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Chemical Composition and Toxicity of Taiwanese Betel Quid Extract

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Abstract—In this genotoxic study, the Ames *Salmonella* microsome test showed that an aqueous extract of betel quid did not induce mutagenicity in *Salmonella typhimurium* strains TA98 and TA100. Mammalian cell studies (Chinese hamster ovary K1 cell; CHO-K1 cell) revealed that only higher concentrations (100 and 1000 μg/ml) of aqueous extract weekly increased the frequencies of sister-chromatid exchange (SCE) in the absence of S9. Animal (male Sprague–Dawley rat) studies showed that low-dose feeding (0.53 g dry aqueous extract/kg diet) significantly increased the activities of glutathione (GSH) peroxidase and cytoplasmic glutathione *S*-transferase (cGST) of liver, high-dose feeding (26.5 g dry aqueous extract/kg diet) lowered the contents of GSH and total glutathione. The effect of an aqueous extract of betel quid on the oxidation of 2′-deoxyguanosine (2′-dG) to 8-hydroxy-2′-deoxyguanosine (8-OH-dG) evaluated that this aqueous extract may act as a pro-oxidant at lower dosage and may be dependent on the iron ions in the model system. However, the aqueous extract of betel quid showed antioxidant activity at higher doses by the ability of the scavenging effect of the hydroxyl radicals.
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Keywords: betel quid; alkaloids; phenolics; Ames *Salmonella* microsome test; SCE; GSH; cGST; 8-OH-dG.

Abbreviations: AIN = American Institute of Nutrition; BrdUrd = 5-bromo-2′-deoxyuridine; CHO-K1 = Chinese hamster ovary K1; CDNB = 1-chloro-2,4-dinitrobenzene; cGST = cytoplasmic glutathione; 2′-dG = 2′-deoxyguanosine; DMSO = dimethyl sulfoxide; EDTA = ethylenediaminetetraacetic acid; FBS = foetal bovine serum; FDNB = 1-fluoro-2,4-dinitrobenzene; GSH = glutathione; 8-OH-dG = 8-hydroxy-2′-deoxyguanosine; IAA = iodoacetic acid; MNNG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; mGST = microsomal glutathione *S*-transferase; NADP = nicotinamide adenine dinucleotide phosphate, sodium salt; GSSG = oxidized glutathione; PBS = phosphate buffered saline; SGPT = serum glutamic–pyruvic transaminase; SCE = sister chromatid exchange; TBARs = thiobarbituric acid reactive substance.

INTRODUCTION

Betel quid, a natural masticatory in south-eastern Asia, is composed of various components in different countries or areas (Bhonsle *et al.*, 1992). Taiwanese betel quid includes an entire fresh green areca fruit (containing the husk), *Piper betle* (leaf or inflorescence) and slaked lime paste (Chen *et al.*, 1984a). The slaked lime, handled as pasty form, is either white (white lime paste), containing no additives, or brown (red lime paste) due to the addition of catechu, an extract of *Acacia catechu*. An average of 14–23 betel quids are chewed per day by a Taiwanese chewer (Ko *et al.*, 1992), which is relatively higher compared with the amount consumed by chewers in India (Stich *et al.*, 1982b) or the Philippines (Stich *et al.*, 1984).

Betel quid chewing appears to be closely associated with an elevated risk of pre-invasive lesions such as leukoplakia or submucous fibrosis and oral cancer (IARC, 1985). The habit alone or in combination with tobacco chewing has been condemned as the major aetiology of oral cancer in some south Asian countries (Awang, 1988; Kwan, 1976; Sanghri, 1981). Epidemiological investigations show high prevalent rates of betel quid chewing among oral cancer patients, ranging from 32.1% to as high as 86.2% in Taiwan (Chang, 1964; Chen, 1987; Chen *et al.*, 1984b). However, the overall incidence of oral cancer is estimated to constitute only 3% in

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all human cancers in Taiwan, much lower than that of the other south Asian countries. These paradoxical findings implicate the necessity for an advanced investigation of the toxicity of Taiwanese betel quid.

It is not yet determined that betel quid or its various components are capable of inducing tumours or causing oral diseases. Some animal experiments show positive results (Bhide *et al.*, 1979; Ranadive *et al.*, 1976, 1979; Rao, 1984; Suri *et al.*, 1971) and others are negative (Dunham and Herrold, 1964; Hamner, 1972; Weerapradist and Boonpuknavig, 1983), which may be attributed to a great variety of compositions and preparations of betel quid derived from different geographical regions (Stich and Anders, 1989). However, little is known about the Taiwanese betel quid.

