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Safety Evaluation and Antimutagenic Activity of Bamboo/Wood Vinegars Collected at Different Temperatures

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The biological action of bamboo/wood vinegars collected at different temperatures from the exit of chimney of earthen kiln was evaluated by *Salmonella* mutagenesis assay, as a safety evaluation (Ames test) and reverse mutation assay (antimutagenic activity). The compounds of the vinegars were analyzed using gas chromatography–mass spectroscopy analysis. The acid, phenol and ketone compounds of bamboo vinegars were 10.65–20.09%, 57.87–65.98% and 10.13–18.76%, and the compounds of wood vinegars were 4.27–14.51%, 50.23–65.03% and 12.93–25.26%, respectively. The vinegars' safety showed that neither cytotoxicity nor mutagenicity toward *Salmonella typhimurium* TA98 and TA100 with S9 mix (an external metabolic activation system) at the diluting percent content of vinegars were lower than 20.00% or less, and without the S9 mix was at 33.33% or less. The vinegars at a diluting percent content below 20.00% expressed a dose–dependent inhibitory effect against both 4–nitroquinoline–*N*–oxide (NQNO), a direct mutagen, and the mutagenicity of aflatoxin B_1 (AFB_1), an indirect mutagen which requires metabolic activation, in *Salmonella typhimurium* TA98 and TA100. The inhibition of the vinegars against NQNO and AFB_1 toward TA100 was better than those toward TA98. In addition, the main percent of phenol and ketone compounds in the vinegars showed cytotoxicity/mutagenicity and an antimutagenic effect against the strains mentioned above, which may partially account for the biological action of bamboo/wood vinegars.

Key words: Ames test, Antimutagenic Activity, Bamboo/Wood Vinegar, Gas Chromatography–Mass Spectroscopy, Safety Evaluation

INTRODUCTION

Both bamboo and wood vinegar, by–products of the bamboo or wood charcoal manufacturing, are brown–red transparent liquids collected during pyrolysis of bamboo or wood charcoal. Yatagai *et al*. (1988) reported that with the application of bamboo/wood vinegars, even the compounds are complex and different, and are mainly able to be divided into three main portions: acid, phenol and neutral compounds. The vinegars consist of 80–200 compounds: 32% organic acid, 40% phenolic compound, 3% aldehyde, 5% alkone compound, 5% alcohol compound, 4% ester compound and 5% others. When both are dehydrated there is usually 80% water (Ikimoto and Ikeshima, 2000; Nomura, 2004; Uchimura *et al*., 2000; Tsai *et al*., 2009). The organic compounds in bamboo/wood vinegars may have practical applications even when present in only trace quantities (Uchimura *et al*., 2000; Lin *et al*., 2006).

Bamboo vinegar is effective in improving soil, promoting crops and preventing worm growth, as well as reducing agricultural chemicals, compost odor and sterilization (Ikimoto and Ikeshima, 2000). Recently, bamboo vinegar products have been developed that are beneficial for promoting growth of plants to as a plant root growth promoter or a pH value adjuster of cultural media (Huang *et al*., 2011; Lin *et al*., 2011; Ho *et al*., 2013). It is also effective when used against allergies (Hageta, 2004), in healthy drinks (Kobahasi, 2004; Nomura, 2004; Yoshie, 2004), as a virus/fungi/bacterial resistant (Kou, 2004; Lin *et al*., 2006; Lin and Shiah, 2006; Lu *et al*., 2007; Lin *et al*., 2008; Chen *et al*., 2010) and as an agent of antioxidation, especially for a resistant lipid oxidation effect (Tsai *et al*., 2009). The compounds of wood vinegar are much more complex than those of bamboo vinegar (Chen *et al*., 2006; Hwang *et al*., 2008), and can repel termites and disease germs of plants, be fungi resistant, improve the soil, promote crops or prevent worm growth and reduce the use of agricultural chemicals, such as a natural insecticide or soil fungicide (Ishihara, 1996; Nishimiya *et al*., 1998; Yatagai, 2002; Lu *et al*., 2007; Lin *et al*., 2009). As stated in the above references, the commercial production of bamboo/wood vinegar is being increased and highly valued for its various effective uses in Taiwan.

Horne and Paul (1996) reported that bamboo/wood

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vinegars collected at the exit of chimney of earthen or furnace kiln, when the carbonization temperature of bamboo/wood was raised to over 500°C, produced some carcinogenic polycyclic aromatic hydrocarbons (PAHs), such as naphthalene and phenanthrene (Namiki, 1990; Vasavada and Cornforth, 2006). The concentrations of these toxics increased with the increase in temperature as well. Even though the council of agriculture in Taiwan has submitted certified agricultural standards of forest products (2004) to prove that it is necessary for bamboo/wood vinegars to be collected at 80–140°C at an exit of chimney of earthen kiln and below 350°C for a furnace kiln, collected bamboo/wood vinegars from above this range of temperatures are necessary to evaluate the potential of mutagenic and carcinogenic agents, due to the fact that both of them are omnipresent in the human environment and seem impossible to completely eliminate. Ames *et al*. (1975) reported that for screening of environmental mutagens and carcinogens, the Ames test (Safety evaluation), a convenient method to evaluate mutagenic activities of these chemicals, has been developed, and McCann *et al*. (1975) and Shugimura *et al*. (1976) have suggested that the mutagenic activities of a number of chemicals correlate well with the carcinogenic activities.

To expect to be one and/or a reference of functional additives for bamboo/wood vinegars, the safety evaluation, including cytotoxicity and mutagenicity, of the vinegars using the bacterial mutation assay on *Salmonella typhimurium* TA98 and TA100 strains with and without an extrinsic metabolic activation system were evaluated. Moreover, because the antimutagenic properties have an array of prospective applications in human care, such as the increasing application in drinks, food antioxidation, etc., and have not been reported for the antimutagenic activities of bamboo/wood vinegars that have been made so far, the antimutagenic activity of the vinegars are investigated as well. These vinegars, collected at different temperatures from the exit of earthen kiln chimney, were also investigated for their basic compound by using gas chromatography–mass spectroscopy.

