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Toxicological and Analytical Investigations of Noni (*Morinda citrifolia*) Fruit Juice

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Morinda citrifolia (noni) is known to contain genotoxic anthraquinones in the roots. Because of the widespread use of noni juice, the possible genotoxic risk was examined through a battery of short-term tests. Noni juice was also chemically analyzed for the possible presence of anthraquinones. Noni juice extract in the *Salmonella* microsome assay showed a slight mutagenic effect in strain TA1537, due to the presence of flavonoids. No mutagenicity was observed in the mammalian mutagenicity test with V79 Chinese hamster fibroblasts. Rats treated with a noni juice concentrate did not show DNA repair synthesis (UDS) in primary rat hepatocytes, nor could DNA adducts or DNA strand breaks be observed. HPLC analysis of noni juice for anthraquinones was negative, with a sensitivity of <1 ppm. In summary, chemical analysis and genotoxicity tests reveal that noni juice does not have a genotoxic potential and that genotoxic anthraquinones do not exist in noni juice.

KEYWORDS: Noni juice; *Morinda citrifolia*; genotoxicity; mutagenicity; anthraquinones; lucidin; rubiadin; alizarin

INTRODUCTION

The tropical plant *Morinda citrifolia* L., belonging to the family Rubiaceae, is known in the Polynesian Islands as nono (Society Islands), noni (Marquesa Islands, Hawaii), nona (Samoa), or kura (Fiji). Native Polynesians use different parts of the plant for food, cosmetics, or medicinal purposes. The plant, an important part of their culture, was among the so-called "canoe plants", that is, plants carried in their canoes when they discovered new islands for settlement. Juice prepared from ripe fruits of the noni plant was very popular because of its many health benefits (*1*, *2*). About 10 years ago, American scientists became aware of the traditional use of noni juice in French Polynesia. They founded a company and introduced noni juice into North America and later to more than 50 countries of the world. In the European Union, noni juice was approved and registered as a "Novel Food" in May 2003 (*3*). However, notwithstanding the legal registered brands of noni juice, there are numerous nonregistered illegal brands on the market, which make quality control difficult.

Recently two publications reporting three cases of liver toxicity were linked to the usage of noni juice (*4*, *5*). The authors speculated that toxic anthraquinones present in noni juice could be responsible for the liver toxicity. In the aftermath of these publications, the occurrence of hydroxyanthraquinones (HAs) has been reported in noni fruits. However, the very low concentrations make a liver toxic effect very unlikely (*6*, *7*). In addition to toxic effects on elimination organs, such as the liver or kidneys, which are normally observable only at doses very close to the acute toxicity, genotoxic effects could also lead to genetic alterations and cancer at doses that do not show acute toxicity. Therefore, the exclusion of genotoxic effects is important in products used for human foods or drugs.

Hydroxyanthraquinones are present in many species of the Rubiaceae family, occurring mainly in the roots (*8*). The synthesis of HAs in Rubiaceae plants follows mainly the shikimic acid pathway, leading to HAs with substitutions in only one of the aromatic ring systems (*9*, *10*; **Figure 1**). A survey of HAs identified derivatives such as lucidin, rubiadin, purpurin, and xanthopurin, which have a genotoxic potential in bacterial and mammalian systems, due to their specific structural properties. However, others, such as alizarin, demonstrate no genotoxicity (*11*). The most critical HA is lucidin (1,3-dihydroxy-2-hydroxymethyl-9,10-anthraquinone), which was positive in a variety of in vitro genotoxicity assays (*12*). The two phenolic hydroxy groups facilitate the elimination of a water molecule from the hydroxymethyl group after protonation. This leaves a very reactive electrophilic carbenium ion (**Figure 2**), which forms adducts after contact with DNA. Such adducts were observed after incubation of lucidin with cells in culture, as well as in treated laboratory animals (*13*). Carcinomas of the liver and kidneys were observed in rats treated over a period of 2 years with madder root (*Rubia tinctorum* L.), which contains high concentrations of lucidin (*14*). A dose-dependent increase