Taiwanese betel quid contains no tobacco, and the chewers never place tobacco in their oral cavities during chewing sessions. The aim of this investigation is to understand the toxicity of Taiwanese betel quid by the *Salmonella* microsome test, mammalian cell study, and animal experiments.

MATERIALS AND METHODS

Chemicals

D-Biotin (CAS No. 58-85-5), 5-bromo-2′-deoxyuridine (BrdUrd; CAS No. 59-14-3), 1-chloro-2,4-dinitrobenzene (CDNB; CAS No. 97-00-7), cinchonine sulfate (CAS No. 5949-16-6), 2′-deoxyguanosine (2′-dG; CAS No. 961-07-9), ethylenediaminetetraacetic acid (EDTA; CAS No. 60-00-4), 1-fluoro-2,4-dinitrobenzene (FDNB; CAS No. 70-34-8), glutathione reductase (CAS No. 9001-48-3), L-histidine monohydrochloride, monohydrate (CAS No. 7048-02-4), iodoacetic acid (IAA; CAS No. 64-69-7), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; CAS No. 70-25-7), nicotinamide adenine dinucleotide phosphate, sodium salt (NADP; CAS No. 1184-16-3), oxidized glutathione (GSSG; CAS No. 27025-41-8), 1,10-phenanthroline (CAS No. 5144-89-8), phosphotungstic acid (CAS No. 12501-23-4), potassium ferricyanide (CAS No. 13746-66-2), reduced glutathione (GSH; CAS No. 70-18-8), sodium azide (CAS No. 26628-22-8), sodium dodecyl sulfate (CAS No. 2044-56-6), 1,1,3,3-tetramethoxy-propane (CAS No. 102-52-3), 2-thiobarbituric acid (CAS No. 504-17-6), trisodium citrate (CAS No. 6132-04-3), and trypsin–EDTA were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dimethyl sulfoxide (DMSO; CAS No. 67-68-5), D-glucose 6-phosphate, disodium salt (CAS No. 3671-99-6), hydrogen peroxide (CAS No. 7722-84-1) and Giemsa stain were obtained from E. Merck (Darmstadt, Germany). Difco-Bacto agar (CAS No. 9002-18-0) was purchased from Difco Laboratories (Detroit, MI, USA). Nutrient broth No. 2 was purchased from Oxoid Ltd (Basingstoke, UK). Aroclor 1254-induced rat liver S9 was obtained from Organon Teknika Corp. (Durham, NC, USA). Colcemid (CAS No. 477-30-5), foetal bovine serum (FBS), McCoy's 5A, and phosphate buffered saline (PBS) were obtained from Gibco BRL, Life Technologies, Inc. (Gaithersburg, MD, USA).

Betel quid

Areca fruits and *Piper betle* inflorescences were obtained from the farm of Nantou county, Taiwan. The red lime paste was purchased from Taichung City, Taiwan. A whole betel quid was composed of fresh areca fruit, *Piper betle* inflorescence, and red lime paste (80.5:12.5:7, by weight).

Preparation of an aqueous extract of betel quid

Betel quids were extracted with deionized water (1:2, w/v) in a Waring blender for 3 min, followed by soaking 6 hr before filtration. Three batches of filtrates were collected and kept at 4°C for further freeze-drying within 2 wk. The yield of the freeze-dried powder was 15.3%. The freeze-dried powder was stored in cold room (−18°C), and was dissolved in PBS for the *Salmonella* microsome test and mammalian cell study.

Estimation of different phenolic groups

The contents of simple phenolics, non-tannin flavans, hydrolysable and condensed tannins in an aqueous extract of betel quid were separately determined by the method of Peri and Pompei (1971), and expressed as milligrams of catechin equivalent per gram of sample.

Analysis of alkaloids

The contents of alkaloids in the aqueous extract of betel quid were measured by the method of HPLC as described in our previous work (Wang and Hwang, 1993a).

Salmonella microsome test

Test strains of *S. typhimurium* TA98 and TA100 were kindly provided by Dr B.N. Ames, University of California at Berkeley. Strain TA98 detects mutagens that cause a frameshift in a guanine-cytosine base pair region. Strain TA100 detects mutagens that cause base-pair substitution at guanine-cytosine pairs. The mutagenic effect of test sample was assayed according to the Ames test using *S. typhimurium* strains TA98 and TA100 (Maron and Ames, 1983). In one experiment, a preincubation modification of the Ames test was employed (Yahagi *et al.*, 1977). The His^+ revertants were counted after incubation at 37°C for 48 hr, and the sample that induced dose-dependent increases of more than twice the number of spontaneous revertants were considered mutagenic. Each sample was assayed using triplicate plates. The testing amounts of each sample were obtained after the tests of cell survival by measuring the total colonies (more than

70% of the control) with two *S. typhimurium*. The maximum test amount for the aqueous extract of betel quid (with and without S9) and MNNG was 100 μg/plate and 10 μg/plate, respectively.