MATERIALS AND METHODS

Specimen preparation

Bamboo/wood vinegars

The vinegars were collected at different temperatures, ranging from 80°C to 150°C (Uchimura *et al*., 2000), during the manufacturing process (pyrolysis) of charcoal from Moso bamboo (*Phyllostachys heterocycla* Milf) and branches/tree tops (BTW) of *Cryptomeria japonica* and were provided by the Division of Forest Utilization, TFRI Taipei, Taiwan (Hwang *et al*., 2006; Hwang *et al*., 2008). The bamboo and wood vinegars were based on the temperatures measured by a thermocouple at the exit of earthen kiln chimneys during bamboo/wood charcoal pyrolysis. The different groups of bamboo vinegar were collected at 80–150°C, and with categories over 80, 90–92, 99–102, 120–123 and 145–150°C. The various collected temperatures of wood vinegar were 80–159°C, and with categories 80–90, 91–100, 101–120, 121–140 and 141–159°C. The basic properties of the bamboo/wood vinegars refer to (Lin *et al*., 2008; Lin *et al*., 2009).

Test strains

Salmonella typhimurium (*S. typhimurium*), including TA98 and TA100, was bought from the Bioresource Collection and Research Center, Food Industry Research and Development Institute.

Rat liver mixture

The rat liver mixture (S9 mix) (Organ Teknika Co., Switzerland) was prepared from Sprague–Dawley male rats treated with Aroclor 1254.

Mutagens

The mutagens are 4–nitroquinoline–*N*–oxide (NQNO) and aflatoxin B1 (AFB1). They were obtained from the Sigma Chemical Co. (Steinheim, Germany). All reagents used in the test were of analytical grade.

Experimental methods

Compounds analysis

Gas chromatography–mass spectroscopy (GC–MS) analysis was performed with a Varian CP–3800 chromatography instrument, combined with a Saturn–2000 mass spectrometer equipped with an electron ionization and quadrupole analyzer. The vinegars collected at different temperatures were diluted 10 times using acetone. The flow rate was set at 1.0 mL/min and the carrier gas was nitrogen. The size of the capillary column (DB–5MS, J&W Scientific, Folsom, CA, USA) was 30.0 m×250 μm i.d. and its film thickness was 0.25 μm. The temperatures of the ion source and interface were set at 280°C, and the mass scan range was from 40 to 550 amu. The electron energy was set at 70 eV. The oven temperature was programmed to hold at 40°C for 5 min and then, increased to 280°C at a rate of 12°C/min. The GC–MS chromatographic spectra of various bamboo/wood vinegar compounds were analyzed by the software of MS Workstation (Tsai *et al*., 2009).

Ames test

Cytotoxicity

0.1 mL of bamboo/wood vinegars were diluted to a percent content of 50, 33.33, 25, 20, 13.33 and 10%. The specimens were put in the test tube and mixed with 0.1 mL phosphate buffer saline and 0.1 mL *S. typhimurium* TA98 and TA100, and cultured overnight in nutrient broth. If there was additional S9 mix, the aforesaid addition level was changed to 0.2 mL phosphate buffer saline, 0.1 mL *S. typhimurium* TA98 and TA100 and cultured overnight in nutrient broth and 0.5 mL S9 (with S9 mix) or zero S9 (without S9 mix). Afterwards, the test tube was pre–cultured at 37°C for 20 min. The mixed diluent was taken out, and then, 1 mL diluent was put in the plate, mixed with nutrient agar and shaken up. When the mixture solidified, the plate was placed in an incubator at 37°C for 48 h. The colony (bacterial) count was calculated; if the bacterial count of the test group (including with or without S9 mix) was greater than the bacterial count of the control group by 80% (the bacterial count rate, Survival; %), there was no toxicity (Ames and

McCann, 1975). The survival of cytotoxicity is calculated as follows:

Survival (%) = (the bacterial count of test group / the bacterial count of control group) * 100

Mutagenicity

The mutagenicity is analyzed by using the method proposed by Maron and Ames (1983). The test vinegars for this mutagenicity test, with or without S9 mix, are the same as for the cytotoxicity test. 0.1 mL phosphate buffer, 0.1 mL *S. typhimurium* TA98 and TA100 cultured overnight in oxoid nutrient broth No.2 were put into the test tube, mixed with 0.5 mL phosphate buffer saline and cultured at 37˚C for 20 min. The test mixture was mixed with 2 mL Molten Top Agar (including 0.05 mM L–histidine, 0.05 Mm Biotin and 0.09 M NaCl) uniformly; the nutrient agar then was poured into the plate. When the mixture solidified, the plate was put in the incubator at 37˚C for 48 h, and then, the colony count was calculated. In addition, the phosphate buffer saline is only a pair of control groups (Blank). If the colony count of the TA98 and TA100 test groups is greater than that for the control group by more than two times, the specimen has mutagenicity. In other words, the Mutagenicity ratio (MR) = induced revertants per plate/spontaneous revertants per plate (Blank).

Antimutagenic activity

The test vinegars of the antimutagenic activity were assayed according to the Ames method (Maron and Ames, 1983). The mutagens that were diluted with dimethyl sulfoxide (DMSO) were NQNO (1 μg/plate for TA 98 and TA 100, respectively), a direct–acting mutagen; and AFB_1 (5 μg/plate for TA 98 and TA 100, respectively), which required S9 mix for metabolic activation. A mutagen (0.1 mL; contained 1 μg NQNO or 5 μg AFB1) was added to the mixture of a strain (TA 98 or TA 100), and 0.1 mL of each test vinegar was added to the S9 mix for AFB1 or to the phosphate buffer (0.1 mol/L, pH 7.4) for NQNO. The mutagenicity of each mutagen in the absence of an extract is defined as 100%. The inhibition (%) of mutagenicity of test vinegar is calculated as follows: Inhibition (%) = [1 – (Number of his+revertants in the presence of the test vinegar – Number of spontaneous revertants)/ (Number of his^+ revertants in the absence of the test vinegar – Number of spontaneous revertants)] × 100

Statistical analysis

The test results of the cytotoxicity, mutagenicity and antimutagenicity are represented by an average value (standard deviation), and the control group and test group are compared by Ducan's analysis. If the ρ value is smaller than 0.05, meaning a significant difference between the test group and the control group, it is represented by different superscript upper case letters.