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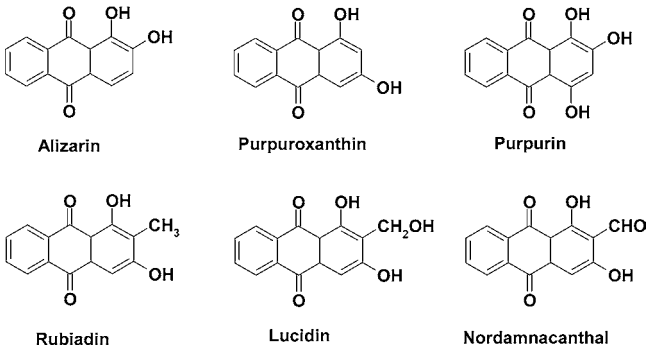

Figure 1. Anthraquinones occurring in plants of the family Rubiaceae. A characteristic feature of these compounds is that functional groups occur in one of the aromatic ring systems only.

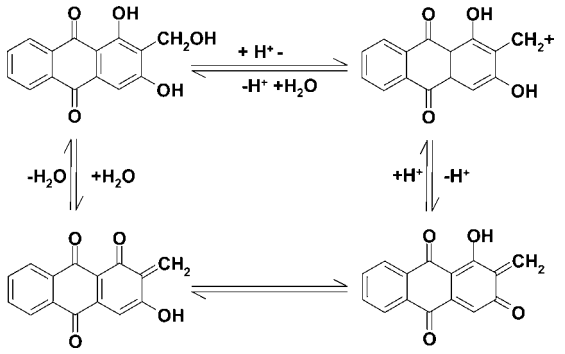

Figure 2. Formation of reactive intermediates of lucidin. The lucidin molecule easily eliminates water by forming a carbenuim ion, which is stabilized by elimination of a proton. The intermediates are strongly electrophilic and can react with nucleic acids to form adducts.

of lucidin−DNA adducts was observed in the liver and kidney of these animals.

The aim of the present investigation was to analyze the toxic, and in particular the genotoxic, potential of noni juice in correlation with the occurrence of HAs. A combination of chemical analysis by high-performance liquid chromatography (HPLC) and a battery of tissue culture short-term tests was used for this purpose.

MATERIALS AND METHODS

Materials. Tahitian noni juice (TNJ) used for this study was provided by Tahitian Noni International Inc., Provo, UT. The juice was prepared from ripe fruits harvested on different islands in French Polynesia. Ripe fruit is pureed, and the seeds and skin are removed. It is then pasteurized. The puree is later blended with blueberry and red grape juice, pasteurized again, and then bottled. Three different batches, prepared in 2004, were used for the investigations. Two were from Austria (T01, T02) and one from Germany (T03). The juice was either directly used in the assays, or extracts were prepared with ethyl acetate as follows: equal amounts of juice and solvent were mixed gently, and the two phases were separated by centrifugation. The organic layer was removed and the solvent evaporated. The residue was dissolved in ethanol or DMSO to yield a 50- or 100-fold concentrated solution with respect to the original juice.

A 10-fold concentrated noni juice syrup (NJC), prepared from pure noni juice (noni puree without solid particles from fruits harvested in French Polnesia) by a freeze-drying method, was also supplied by Tahitian Noni International Inc. This syrup was used directly to treat the rats used in the ex vivo UDS assay or extracted with ethyl acetate as described for TNJ.

Lucidin and rubiadin were synthesized as described by Murti et al. (*15*) and Bloemeke (*16*), respectively. The identity of the compounds was confirmed by mass spectrometry (Varian MAT 311 A) and NMR spectrometry (Bruker, 400 MHz). Alizarin was purchased from Carl Roth GmbH (Karlsruhe, Germnay). Stock solutions were prepared by dissolving 10 mg/mL of the compounds in dimethyl sulfoxide (DMSO).

