in vegetables (e.g., onion and broccoli), fruits (e.g., apples, grapes, cherries, and berries), tea, red wine, and herbs (e.g., Ginkgo and sophora). Our study showed that quercetin (1.8% in PD) is a potent mutagen in the Ames test but not in the chromosome aberration test. Although the mutagenic effects of quercetin have been reported in many cell types (Caria et al., 1995; Oliveira et al., 1997; van Duursen et al., 2004), the underlying mechanism of its action is still unclear.

Like quercetin, kampferol is a well-known antioxidant that prevents ROS-mediated apoptosis in cerebellar granule cells of Wistar rats (Samhan-Arias et al., 2004). Kampferol (3.2% in PD) exhibited a mutagenic effect in the Ames test but not in the chromosome aberration test. From the results of our study, quercetin and kampferol both induced bacterial revertants with or without S9 but had virtually no effect in the chromosome aberration test. Direct ROS generation was measured by flow cytometry with DCF fluorescence and showed that PD, but not quercetin and kampferol, significantly induced ROS generation in CHO-K1 cells.

There are a number of contradicting results examining the role of quercetin in ROS generation. Quercetin induced ROS generation in WIL2-NS cells (Saito et al., 2004; Mertens-Talcott et al., 2005) and suppressed ROS generation in HL-60 and PC12 cells (Wang et al., 1999; Wang and Joseph, 1999). Most researchers agree that ROS play a key role in human cancer development (Monari et al., 2006; ten Kate et al., 2006, Knaapen et al., 2006; Jenkins et al., 2007). ROS include oxygen radicals (e.g., superoxide, hydroxy, and peroxyl and alkoxyl radicals) and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals (e.g., hypochlorous acid, ozone, and peroxynitrite), singlet oxygen, and hydrogen peroxide. Several *in vitro* studies have reported the characteristic DNA damage caused by ROS. Quercetin can produce H_2O_2 in the medium, and chromosome damage induced by H_2O_2 has been demonstrated in WIL2-NS cells (Saito et al., 2004). Further, quercetin and kampferol also increase 8-oxodG formation (Yamashita, et al., 1999). Previous studies have shown that ROS might be involved in the mutagenic mechanism induced by quercetin and kampferol. In our study, ROS were not involved in quercetin- and kampferol-induced mutagenesis either in the Ames test or in abnormal chromosomal structure assays, since no ROS were detected by DCFH-DA fluorescence probe.

The *in vivo* micronucleus test (Table 4) showed that PD induced micronucleus formation. Surprisingly, quercetin exerted a genotoxic effect in the Ames test but did not show a clastogenic effect in the *in vivo* micronucleus test. These data suggest a minimal role of quercetin in PD-induced mutagenesis. A safety review of quercetin for clinical applications was reported in 2005 (Okamoto, 2005). The International Agency for Research on Cancer (IARC) concluded in 1999 that quercetin could not be classified as carcinogenic to humans. Although quercetin is genotoxic to *Salmonella*, its safety in certain human applications has been approved. Although one report has indicated that quercetin and kampferol could induce micronuclei *in vivo* (Sahu et al., 1981), this effect did not occur in our study.

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Conclusions

We conclude that PD could induce genotoxicity *in vitro* and *in vivo*, and this effect might not come from quercetin or kampferol. However, there are other constituents that could have contributed to the PD-induced mutagenesis. The lack of information on the carcinogenesis, bioavailability, metabolism, and tissue accumulation of PD in the animal requires further study. Although the genotoxic effect might not equal the carcinogenic effect, we have drawn attention to the mutagenic effects of PD and will conduct further investigations to identify the precise mutagen in PD. Although this medicinal plant derivative is easily obtained, these data suggest that the use of PD should be done with caution.

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