RESULTS AND DISCUSSION

Compounds of bamboo/wood vinegars

The identified compounds of bamboo/wood vinegars collected from different temperatures were analyzed with GC–MS. The acid, phenol, ketone and other compounds were found in bamboo/wood vinegars. In general, the main fraction of bamboo/wood vinegars for an acid compound is acetic acid (Yatagai *et al.*, 1988). However, the retention time cannot be measured from the beginning point (zero) and is about 5 min, so that the acetic acid is not shown in the results of the study. In other words, the fraction percents of compounds from bamboo/wood vinegars are calculated only from the measured compounds, shown in Tables 1 and 2.

The acid, phenol, ketone and other compounds of bamboo vinegars were 10.65–20.09%, 57.87–65.98%, 10.13–18.76% and 9.66–15.30, respectively (Table 1). The acid compounds included butanoic acid, 2–methoxyethyl acetate, 4–hydroxy–butanoic acid and 4–hydroxy–3–methoxy–butanoic acid. The maximum fraction of acid compounds was the bamboo vinegar collected from 80–150˚C. The phenol (5.93–16.60%), 2–methoxy–phenol (8.27–16.39%) and 4–ethyl–phenol (3.68–9.48%) were the main fractions of phenol compounds for bamboo vinegars. For ketone compounds, the 2–hydroxy–3–methyl–2–cyclopentenone–1–one, 2,3–dimethyl–2–cyclopentenone–1–one and maltol could be measured for bamboo vinegars collected at all temperatures. The maximum fraction of ketone compounds was the bamboo vinegar collected from 120–123˚C.

For wood vinegars (Table 2), the acid, phenol, ketone and other compounds of wood vinegars were 4.27–14.51%, 50.23–65.03%, 12.93–25.26% and 9.57–19.92%, respectively. The main fractions of phenol compounds were phenol (3.36–11.30%), 2–methoxy–phenol (11.70–22.90%) and 2–methoxy–4–methyl–phenol (7.09–20.10%). Compared to both vinegars, the maximum fraction of ketone compounds for wood vinegar was also collected at temperatures from 120–123˚C, and was higher than those for bamboo. Moreover, the range of the phenol compounds fraction for bamboo/wood vinegar was around 50–60%; among which, the maximum fractions of phenol compounds were phenol and 2–methoxy–phenol.

Cytotoxicity of bamboo/wood vinegars

According to the former results of compound analysis, phenol, ketone and other compounds are present in bamboo/wood vinegars. Some phenol compounds displayed antimicrobial activity (Yam *et al.*, 1997). If mutagenicity occurred in a treated material, the results of the antimutagenic assay would be affected and confused due to increased or decreased numbers of revertants of TA98 and TA100 (Duh and Yen, 1997). The phenol compounds of bamboo vinegars were 57.87–65.98%, and the compounds of wood vinegars were 50.23–65.03%. Therefore, the cytotoxicity of bamboo/wood vinegars must be determined before testing the mutagenicity and antimutagenicity of bamboo/wood vinegars. Cytotoxicity results for the bamboo/wood vinegars collected at temperatures of 90–92/105–109˚C with the original vinegar (no diluting) and a range of diluting percent contents of 50.00, 33.33, 25.00, 20.00, 13.33 and 10.00% for *S. typhimurium* TA98 and TA100 without the S9 mix, as well as for with the S9 mix with diluting percent contents of 33.33,

Table 1. Various Compounds of bamboo vinegars collected from different temperatures by using Gas chromatography–mass spectroscopy

Identified compound	Collection temperature (˚C)					
	80	90–92	99–102	120–123	145–150	80–150
	Compound percent (%)					
Acid compound						
butanoic acid	1.91	1.77	1.37	1.79	2.23	4.58
2–methoxyethyl acetate	1.50	1.38	1.11	1.26	1.63	5.98
4–hydroxy–butanoic acid	8.98	9.02	8.17	6.84	7.89	8.11
4–hydroxy–3–methoxy–butanoic acid	–	–	–	1.44	0.84	1.42
Total	12.39	12.17	10.65	11.33	12.59	20.09
Phenol compound						
phenol	14.69	16.60	15.20	12.48	15.79	5.93
4–methyl–phenol	2.78	4.47	3.88	3.13	5.32	2.04
3–ethyl–phenol	4.60	6.44	6.06	8.35	8.81	6.69
2–methoxy–phenol	16.39	15.40	11.70	9.33	8.27	12.60
2,4–dimethyl–phenol	–	1.41	1.56	1.09	1.73	–
4–ethyl– phenol	8.26	9.48	8.60	6.64	6.98	3.68
2–methoxy–4–methyl–phenol	4.06	4.20	3.59	2.92	2.40	3.41
4–ethyl–2–methoxy–phenol	2.97	3.16	2.75	2.03	1.73	3.46
2,6–dimethoxy–phenol	5.77	4.82	9.69	11.90	8.76	20.20
Total	59.52	65.98	63.03	57.87	59.79	58.06
Ketone compound						
4–hydroxy–4–methyl–2–pentanone	3.07	2.80	2.28	2.14	5.73	–
1–hydroxy–3–methyl–2–butanone	–	–	1.66	1.46	1.51	–
1–methyl–2–propanone	–	–	0.86	0.94	–	–
3–methyl–2–cyclopentenone–1–one	1.19	–	0.90	2.31	2.22	1.38
2,5–dihydro–3,5–dimethyl–2–furanone	2.11	–	1.57	1.65	1.41	1.78
2–hydroxy–3–methyl–2–cyclopentenone–1–one	3.37	3.40	3.76	3.46	1.25	2.07
2,3–dimethyl–2–cyclopentenone–1–one	2.10	2.28	2.00	1.76	1.69	1.79
maltol	0.95	1.17	1.74	3.30	2.53	1.88
2–nonanone	–	1.05	–	0.89	1.02	–
1–(2,6–dihydroxy–4–methoxyphenyl)–ethanone	–	–	–	0.84	0.60	1.28
Total	12.79	10.70	14.77	18.76	17.96	10.13
Other compounds						
pyridine	9.36	6.46	5.86	5.02	4.32	5.87
4–methyl–1–penten–3–ol	4.92	3.65	3.08	2.35	–	–
3–propoxy–1–propene	–	–	–	1.17	1.72	1.96
tetrahydro–2,5–dimethyl–furan	–	–	1.30	1.50	1.66	–
2,6–dimethyl–heptane	1.02	1.04	1.31	2.00	1.96	3.89
Total	15.30	11.15	11.55	12.04	9.66	11.72