Positive controls and other chemicals for the genotoxicity tests were purchased from (1) Sigma, Munich, Germnay [2-aminoanthracene, 2-nitrofluorene, 7,12-dimethylbenz[*a*]anthracene (DMBA), *N*,*N*-dimethylnitrosamine (DMN), acetylaminofluorene (AAF), 6-thioguanine]; (2) EGA-Chemie, Steinheim, Germany [*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)]; (3) Amersham, Braunschweig, Germany [[^{3}H]thymidine (specific activity = 70 Ci/mmol)]; (4) Grand Island Biological, Karlsruhe, Germany [Williams medium E (WME), Dulbecco's medium, penicillin/streptomycin solution]; (5) Fresenius Co., Munich, Germany [fetal calf serum (FCS)]; (6) Merck, Darmstadt, Germany [sodium azide, DMSO (ultrapure), methanol (p.a.), ethanol (absolute) ethyl acetate (p.a.), acetic acid (100%, p.a.), acetonitrile (HPLC grade), water (HPLC grade), Giemsa's solution]; (7) Eastman Kodak, Rochester, NY (NTB-photo emulsion, Kodak D10 developer); (8) Cambrex Bio Science Rockland Inc., Rockland, ME (SYBR Green); and (9) Oxoid, Basingstoke, Hampshire, U.K. (purified agar, nutrient broth).

Tissue Culture Tests. *Rat Hepatocytes.* Cultures of primary rat hepatocytes (PRH) are very close to the in vivo situation and maintain most of their metabolizing capacity, at least for a few hours after isolation. Under normal circumstances the cells do not proliferate in culture. Male Wistar rats (Zentralinstitut für Versuchstierkunde, Hannover, Germany) were fed a standard diet (Altromin, Lage, Germany) and water ad libitum. They were housed under controlled conditions. Hepatocytes were isolated by collagenase perfusion from rats (150−200 g) as described in an earlier study (*17*). A concentration of 10^6 cells per well in six-well dishes (one dish/concentration) was plated in WME with 10% FCS and then treated with either TNJ directly, at concentrations in medium between 0.1 and 10%, or ethyl acetate extracts (50-fold concentrated) at concentrations between 0.001 and 0.3%.

The Neutral Red assay was performed with PRH according to the method of Borenfreund et al. (*18*). The test is based on the assumption that the dye Neutral Red accumulates in the lysosomes of viable cells. The compound enters the cell by passive diffusion through the plasma membrane. Damage to the cell surface or lysosomal membranes by xenobiotics results in decreased uptake of the dye. It was demonstrated that the reduction of the intracellular accumulation of Neutral Red in human and rat hepatocytes correlates well with other parameters of cytotoxicity, such as lactate dehydrogenase (LDH) release, protein content, and morphological changes (*19*).

H4IIE Rat Hepatoma Cells. H4IIE cells (Reuber hepatoma cells) were purchased from American Type Culture Collection, Manassas, VA. This cell line is suitable for the screening of hepatotoxic compounds. The cells express a variety of xenobiotic metabolizing enzymes, similar to PRH. In contrast to primary hepatocytes, H4IIE cells proliferate in culture.

Plating Efficiency Assay. Two hundred H4IIE cells per dish were plated in 10 cm^2 dishes with WME and 10% FCS. Five cultures were prepared for each test concentration. After 24 h of incubation in a tissue culture incubator (37 °C, 5% CO_2), the medium was replaced by WME without FCS. The test compounds [TNJ or ethyl acetate extracts of TNJ (50-fold)] were added to the medium, and the cells were incubated for 24 h. Thereafter, the medium was removed and replaced by fresh WME with FCS. After 1 week of incubation, the medium was removed, and the cells were washed with isotonic phosphate buffer and fixed with methanol. Colonies were stained with Giemsa's solution and counted.