Mammalian cell culture

Chinese hamster ovary K1 (CHO-K1) cells were grown in McCoy's 5A medium supplemented with 10% FBS. A total of 100 cells/5 ml was incubated in a petri dish (40 mm diameter) for 24 hr at 37°C in a humidified 5% CO_2 atmosphere; 0.1 ml test sample was added to each culture and incubated for 24 hr (12 hr for one cell cycle). For the activation of S9, 0.1 ml test sample and 0.5 ml of a 10% S9 solution were added to each culture and incubated for 2 hr. New medium was added and the cells were incubated for 7 days. The medium was removed and the cells were treated with methanol. Methanol-treated cells were stained with 10% Geimsa for 5 min and counted (>50 cells/colony). The concentrations of each test sample depressed by less than 50% colony formation (as compared with the control), were used to evaluate the chromosomal aberration and SCE. The maximum test concentration for the aqueous extract of betel quid (with and without S9) and MNNG was 1000 μg/ml and 0.1 μg/ml, respectively.

For the measurement of chromosomal aberration and SCE, cells (2×10^5/10 ml) were prepared in a 25-cm^2 flask, and incubated for 24 hr (37°C, 5% CO_2). Then 0.2 ml test sample and 0.1 ml 10 μM BrdUrd were added to each culture, and the resultant mix was incubated for 24 hr. For the metabolic activation of S9, 0.2 ml test sample and 1 ml of a 10% S9 solution were added to each culture and incubated for 2 hr, new medium was replaced and 0.1 ml 10 μM BrdUrd were added, and the cells were incubated for 22 hr. At 1.5 hr before harvesting, 0.1 ml of a 10 μg/ml solution of colcemid was added to each culture. After gentle mixing, the cultures were returned to the incubator. At the end of the incubation period, each flask was shaken gently to dislodge mitotic cells and the cell suspension was transferred to capped 15-ml centrifuge tubes; 0.5 ml trypsin–EDTA (37°C, 3 min) was added to each flask to suspend the cells again. The cell suspension mixtures were centrifuged at 800 rpm for 10 min. The supernatant was discarded and the pellet of cells was resuspended in 8 ml 0.075 M potassium chloride solution. The suspension was left for 7 min at room temperature and then the centrifugation process was repeated. In a dropwise manner, 8 ml freshly made methanol–acetic acid (3:1, v/v) fixative was added and allowed to stand for 30 min at room temperature; the tube was gently shaken to resuspend the cells. They were then centrifuged at 800 rpm for 10 min and the supernatant was discarded. The cells in the centrifuge tube were resuspended with 8 ml of fixative for 10 min. The centrifugation procedure was repeated twice more, each time discarding the old fixative and resuspending the cells in fresh fixative. After the final addition of fixative, the tubes were centrifuged as above, the fixative was discarded, and the cell button was resuspended in three to four drops of fixative. Slides were prepared by the air-drying technique and stained with 3% Giemsa for 10 min. Chromosomal aberration and SCE were measured by the microscopic analysis on the 90-sec metaphases of each treatment (Wang and Lee, 1996).

Animal study

Thirty-two 4-wk-old male Sprague–Dawley rats, weighing 70–90 g were randomly assigned to the following four groups: one control group (feeding with AIN-76 diet) and three experimental groups. AIN-76 diet contained the following (g/100 g diet): casein, 20; sucrose, 50; corn starch, 15; alphacel, 5; corn oil, 5; AIN mineral mix, 3.5; AIN vitamin mix, 1; DL-methionine, 0.3; choline bitartrate, 0.2 (ICN Bio-chemicals, USA). Within the experimental groups, low-dose feeding (0.53 g dry aqueous extract/kg diet), mid-dose feeding (5.3 g dry aqueous extract/kg diet), and high-dose feeding (26.5 g dry aqueous extract/kg diet) were designed. The alkaloids of betel quid exhibit physiological activities. Each gram of dry matter of the aqueous extract of betel quid contained 19.78 mg alkaloids. An average user chews 14–23 betel quids/day (Ko *et al*., 1992), and the amount of alkaloid intake is 3.8 mg when an individual chews one betel quid (data not shown). The low-dose feeding is determined according to the calculation below: one rat (body weight; 250 g) as compared with one person (60 kg) chewing 20 betel quids/day. Animals were housed in a room maintained at a constant temperature (23°C) and humidity (50%), with alternating 12-hr periods of light and dark. The amount of food consumption and body weight were recorded weekly. After 8 wk of feeding, the livers and serum were isolated from the rats. The hepatic homogenates were obtained by homogenizing the liver with 20 mM phosphate buffer (pH 7; 1:10, w/v), then centrifuged (10,000 ***g***, 30 min). The resultant supernatant was ultracentrifuged (105,000 ***g***, 43 min) again to separate the supernatant (cytoplasmic fragment) and the sediment; the sediment was resuspended with phosphate buffer to obtain the microsomal fragment. The homogenates, cytoplasmic and microsomal fragments of liver and serum were taken for the following measurements. With hepatic homogenate, the lipid peroxidation was determined by the values of thiobarbituric acid reactive substance (TBARs; Fraga *et al*., 1988), and GSH and GSSG were measured by HPLC (Reed *et al*., 1980). With cytoplasmic fragment of liver, GSH peroxidase activity was determined spectrophotometrically by a couple of procedures using hydrogen peroxide as a substrate (Lawrence and Burk, 1976). GSH reductase activity was measured as described by Bellomo *et al*.