– : Trace amount (can not be measured)

25.00, 20.00, 13.33 and 10.00% are shown in Table 3.

The residual bacterial count of the control group (Blank) toward *S. typhimurium* TA98 and TA100 was 1838 and 1500. For bamboo vinegars collected at temperatures of 90–92˚C, the residual bacterial count without S9 for various diluting percent contents was 0–1830 for TA98 and 87–1641 for TA100; for those with S9, it was 1605–2000 for TA98 and 1736–2249 for TA100. For wood vinegars at 105–109˚C, the range of the residual bacterial count without S9 was 607–2071 for TA98 and 1558–1917 for TA100, and with S9 was 1864–2242 for TA98 and 3181–3448 for TA100. Waleh *et al*. (1982) indicated that the residual bacteria rate of *S. typhimurium* must be over 80% of the control group to determine that the test group has no cytotoxicity for *S. typhimurium*. The residual bacteria rate (Survival, %) toward TA98 and TA100 for bamboo vinegar without the S9 mix at a diluting percent content 33.3 % or less, and with the

Table 2. Various Compounds of wood vinegars collected from different temperatures by using Gas chromatography–mass spectroscopy

Identified compound	Collection temperature (˚C)					
	80–81	90–97	105–109	125–133	153–159	80–159
	Compound ratio (%)					
Acid compound						
butanoic acid	4.74	2.87	2.70	1.48	6.80	3.10
2–methoxyethyl acetate	3.96	3.29	2.66	1.27	2.43	4.36
4–hydroxy–butanoic acid	5.81	4.69	4.15	1.52	5.01	3.01
Total	14.51	10.85	9.51	4.27	14.24	10.47
Phenol compound						
phenol	8.12	8.70	9.48	10.88	11.30	3.36
4–methyl–phenol	2.94	3.60	4.46	4.81	4.36	1.60
3–ethyl–phenol	4.26	5.28	6.50	6.33	6.94	7.21
2–methoxy–phenol	18.20	13.39	12.40	14.98	11.70	22.90
2,4–dimethyl–phenol	–	1.75	2.05	1.29	1.98	–
4–ethyl–phenol	–	1.17	1.32	1.45	1.09	–
2–methoxy–4–methyl–phenol	13.80	11.29	10.30	7.09	9.83	20.10
4–ethyl–2–methoxy–phenol	4.97	4.02	3.77	3.15	3.90	7.50
2,6–dimethoxy–phenol	–	1.03	1.00	0.54	1.04	2.36
Total	52.29	50.23	51.28	50.55	52.11	65.03
Ketone compound						
4–hydroxy–4–methyl–2–pentanone	3.75	2.07	2.01	1.10	3.62	–
1–hydroxy–3–methyl–2–butanone	–	0.89	1.37	1.15	1.39	–
1–methyl–2–propanone	2.24	1.79	1.51	2.18	1.13	–
3–methyl–2–cyclopentenone–1–one	2.26	1.97	2.26	0.81	1.50	–
5–methoxy–2–pentanone	2.08	1.62	1.54	2.15	1.55	–
2,5–dihydro–3,5–dimethyl–2–furanone	2.26	2.36	2.08	3.12	1.44	2.21
2–hydroxy–3–methyl–2–cyclopentenone–1–one	6.98	6.45	6.57	5.70	5.50	5.21
2,3–dimethyl–2–cyclopentenone–1–one	2.08	1.52	1.51	1.87	1.63	2.83
maltol	1.98	3.58	4.09	5.00	3.10	2.68
2,9–decanedione	–	1.80	1.87	2.18	1.73	–
Total	23.63	24.05	24.81	25.26	22.59	12.93
Other compounds						
pyridine	3.14	3.88	3.22	4.57	2.70	3.43
4–methyl–1–penten–3–ol	4.25	3.55	2.70	4.68	–	–
2,5–dimethyl–furan	2.18	1.25	1.04	1.51	2.62	–
2–(1–methylethoxy)–1–propene	–	1.15	1.42	1.75	–	–
1–methoxy–cyclobutane	–	2.89	3.57	4.61	3.30	3.65
2,6–dimethyl–heptane	–	2.15	2.45	2.80	2.44	4.49
Total	9.57	14.87	14.40	19.92	11.06	11.57

– : Trace amount (can not be measured)

S9 mix at 25.00% or less was greater than 80%. The wood vinegars for survival without the S9 mix at a diluting percent content of 50.00% or less toward TA98 but for TA100 at 100% (original vinegar), and with the S9 mix at 33.33% or less toward TA98 and TA100 was higher than 80%.