Salmonella/Microsome Assay. This reverse mutation assay was performed according to the method of Yahagi et al. (*20*). The bacteria are auxotrophic with respect to histidine. A single mutation can convert a cell back to histidine prototrophy. Mutants can thus be selected on histidine-free agar plates. Three strains were used: TA98, TA1537 (frame shift), and TA100 (point mutation). The strains were provided by Dr. Bruce Ames, University of California, Berkeley, CA. Samples were incubated in nutrient broth with and without the addition of rat liver S9 mix for 30 min prior to plating on histidine-free agar. Sodium azide (1 μg/plate), 2-aminoanthracene (1 μg/plate), and 2-nitrofluorene (2 μg/plate) were used as positive controls. The extracts (20 μL) were tested in a half-logarithmic concentration range. Duplicate plates were

run for each concentration. A test is considered to be positive if the mutation frequency doubles the background mutation frequency. No further statistical method is used to characterize positive effects.

V79-HPRT Mutagenicity Assay. This forward mutation test was performed according to the OECD guideline 476, described by Jensen (*21*). The V79 Chinese hamster fibroblasts used were provided by Dr. E. H. Y. Chu (Department of Human Genetics, University of Michigan, Ann Arbor, MI). Cells were treated for 3 h in the presence or absence of rat liver S9 mix in Dulbecco's medium, without FCS. Surviving cells are selected for hypoxanthin-phosphoribosyl-transferase (HPRT) negative mutants by the addition of 6-thioguanin (TG, 10 μg/mL) to the medium. Only HPRT-negative mutants survive in the presence of TG. A total of 500 000 cells per test concentration is plated in each of five tissue culture flasks (75 cm^2). The direct-acting carcinogen MMNG (1 μg/mL) and the procarcinogen DMBA (10 μg/mL) are used as positive controls for tests with and without the S9 rat liver mix. The mutation frequency is expressed in mutations per 10^5 surviving cells. A test is considered to be positive if the mutation frequency doubles the background value. No further statistical method is used to characterize positive effects.

Ex Vivo UDS Assay in Primary Rat Hepatocytes. The UDS monitors DNA repair synthesis following DNA damage. The target for the test compounds is liver cells, which have the highest capacity of xenobiotic metabolism. Animals are treated with the test compound, and liver cells are separated after a certain time. These cells are then incubated in the presence of radioactive thymidine, which is incorporated during the DNA repair synthesis. The test was performed in accordance with the OECD guideline 486, described by Ashby et al. (*22*), using male Wistar rats (three per dose). The animals received 100 or 1000 mg/kg of a 10-fold concentrate of pure noni juice (NJC) via a stomach tube, either 2 or 12 h before sacrifice. Control rats received 1 mL of water with 10% DMSO per kilogram of body weight (BW). Two different positive controls were used for the short- and long-term treatments. The fast direct-acting carcinogen, *N*,*N*-dimethylnitrosamine (DMN, 10 mg/kg), was used for the 2 h treatment period, whereas the procarcinogen acetylaminofluorene (AAF, 50 mg/kg) was given to rats in the 12 h treatment cycle. DMN and AAF were first dissolved in DMSO at concentrations of 100 and 500 mg/mL and diluted 1:10 with water prior to administration at 1 mL/kg to the animals.

The liver perfusion was performed as described in an earlier study (*17*). Five dishes with liver cells were prepared for each rat. Petri dishes containing 10^6 cells per dish were incubated for 4 h in the presence of [^{3}H]thymidine (10 μCi/mL). A computer-based analyzing system (BioSys, Karben, Germany) was used for silver grain counting after autoradiography. One hundred nuclei and 300 adjacent cytoplasmic areas per slide were counted. A test is considered to be positive if three conditions are satisfied: (I) mean of net silver grain count over cell nuclei >5, (II) cells in repair per slide >50%, and (III) *p* value of mean of net silver grain count over cell nuclei of test animals compared to control <0.001.