Table 1. The contents of alkaloids and phenolics in the aqueous extract and its freeze-dried powder of betel quid

Sample	Alkaloids				Phenolics			
	Guvacine	Arecaidine	Guvacoline	Arecoline	Hydrolysable tannins	Condensed tannins	Non-tannin flavans	Simple phenolics
	(mg/g dry weight)				(mg catechin equivalent/g dry weight)			
Extract	5.84 ± 0.4	7.53 ± 1.2	0.26 ± 0.04	5.47 ± 0.18	0.42 ± 0.03	10.27 ± 1.1	0.37 ± 0.06	0.18 ± 0.01
Powder	5.79 ± 0.3	7.84 ± 0.8	0.29 ± 0.02	5.86 ± 0.25	0.44 ± 0.01	10.44 ± 0.7	0.40 ± 0.01	0.17 ± 0.01

(1987). Both the activities of cytoplasmic and microsomal glutathione *S*-transferase (cGST; mGST) were determined according to the method of Bellomo *et al*. (1987). With the serum sample, serum glutamic–pyruvic transaminase (SGPT) activity as assayed by the GPT assay kit spectrophotometrically at 340 nm.

Formation of 8-OH-dG in vitro

The method was adapted from that described by Stadler *et al*. (1994) and modified accordingly. The standard reaction mix was prepared in 0.1 M potassium phosphate buffer (pH 7.4) and contained 2′-dG (0.427 mM), hydrogen peroxide (8.57 μM), ferric chloride (1.23 mM), EDTA (4 mM), ascorbic acid (2.06 mM) and the aqueous extract of betel quid. The reaction mix was incubated for 25 min at 37°C and then terminated by placing the samples on ice. Routine analyses of 8-OH-dG formation were conducted with HPLC with a Hitachi LC 6200A delivery system equipped with a Hitachi LC 4200 photodiode array detector, and employing a Lichrospher RP-18 endcapped column (Merck, 5 μm, 4.6 × 250 mm). The column was equilibrated with mobile phase of 88.8% 50 mM potassium phosphate and 11.2% methanol at a flow rate of 0.8 ml/min. 2′-dG (retention time = 18.54 min) and 8-OH-dG (retention time = 27.97 min) were monitored at 254 nm. The 8-OH-dG formation was expressed as: (the area of 8-OH-dG formation in the reaction mix containing sample/the area of 8-OH-dG formation in the reaction mix containing no sample) × 100%.

Statistics

The means and standard deviations were calculated for each treatment. The data were assessed by analysis of variance. Multiple comparisons were made by using Duncan's new multiple range test to determine significant difference among the means of different treatments.

RESULTS

Changes of alkaloids and phenolics during sample preparation

Arecoline, arecaidine, guvacoline and guvacine were included in an aqueous extract of betel quid; arecaidine is the most abundant (7.53 mg/g dry weight) and guvacoline is the least (0.26 mg/g dry weight). The aqueous extract of betel quid contained high levels of condensed tannins (10.27 mg catechin equivalent/g dry weight) and fairly low levels of simple phenolics (0.18 mg catechin equivalent/g dry weight). No change in the contents of alkaloids and phenolics was observed during the low temperature storage. In addition, both alkaloids and phenolics were also unchanged by the process of freeze-drying (Table 1).

Table 2. Mutagenic activities of the aqueous extract of betel quid and MNNG in *Salmonella typhimurium* strains TA98 and TA100

Amount per plate (μg)	His^+ revertant count per plate[1]			
	TA98		TA100	
	−S9	+S9	−S9	+S9
Spontaneous revertants[2]	36 ± 5	38 ± 4	177 ± 10	186 ± 9
Aqueous extract				
1	35 ± 2	37 ± 2	225 ± 21	168 ± 8
10	32 ± 4	41 ± 1	189 ± 13	160 ± 8
100	30 ± 2	41 ± 3	194 ± 14	179 ± 9
MNNG[3]				
0.1	N.D.[4]	N.D.	289 ± 13	N.D.
1	N.D.	N.D.	645 ± 25	N.D.
10	N.D.	N.D.	2389 ± 67	N.D.