For vinegars collected at all different temperatures, the Survival results are shown in Table 4. The Survival of all bamboo vinegars collected from 80–150, over 80, 90–92, 99–102, 120–123 and 145–150˚C without the S9 mix at a diluting percent content of 33.33% or less were all higher than those for Blank by more than 80%. However, the Survival of the bamboo vinegar collected at temperatures of 99–102°C at a diluting percent content of 25% showed that the cytotoxicity toward *S. typhimurium* TA98 and TA100 with the S9 mix was lower than 80%, indicating "with toxicity". For the collected temperatures of all wood vinegars at 80–159, 80–90,

Table 3. Cytotoxicity of bamboo vinegar collected at the temperature of 90–92°C and wood vinegar collected from the temperature at 105–109°C toward *Salmonella typhimurium* TA98 and TA100 without the S9 or with the S9 mix

S9 mixture	Diluting percent content (%)	Bamboo vinegar (90–92°C)				Wood vinegar (105–109°C)			
		TA98	Survival [1] (%)	TA100	Survival (%)	TA98	Survival (%)	TA100	Surviva (%)
	Blank [2]	1838 (127.50)[c3]	100.00	1500 (145.26)[de]	100.00	1838 (127.50)[cd]	100.00	1500 (145.26)[a]	100.00
–S9	Original vinegar	0 (00.00)[a]	0.00	87 (19.63)[a]	5.82	607 (96.34)[a]	33.01	1558 (130.18)[a]	103.87
	50.00	336 (70.53)[b]	18.26	285 (15.13)[b]	19.00	1938 (110.02)[de]	105.46	2093 (81.40)[c]	139.51
	33.33	1652 (176.22)[c]	89.86	1284 (111.72)[c]	85.58	1602 (147.16)[b]	87.18	1604 (222.67)[a]	106.93
	25.00	1822 (67.02)[c]	99.13	1201 (66.98)[c]	80.04	1653 (89.29)[bc]	89.95	1525 (184.19)[a]	101.69
	20.00	1736 (50.01)[c]	94.43	1264 (12.29)[c]	84.27	1844 (135.19)[cd]	100.31	1637 (29.69)[a]	109.11
	13.33	1829 (147.70)[c]	99.53	1353 (150.01)[cd]	90.20	1626 (114.02)[bc]	88.48	1724 (104.84)[ab]	114.93
	10.00	1830 (155.52)[c]	99.56	1641 (120.79)[e]	109.42	2071 (128.16)[e]	112.68	1917 (156.16)[bc]	127.82
S9	Blank	2165 (75.75)[c]	100.00	2585 (77.60)[c]	100.00	2165 (75.75)[cd]	100.00	2585 (77.60)[a]	100.00
	33.33	1605 (43.92)[a]	74.16	1736 (137.58)[a]	67.15	1864 (93.25)[a]	86.13	3181 (122.27)[d]	123.03
	25.00	1960 (104.01)[b]	90.53	2209 (131.53)[b]	85.46	2145 (82.85)[b]	99.11	3139 (151.73)[cd]	121.43
	20.00	1938 (129.55)[b]	89.53	2243 (79.43)[b]	86.75	2640 (119.93)[d]	121.96	3001 (74.71)[bc]	116.08
	13.33	2046 (117.96)[bc]	94.53	2885 (98.43)[d]	111.60	2337 (104.64)[c]	107.98	2938 (22.90)[b]	113.65
	10.00	2000 (113.21)[bc]	92.39	2249 (139.96)[b]	87.00	2242 (107.34)[cd]	103.59	3448 (41.04)[e]	133.38

[1] Survival (%) = (the bacterial count of test group / the bacterial count of control group) * 100
[2] Blank (the control group) was added without either bamboo or wood vinegars
[3] Values (standard deviation) within a transverse with the different superscripts are significantly different ($\rho < 0.05$) by Duncan's multiple range tests

Table 4. Survival of various bamboo/wood vinegars collected at different temperatures toward *Salmonella typhimurium* TA98 and TA100 without the S9 or with the S9 mix

Unit: %

Specimens		Diluting percent content (%)	Collection temperature (°C)											
			80		90–92		99–102		120–123		145–150		80–150	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Bamboo vinegars	–S9	33.33	95.16	85.42	89.86	85.58	91.69	90.18	109.12	90.76	95.92	112.16	109.79	104.98
		25.00	115.54	88.91	99.13	80.04	99.13	105.82	104.13	115.42	90.86	99.82	112.82	102.42
		20.00	86.43	109.11	94.43	84.27	84.64	86.20	111.19	115.69	107.04	123.40	124.23	108.62
		13.33	95.30	88.11	99.53	90.20	99.53	90.56	114.93	118.91	109.61	102.42	95.88	128.20
		10.00	106.58	94.69	99.56	109.42	101.16	84.53	113.75	111.47	122.63	96.44	127.98	119.71
	S9	25.00	88.81	92.83	90.53	85.46	76.36	70.81	89.79	96.39	103.87	122.07	86.94	89.12
		20.00	95.40	84.84	89.53	86.75	86.53	106.65	113.32	90.36	89.11	89.27	85.19	89.74
		13.33	92.12	120.27	94.53	111.60	98.66	104.95	126.73	115.78	143.39	140.28	84.85	97.83
		10.00	84.19	99.79	92.39	87.00	94.73	83.29	117.83	99.02	120.54	98.09	102.71	131.10

Specimens		Diluting percent content (%)	80–81		90–97		105–109		125–133		153–159		80–159	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Wood vinegars	–S9	33.33	90.01	102.58	100.42	111.73	87.18	106.93	90.13	133.16	83.26	140.98	85.46	111.56
		25.00	103.97	106.67	109.56	107.64	89.95	101.69	103.16	138.31	81.72	133.07	88.08	129.24
		20.00	110.83	110.93	117.45	94.67	100.31	109.11	96.52	132.09	117.75	177.20	99.87	136.58
		13.33	113.04	96.71	102.90	94.40	88.48	114.93	83.21	130.80	81.05	131.64	101.29	165.96
		10.00	108.52	131.07	91.53	156.44	112.68	127.82	105.24	139.91	115.27	145.33	113.15	162.02
	S9	25.00	93.36	93.62	109.47	100.49	99.11	121.43	122.68	126.99	104.80	109.99	86.71	94.26
		20.00	115.51	91.06	92.98	93.26	121.96	116.08	90.50	94.58	84.96	99.43	90.78	85.82
		13.33	131.04	104.53	121.68	108.55	107.98	113.65	103.05	136.98	100.75	142.46	98.68	88.24
		10.00	101.37	107.14	111.80	130.17	103.59	133.38	102.57	94.91	102.56	128.98	95.61	108.64

91–100, 105–109, 101–120, 121–140 and 141–159°C without the S9 mix, the Survival for the diluting percent content at 33.33% or less were all higher than those for the control by more than 80% and for those with the S9 mix at 25% or less. It is said that the Survival of bamboo/wood vinegars collected at all different temperatures at a diluting percent content of 20.00% less for either with or without S9 mix is no cytotoxicity absolutely, and the dose for the mutagenicity test can be selected according to the results above.

Mutagenicity of bamboo/wood vinegars

Ames *et al*. (1975) reported that the number of spontaneous revertants induced by the specimen is less than for the control group by more than two times; the specimen has no mutagenicity. Table 5 shows the muta-

Table 5. Mutagenicity of bamboo/wood vinegars collected at the temperatures of 90–92/105–109°C toward *Salmonella typhimurium* TA98 and TA100 without the S9 or with the S9 mix

S9 mixture	Diluting percent content (%)	Bamboo vinegar (90–92°C)				Wood vinegar (105–109°C)			
		TA98	MR[1)]	TA100	MR	TA98	MR	TA100	MR
	Blank[2)]	65 (4.93)[b3)]	1.00	132 (4.36)[b]	1.00	65 (4.93)b	1.00	132 (4.36)[d]	1.00
–S9	25.00	53 (8.19)[a]	0.81	129 (3.00)[b]	0.98	54 (5.29)[a]	0.83	98 (6.11)[b]	0.74
	20.00	42 (6.66)[a]	0.64	121 (4.51)[ab]	0.91	51 (1.73)[a]	0.78	120 (10.12)c	0.91
	13.33	53 (4.04)[a]	0.81	109 (5.69)[a]	0.83	50 (6.03)[a]	0.76	86 (7.81)[ab]	0.65
	10.00	41 (8.00)[a]	0.63	115 (10.26)[a]	0.87	51 (8.50)[a]	0.79	84 (2.31)[a]	0.63
S9	Blank	76 (0.58)[b]	1.00	140 (12.06)[a]	1.00	76 (0.58)[a]	1.00	140 (12.06)[ab]	1.00
	20.00	69 (4.51)[ab]	0.90	195 (10.97)[b]	1.39	81 (9.64)[b]	1.06	160 (3.51)[bc]	1.14
	13.33	73 (6.66)[b]	0.96	204 (13.23)[b]	1.45	92 (3.21)[bc]	1.20	188 (15.52)[d]	1.34
	10.00	74 (5.20)[b]	0.97	206 (3.06)[b]	1.47	82 (7.21)[b]	1.07	182 (15.82)[cd]	1.29

[1)] MR (Mutagenicity ratio) = induced revertants per plate/spontaneous revertants per plate (Control);
[2)] Blank (the control group) was added without either bamboo or wood vinegars
[3)] Values (standard deviation) within a transverse with the different superscripts are significantly different ($\rho < 0.05$) by Duncan's multiple range tests

Table 6. Survival of various bamboo/wood vinegars collected at different temperatures toward *Salmonella typhimurium* TA98 and TA100 without the S9 or with the S9 mix

Specimens		Diluting percent content (%)	Collection temperature (°C)											
			80		90–92		99–102		120–123		145–150		80–150	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Bamboo vinegars	–S9	25.00	0.72	1.08	0.81	0.98	–	–	0.74	0.63	0.68	0.50	0.62	0.80
		20.00	0.68	1.02	0.64	0.91	0.65	0.90	0.81	0.62	0.59	0.54	0.67	0.76
		13.33	0.70	0.86	0.81	0.83	0.72	0.78	0.52	0.57	0.57	0.50	0.68	0.72
		10.00	0.61	0.93	0.63	0.87	0.75	1.00	0.69	0.77	0.69	0.56	0.73	0.60
	S9	20.00	1.10	1.47	0.90	1.39	0.88	1.57	1.18	1.19	1.32	1.32	1.16	0.83
		13.33	0.91	1.51	0.96	1.45	0.89	1.62	1.14	1.29	1.22	1.26	1.21	1.04
		10.00	1.04	1.42	0.97	1.47	0.96	1.66	1.00	1.28	1.20	1.40	1.35	1.05
Specimens		Diluting percent content (%)	80–81		90–97		105–109		125–133		153–159		80–159	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Wood vinegars	–S9	25.00	0.84	1.19	1.06	0.90	0.83	0.74	0.75	0.76	0.69	0.50	0.70	0.98
		20.00	0.88	1.04	0.82	0.90	0.78	0.91	0.89	0.74	0.87	0.54	0.73	0.99
		13.33	0.98	0.80	0.89	0.66	0.76	0.65	0.95	0.58	0.86	0.49	0.93	0.62
		10.00	0.73	0.82	0.83	0.68	0.79	0.63	0.85	0.71	0.82	0.49	0.82	0.76
	S9	20.00	1.07	1.27	0.99	1.14	1.06	1.14	1.07	1.22	1.22	1.32	1.06	1.15
		13.33	1.11	1.39	1.16	1.39	1.20	1.34	1.14	1.22	1.28	1.32	1.14	1.26
		10.00	1.09	1.25	1.08	1.26	1.07	1.29	1.06	1.26	1.11	1.34	1.15	1.28

genicity results for the bamboo/wood vinegars collected at temperatures of 90–92/105–109°C with a diluting percent content of 25.00, 20.00, 13.33 and 10.00% for *S. typhimurium* TA98 and TA100 without the S9 mix and with the S9 for a diluting percent content of 20.00, 13.33 and 10.00%. The spontaneous revertants of Blank were 65 for TA98 and 132 for TA100, and of bamboo/wood vinegars without the S9 mix were from 41 to 54 for TA98