[1]Data are means ± SD of three plates.
[2]The number of spontaneous revertants was determined without samples.
[3]*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.
[4]No data.

Salmonella microsome test

MNNG is a strong mutagen, which causes the base-pair substitution (McCann *et al.*, 1975). In this study, MNNG was used as a positive control and showed strong mutagenic response in the TA100 strain. The mutagenicity induced by an aqueous extract of betel quid was performed in both strains TA98 and TA100 with and without rat liver S9 fraction. As presented in Table 2, the aqueous extract of betel quid did not directly induce mutagenicity towards TA98 and TA100 in both the presence and absence of S9.

Mammalian cell test

The effects of the aqueous extract of betel quid and MNNG on the colony formation in CHO-K1 cells were assayed. As shown in Fig. 1, MNNG depressed, by 35%, colony formation at 0.1 μg/ml. At a concentration of 10 μg/ml, MNNG inhibited cell colony formation completely. The aqueous extract of betel quid depressed the colony formation at 1000 μg/ml, by 48% without S9 and 35% with S9.

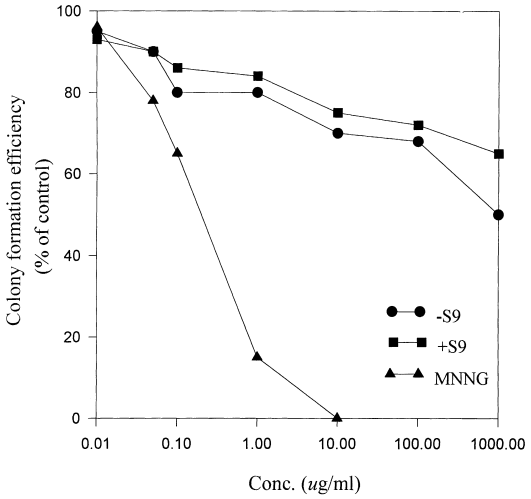

Fig. 1. Plating efficiency assay of an aqueous extract of betel quid and MNNG in CHO-K1 cells.

The effects on the chromosomal aberrations and SCE are presented in Table 3 and 4. MNNG induced strong chromosomal aberrations and sig-

Table 3. Chromosome aberrations induced by treatment of CHO cells with an aqueous extract of betel quid and MNNG

	Type of aberrations (%)							
	Gaps		Breaks		Exchanges		Aberrant metaphase (%)	
Concn (μg/ml)	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Control	2	2	0	0	0	0	2	2
Aqueous extract								
0.1	2	2	0	0	0	0	2	2
1	2	2	0	0	0	0	2	2
10	2	2	0	0	0	0	2	2
100	2	2	0	0	0	0	2	2
1000	2	2	0	0	0	0	2	2
MNNG[2]								
0.01	2	N.D.[1]	13	N.D.	5	N.D.	20	N.D.
0.05	4	N.D.	28	N.D.	15	N.D.	47	N.D.
0.1	7	N.D.	56	N.D.	24	N.D.	87	N.D.

[1]No data.
[2]*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

Table 4. Sister-chromatid exchange (SCE) frequencies of CHO cells exposure to the aqueous extract of betel quid and MNNG

	SCEs/metaphase[1]		
Concn (μg/ml)	−S9	+S9	MNNG (−S9)
Control	8.1 ± 0.3^{b2}	8.3 ± 0.5^{a}	8.5 ± 0.7^{d}
0.01	N.D.[3]	N.D.	10.3 ± 0.2^{c}
0.05	N.D.	N.D.	11.3 ± 0.6^{b}
0.1	7.8 ± 0.3^{b}	8.4 ± 0.2^{a}	15.8 ± 0.7^{a}
1	8.0 ± 0.3^{b}	8.7 ± 0.4^{a}	N.D.
10	8.3 ± 0.5^{b}	8.5 ± 0.1^{a}	N.D.
100	9.3 ± 0.2^{a}	9.0 ± 0.5^{a}	N.D.
1000	9.8 ± 0.4^{a}	8.7 ± 0.4^{a}	N.D.

[1]Data are means ± SD of three plates.
[2]Values of the same column with different superscript letters are significantly different ($P < 0.05$).
[3]No data.