Table 7. Antimutagenicity of bamboo/wood vinegars collected at the temperatures of 90–92/105–109°C toward *Salmonella typhimurium* TA98 and TA100 without the S9 or with the S9 mix

Mutagens	Diluting percent content (%)	Bamboo vinegar (90–92°C)				Wood vinegar (105–109°C)			
		TA98	Inhibition[1] (%)	TA100	Inhibition (%)	TA98	Inhibition (%)	TA100	Inhibition (%)
NQNO (1 μg/plate)	Blank[2]	1128 (93.15)[c3]	0.00	1445 (75.80)[c]	0.00	1128 (93.15)[b]	0.00	1445 (75.8)[e]	0.00
	25.00	807 (48.22)[a]	30.18	425 (52.79)[a]	77.65	906 (43.27)[a]	20.89	579 (61.13)[b]	65.92
	20.00	841 (71.59)[a]	27.04	446 (35.80)[a]	76.05	925 (70.27)[a]	19.07	620 (39.95)[b]	62.82
	13.33	891 (85.05)[ab]	22.33	594 (32.92)[b]	64.80	1058 (64.71)[b]	6.59	794 (57.58)[c]	49.57
	10.00	985 (64.26)[b]	13.49	616 (42.71)[b]	63.15	1084 (64.16)[b]	4.14	977 (61.78)[d]	35.65
Spontaneous revertants		65 (4.93)		132 (4.36)		65 (4.93)		132 (4.36)	
AFB1 (5 μg/plate)	Blank	1824 (57.86)[d]	0.00	2406 (86.00)[e]	0.00	1824 (57.86)[c]	0.00	2406 (86.00)[d]	0.00
	20.00	1114 (6.93)[b]	40.63	843 (25.48)[b]	68.97	1498 (83.21)[ab]	18.65	619 (21.39)[a]	78.89
	13.33	1292 (73.32)[c]	30.44	1012 (49.27)[c]	61.53	1510 (45.03)[b]	17.97	769 (45.71)[b]	72.27
	10.00	1319 (44.38)[c]	28.88	1202 (35.04)[d]	53.14	1587 (77.60)[b]	13.58	1051 (55.18)[c]	59.79
Spontaneous revertants		76 (0.58)		140 (12.06)		76 (0.58)		140 (12.06)	

[1] Inhibition (%) = [1 – (Number of his$^+$ revertants in the presence of the test vinegar – Number of spontaneous revertants) / (Number of his$^+$ revertants in the absence of the test vinegar – Number of spontaneous revertants)] × 100

[2] Blank (the control group) was added without either bamboo or wood vinegars

[3] Values (standard deviation) within a transverse with the different superscripts are significantly different ($\rho<0.05$) by Duncan's multiple range tests

Table 8. Inhibition of various bamboo/wood vinegars collected at different temperatures toward *Salmonella typhimurium* TA98, TA100 without the S9 or with the S9 mix

Unit: %

Specimens		Diluting percent content (%)	Collection temperature (°C)											
			80		90–92		99–102		120–123		145–150		80–150	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Bamboo vinegars	NQNO	25.00	25.03	70.14	30.18	77.65	–	–	27.85	74.45	2.76	81.77	22.96	68.97
		20.00	23.15	65.34	27.04	76.05	23.21	75.22	11.67	64.45	1.19	60.23	4.20	61.38
		13.33	12.80	60.64	22.33	64.80	20.58	58.91	4.08	61.55	1.00	40.71	0.75	57.31
		10.00	11.29	59.45	13.49	63.15	15.18	57.97	1.07	28.52	0.63	18.18	0.19	42.74
	AFB_1	20.00	24.38	62.94	40.63	68.97	37.80	66.35	20.03	75.24	11.63	73.06	20.14	74.86
		13.33	21.13	57.88	30.44	61.53	26.36	66.00	18.73	68.97	8.54	66.41	17.09	73.27
		10.00	18.31	37.43	28.88	53.14	24.26	54.88	13.20	59.88	0.92	60.50	7.44	58.44
Specimens		Diluting percent content (%)	80–81		90–97		105–109		125–133		153–159		80–159	
			TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
Wood vinegar	NQNO	25.00	16.88	43.37	19.39	41.26	20.89	65.92	22.02	52.77	5.02	67.19	31.43	54.95
		20.00	5.46	40.58	12.23	39.77	19.07	62.82	8.59	48.81	1.76	53.94	22.02	52.82
		13.33	4.89	32.86	8.72	37.07	6.59	49.57	1.69	33.42	–0.19	47.99	7.72	49.87
		10.00	2.32	32.10	5.40	35.63	4.14	35.65	0.75	32.96	–4.45	42.00	4.71	43.12
	AFB_1	20.00	18.96	61.88	20.71	71.68	18.65	78.89	16.94	69.18	7.13	70.56	25.06	71.15
		13.33	15.75	50.14	17.09	62.28	17.97	72.27	15.22	64.76	6.52	62.67	15.60	66.32
			11.67	12.03	8.93	61.88	13.58	59.79	14.99	59.06	3.62	59.35	11.56	64.53

and from 84 to 129 for TA100. With the S9 mix, the spontaneous revertants of bamboo/wood vinegars were 69–92 for TA98 and 160–206 for TA100. The results on the same Table 5 also show that the bamboo/wood vinegars in the test range did not exceed spontaneous revertants by more than two times for TA98 and TA100 without/with S9 mix; that is, the mutagenicity ratio (MR) was less than 2. The spontaneous revertants of the other bamboo/wood vinegars collected at various temperatures at the ranges of the diluting percent content were smaller than those of the control group by more than two times; that is, the MR was also less than 2 (results not shown in this paper). The bamboo/wood vinegars, therefore, have no mutagenicity toward S. typhimurium TA98 and TA100.