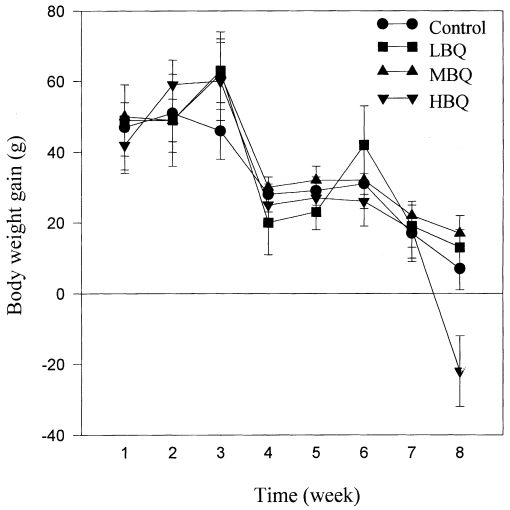

Fig. 2. Effect of an aqueous extract of betel quid on body weight gain. Control AIN-76 diet (n = 8). LBQ 0.53 g dried aqueous extract of betel quid/kg diet (n = 8). MBQ 5.3 g dried aqueous extract of betel quid/kg diet (n = 8). HBQ 26.5 g dried aqueous extract of betel quid/kg diet (n = 8).

Table 5. Effect of the aqueous extract of betel quid on relative liver weight (liver weight/body weight)

Treatment[1]	Body weight (g)	Liver weight (g)	Relative liver weight
Control	356 ± 32	15.7 ± 1.3	$0.044 \pm 0.011^{a*}$
LBQ	412 ± 51	16.1 ± 2.2	0.039 ± 0.017^{ab}
MBQ	396 ± 45	15.3 ± 3	0.039 ± 0.014^{ab}
HBQ	396 ± 32	11.7 ± 2.7	0.030 ± 0.012^{b}

[1]As in Fig. 2.
*Values of the same column with different superscript letters are significantly different ($P < 0.05$).

Table 6. Effect of the aqueous extract of betel quid on the production of thiobarbituric acid reactive substance (TBARs) in rat hepatocytes

Treatment[1]	TBARs (nmol/mg protein)
Control	$0.23 \pm 0.03^{a*}$
LBQ	0.25 ± 0.06^{a}
MBQ	0.26 ± 0.12^{a}
HBQ	0.25 ± 0.11^{a}

[1]As in Fig. 2.
*Values of the same column with different superscript letters are significantly different ($P < 0.05$).

nificantly elevated the frequency of SCE. However, no difference in the chromosomal aberration and no high frequencies of SCE were observed in cells treated with the aqueous extract of betel quid. Except that a slight increase in the frequency of SCE occurred at the treatments of 100 and 1000 μg/ml in the absence of S9.

Animal test

As presented in Fig. 2, an aqueous extract of betel quid showed no effect on body weight gain. The high-dose feeding had no effect on body weight gain in rats for 7 wk, but there was a decrease at wk 8. The high-dose feeding also reduced the relative liver weight (Table 5). The effect of an aqueous extract of betel quid on the lipid peroxidation of liver was determined by measuring the TBARs values. As shown in Table 6, there was no significant difference among all groups. GSH protects cells from the toxic effects of many reactive oxygen species. The effect of an aqueous extract of betel quid on intracellular levels of glutathione in rat hepatocytes is illustrated in Table 7. The high-dose feeding markedly reduced the contents of GSH and total glutathione (GSH + 2 × GSSG), but did not affect the content of GSSG and the ratio of GSH to GSSG.

GSH peroxidase and reductase were important enzymes for the removal of reactive oxygen species. The activity of GSH reductase was not changed by the aqueous extract of betel quid, but the activity of GSH peroxidase was found to increase in the low-dose feeding group (Table 8). The activities of detoxifying enzymes in hepatocytes (mGST, cGST and SGPT) are presented in Table 9. Only the activity of cGST was significantly increased in the low dosage feeding group.

Formation of 8-OH-dG in vitro

The effect of an aqueous extract of betel quid on the formation of 8-OH-dG in the presence of Fe^{3+}/EDTA/H_2O_2 was analysed by HPLC. The oxi-

Table 7. Effect of the aqueous extract of betel quid on intracellular level of glutathione in rat hepatocytes

	GSH	GSSG	Total glutathione[2]	
Treatment[1]	(nmol/mg protein)			GSH/GSSG
Control	$69 \pm 16^{a*}$	1.0 ± 0.2^{ab}	71 ± 16^{a}	68 ± 1^{a}
LBQ	63 ± 15^{ab}	1.0 ± 0.1^{ab}	65 ± 15^{a}	65 ± 7^{a}
MBQ	71 ± 26^{a}	1.3 ± 0.5^{a}	74 ± 27^{a}	54 ± 15^{a}
HBQ	35 ± 13^{b}	0.6 ± 0.2^{a}	36 ± 13^{b}	60 ± 5^{a}

[1]As in Fig. 2.
[2]Total glutathione = 1GSH + 2GSSG.
*Values of the same column with different superscript letters are significantly different ($P < 0.05$).