The MR of the bamboo/wood vinegars collected at all different temperatures toward *S. typhimurium* TA98 and TA100 without the S9 mix for a diluting percent content of 25.00, 20.00, 13.33 and 10.00% or with the S9 mix for a diluting percent content of 20.00, 13.33 and 10.00% is shown in Table 6. No matter what the vinegars were for all collected temperatures and *S. typhimurium* TA98 and TA100 without/with the S9 mix, the MR was less than 2. Neither mutagenicity nor toxicity was observed for bamboo/wood vinegars collected at various temperatures toward *S. typhimurium* TA 98 or TA 100 without/with the S9 mix. Hence, without the S9 mix a diluting percent content of 25.00 to 10.00% and with S9 mix at 20.00 to 10.00% a diluting percent content was selected for the antimutagenic assay.

Antimutagenicity of bamboo/wood vinegars

In the present study, NQNO and AFB1 were used as direct mutagens requiring metabolic activation and indirect acting mutagen, respectively. Doses of mutagens, 1 μg for NQNO and 5 μg for AFB1, were selected from a dose–response curve of a preliminary experiment (Yen *et al.*, 2001). The His^+revertants of strain are less than those of the control group, said that with antimutagenic activities. Meanwhile, the inhibitory effect of the specimen is expressed by inhibition (%), and the higher the inhibition, the more effective the antimutagenic activities (Maron and Ames, 1983). The inhibitory effects for one of the antimutagenicity results for the bamboo/wood vinegars collected at temperatures of 90–92/105–109°C with a diluting percent content of 25.00, 20.00, 13.33 and 10.00% for NQNO and at 20.00, 13.33 and 10.00% for AFB_1 are summarized in Table 7.

The His^+revertants of strain against the NQNO in Blank (without bamboo/wood vinegars) were 1128 for TA98, and 1445 for TA100, for AFB1: they were 1824 for TA98, and 2406 for TA100. The spontaneous revertants without NQNO were 65 for TA98 and 132 for TA100, and without AFB1 were 76 for TA98 and 140 for TA100. The His^+revertants of strain (inhibition) against the NQNO for bamboo vinegar at 90–92°C with different diluting percent contents were 807–985 (30.18–13.49%) for TA98 and 425–616 (77.65–63.15%) for TA100, for wood vinegar at 105–109°C they were 906–1084 (20.89–4.14%) for TA98 and 579–977 (65.92–35.65%) for TA100. For AFB1, they were 1114–1319 (40.63–28.88%) for TA98 and 843–1202 (68.97–53.14%) for TA100 from bamboo vinegar at 90–92°C with different diluting percents, from wood vinegar at 105–109°C they were 1498–1587 (18.65–13.58%) for TA98 and 619–1051 (78.89–59.79%) for TA100. The results also showed that the higher diluting percent content, the greater the inhibition; as well as, no matter what the vinegar was, the inhibition for TA100 was greater than that of TA98.

The inhibition of the bamboo/wood vinegars collected at all different temperatures against the NQNO for diluting percent contents of 25.00, 20.00, 13.33 and 10.00% or against the AFB_1 at 20.00, 13.33 and 10.00% is shown in Table 8. The inhibition of the bamboo vinegars to TA98 was 0.19–30.18% for NQNO and 0.92–40.63% for AFB1; they were better than that of wood vinegar, –4.45–31.43% and 3.62–25.06%. For both vinegars with TA100 against NQNO (18.18–81.77%) and AFB_1 (12.03–78.89%), they were better than those for TA98. The antimutagenicity to NQNO was effective for bamboo/wood vinegars collected at various temperatures with a diluting percent content of 25.00% or less, and for AFB_1, it was also effective at a 20.00% or less diluting percent content. Furthermore, the bamboo/wood vinegars showed that the inhibitory effect on NQNO or AFB_1 toward TA100 was greater than that toward TA98. It is also indicated that the inhibition of the vinegars against AFB_1 toward TA98 and TA100 is better than that against NQNO.

In this study, the main percent of the phenol and ketone compounds in the bamboo/wood vinegars collected from various different temperatures was 50.23–65.98% (Table 1) and 10.13–25.26% (Table 2), resulting in no cytotoxicity (Table 4)/mutagenicity (Table 6) and with antimutagenic effect (Table 6) against strains. It is inferred that the phenol and ketone compounds may partially account for the biological action of bamboo/wood vinegars.

CONCLUSIONS

The compounds, safety evaluation and antimutagenic activity of bamboo/wood vinegars were investigated. The compounds of bamboo/wood vinegars include the acid, phenol, ketone and the other compounds. The main percent content of the phenol compounds for bamboo vinegars was phenol (5.93–16.60%) and 2–methoxy–phenol (8.27–16.39%). For wood vinegars, the main compounds were 2–methoxy–phenol (11.70–22.90%) and 2–methoxy–4–methyl–phenol (7.09–20.10%). The diluting percent content of vinegars was lower than 20.00% or less with the S9 mix and 33.33% or less without the S9 mix in cytotoxicity and mutagenicity toward *S. typhimurium* TA98 and TA100 because the rest of the bacterium at these percent contents was higher than 80% of the control, and the mutagenicity ratio was less than for the control group by more than two times. The diluting percent content of vinegars of 20.00% or less, expressed an amount–dependent inhibitory effect against both the mutagenicity of 4–nitroquinoline–N–oxide (NQNO) with 1 μg/plate and aflatoxin B_1 (AFB_1) with 5 μg/plate in *S. typhimurium* TA98 and TA100. It is suggested that

bamboo/wood vinegars with a diluting percent content to the least extent of 20.00% or less had no cytotoxicity and mutagenicity, and their antimutagenicity with NQNO and AFB_1 was effective.

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