Table 8. Effect of the aqueous extract of betel quid on the activities of GSH peroxidase and GSH reductase

Treatment[1]	GSH peroxidase (nmol/mg protein)	GSH reductase (nmol/mg protein)
Control	$132 \pm 17^{b*}$	7.3 ± 0.6^{a}
LBQ	170 ± 25^{a}	8.4 ± 1.7^{a}
MBQ	139 ± 12^{ab}	8.1 ± 1.5^{a}
HBQ	149 ± 40^{ab}	8.3 ± 1.8^{a}

[1]As in Fig. 2.
*Values of the same column with different superscript letters are significantly different ($P < 0.05$).

Table 9. Effect of the aqueous extract of betel quid on the activities of detoxifying enzymes of hepatocytes

Treatment[1]	mGST (nmol/mg protein)	cGST (nmol/mg protein)	SGPT (unit/litre)
Control	$177 \pm 28^{a*}$	350 ± 35^{b}	23 ± 3^{a}
LBQ	184 ± 39^{a}	530 ± 98^{a}	19 ± 4^{a}
MBQ	170 ± 16^{a}	376 ± 73^{b}	16 ± 9^{a}
HBQ	164 ± 24^{a}	401 ± 72^{b}	21 ± 4^{a}

[1]As in Fig. 2.
*Values of the same column with different superscript letters are significantly different ($P < 0.05$).

dation of 2′-dG by an aqueous extract of betel quid into 8-OH-dG is expressed in Fig. 3. The aqueous extract of betel quid accelerated the formation of 8-OH-dG with doses (0.1–0.5 mg) up to a maximum and then decreased.

DISCUSSION

Betel quid (different composition from that of Taiwanese betel quid), areca nut and its constituents (e.g. alkaloids and related *N*-nitrosamines) show mutagenic activity in *S. typhimurium* TA98 and TA100 (Polasa *et al.*, 1993; Shirname *et al.*, 1983, 1984; Wang and Peng, 1996). Both alkaloids and phenolics are physiologically active substances in betel quid (Hwang *et al.*, 1992; Leslie, 1965; Nieschulz, 1967). Betel nut and arecoline are found to be weakly mutagenic towards *S. typhimurium* TA100 (Polasa *et al.*, 1993; Shirname *et al.*, 1983; Wang and Peng, 1996). Whereas the crude phenolic extract (from fresh areca fruit and *Piper betle*) and some substances (e.g. tannin, gallic acid, hydroxychavicol and chlorogenic acid) of betel quid exhibited antimutagenic activities (Abraham *et al.*, 1979; Wang and Lee, 1996; Wang and Wu, 1996) and antinitrosation (Stich *et al.*, 1982a; Wang and Peng, 1996). In this study, no mutagenic response towards both strains of *S. typhimurium* was probably due to the abundant polyphenolics in the aqueous extract of betel quid.

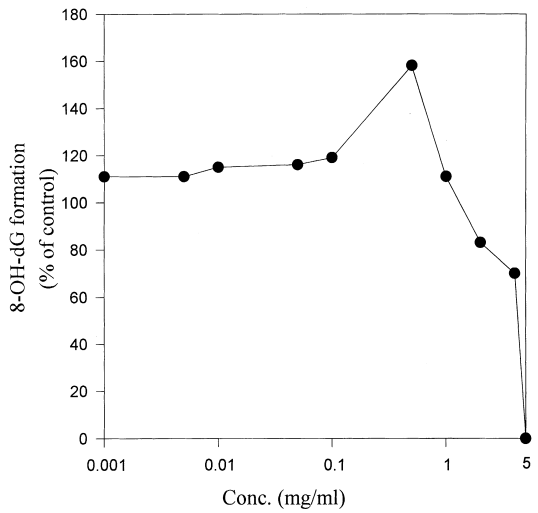

Fig. 3. Effect of an aqueous extract of betel quid on the formation of 8-OH-dG.

Many mutagens induce chromosomal aberrations or increase the frequency of SCE in a dose-dependent manner (Popescu *et al.*, 1991). The DMSO extract of pan masala induces chromosomal aberrations and SCE in the presence or absence of S9 (Patel *et al.*, 1994). Arecoline is also found to have a weak chromosome-damaging effect *in vivo*, and the frequency of chromosomal aberrations in mouse bone marrow cells show a dose–response relationship (Panigrahi and Rao, 1982). Betel quid, areca nut-related compounds and its extracts were toxic to the human buccal epithelial cells (Jeng *et al.*, 1994b; Sundqvist *et al.*, 1989; Van Wyk *et al.*, 1994). In this study, the aqueous extract of betel quid did not elevate the chromosomal aberration, but minimally increase the frequency of SCE at 100 and 1000 μg/ml only in the absence of S9. It indicated that the cytotoxicity of the aqueous extract of betel quid was removed after the function of an S9 metabolic activation system. Mathew and Govindarajan (1964) showed that tender betel nut contained higher levels of polyphenolics than ripe nut. The ripe betel nut is used as the fresh, curing or baking type in India (Bhonsle *et al.*, 1992). Fresh areca fruit (tender nut with husk) is the main ingredient of Taiwanese betel quid, which abundant polyphenolics exhibit marked antioxidant activity and antimutagenic effect (Wang and Hwang, 1993b; Wang and Lee, 1996). The negative biological significance in this assay may be ascribed to the polyphenolics of this aqueous extract, which diminished the chromosome damage. Further research on the role of the antimutagenic ability of the polyphenolics in the aqueous extract of betel quid is required.

In the animal experiment, the body weight gain at wk 8 and the relative liver weight were reduced by high-dose feeding. Such results showed that high-dose feeding probably damaged the liver and the absorption or metabolism of nutrients was

retarded by high amounts of substances (e.g. tannin) in the aqueous extract of betel quid.

The measurement of TBARS is a good indicator for the lipid peroxidation. The negative results revealed that the aqueous extract of betel quid did not induce direct oxidative damage to liver. GSH is known to be broadly distributed and participate in numerous cellular processes, particularly in the detoxification process. GSH removes the intermediates such as hydrogen peroxide and other hydroperoxides by GSH peroxidase and is converted as GSSG. GSH also forms conjugates with a variety of xenobiotics by GST, thereby aiding in detoxification and excretion (Meister, 1989). The high-dose feeding lowered the contents of GSH and total glutathione, but did not affect the GSSG content and the ratio of GSH to GSSG. It seemed that high dosage of aqueous extract of betel quid perhaps depressed the synthesis of GSH or some substances in this extract which conjugated with GSH. Eugenol and hydroxychavicol are phenolic compounds of betel quid (Wang and Hwang, 1993b), which are found to inhibit the lipid peroxidation and depress the content of GSH (Bhide *et al.*, 1991; Jeng *et al.*, 1994a; Mizutani *et al.*, 1991; Wang and Wu, 1996). On the other hand, Singh and Rao (1993) found that areca nut decreased the cGST activity in the liver of mice. However, we showed that the low-dose feeding increased the activities of GSH peroxidase and cGST. The difference was mainly due to the use of different experimental samples (ripe areca nut; whole betel quid) and animals (mice; rat). The increase of GSH peroxidase activity and the promotion of cGST activity by the aqueous extract of betel quid showed that this aqueous extract probably activated the antioxidative effect and detoxification of liver cells to prevent oxidative damage. Rather, the higher cGST activity may be ascribed to a compensatory mechanism consequent to GSH depletion in the liver.

SGPT was closely related to the mechanism of amino acid and the level of liver damage. The aqueous extract of betel quid did not increase the SGPT activity. It was evident that the aqueous extract had no damage to liver. Sarma *et al.* (1992) showed that the activity of SGPT was increased by pan masala. It meant that pan masala was more toxic to liver than Taiwanese betel quid.

All the results clearly showed that the formation of reactive oxygen species induced by the aqueous extract of betel quid played an important role in the oxidative toxicity. Nair *et al.* (1987, 1990, 1992, 1995) reported that the reactive oxygen species, superoxide anion and hydrogen peroxide, can be generated from areca nut extract under alkaline slaked lime (pH $\geqslant$9.5). Stadler *et al.* (1994) indicated that incubation of caffeic acid led to much 8-OH-dG formation, resulting from the reductant ability of caffeic acid. Coffee also accelerated the oxidation of 2′-dG to 8-OH-dG at lower dosages. However, the formation of 8-OH-dG was lowered by increasing the dose of coffee, which may be due to the highly electrophilic hydroxyl radical with coffee constituents. In this study, an aqueous extract of betel quid may act as pro-oxidants at lower dosages and may be dependent on the iron ions that exist in the model system. The aqueous extract of betel quid showed antioxidant activity at higher dosage which might be due to the ability of the scavenging effect of the hydroxyl radicals. The negative results for the mid- and high-dose feeding treatments could be substantiated by the inhibition on the formation of reactive oxygen species.

The toxicity of aqueous extract of betel quid was marginally significant. However, the toxicity was closely correlated with many factors, further research on the toxic responses affected by immune system, genetic abnormalities or nutritional deficiencies are required.

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