Determination of DNA Strand Breaks (Comet Assay). This assay is among the most sensitive tests for the determination of DNA damage caused by oxidative stress. It was performed according to the technique described by Singh et al. (*23*). In this study we employed a standard protocol as reported by Tice (*24*). Liver cells prepared for the ex vivo UDS assay were utilized for this test. After isolation, about 10 000 cells were plated on glass slides covered with an alkaline gel, and an electrophoresis was performed. The alkaline solution results in lysis of the cells and separation of the DNA double strand. Migration of the DNA in the electric field is dependent on the length of the single-strand fragments and reflects, therefore, the number of single-strand breaks caused by the genotoxic agent. The slides are stained with the fluorescent dye SYBR Green. The DNA damage is monitored under a fluorescent microscope. A score of 1 (no damage) to 4 (severe damage) is estimated from the length of the DNA comet. One hundred cells are scored per slide.

^{32}P Postlabeling Assay ex Vivo. Many carcinogens form covalent adducts with DNA bases. The detection of such adducts is, therefore, a sensitive method for the detection of potential carcinogens. This test method can be performed with DNA isolated from cell culture or ex vivo material. After isolation, the DNA is hydrolyzed to single bases, which are then labeled with ^{32}P. Separation of bases with covalent adducts from normal bases is performed by two-dimensional thin-layer chromatography (TLC). Modified bases are visualized by autoradiography. Quantitation is possible after cut of the radioactive areas and counting in a liquid scintillation counter.

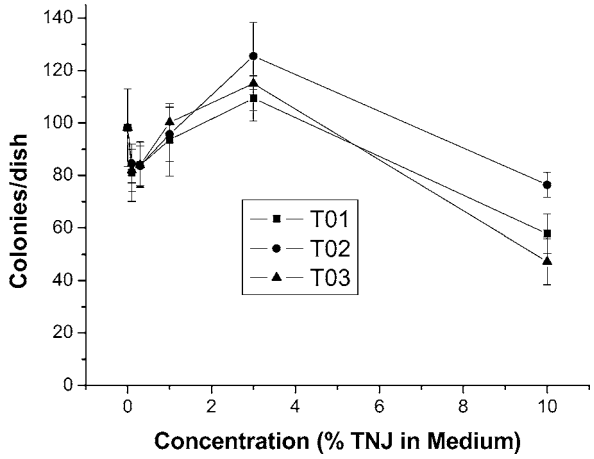

Figure 3. Toxicity of Tahitian noni juice (TNJ) in H4IIE rat hepatoma cells. Sterile filtered juice was directly added to the culture medium. Two hundred H4IIE cells per dish were plated in 10 cm^2 dishes with Williams medium E (WME) and 10% FCS. Five dishes were prepared for each test concentration. Colonies were fixed and counted after 1 week.

Primary rat hepatocytes isolated from rats treated with NJC (100 or 1000 mg/kg), 2 or 12 h prior to sacrifice, were used for the determination of DNA adducts. The cells were the same as used for the UDS assay and the comet assay. Triplicate batches with 10^6 cells per rat from three rats per dose were analyzed. The lysis of the cells, the preparation of the DNA, and the labeling with ^{32}P were performed as described by Pfau et al. (*25*).

Chemical Analysis. Twenty milliliters of the test samples of TNJ was extracted with 20 mL of ethyl acetate. A parallel sample was additionally spiked with 20 μg of lucidin, alizarin, and rubiadin, yielding concentrations of 1 ppm each. The organic layer was removed after centrifugation and evaporated to dryness. The residues were dissolved in 400 μL of methanol, producing extracts concentrated 50-fold, with respect to the juice. Ten microliters of these extracts was injected on a Nucleosil 100 RP-18/10μ HPLC column, length = 25 cm, i.d. = 4.6 mm (Machery & Nagel), equipped with a Thermo Finnigan gradient pump and a diode array detector. It was eluted at a flow rate of 2 mL/min at a temperature of 22 °C with a gradient of acetonitrile (MeCN) and 0.1% acetic acid: 0−5 min, 20% MeCN; 5−10 min, 45% MeCN; 10−15 min, 45% MeCN; 15−30 min, 100% MeCN.

RESULTS

Toxicity of TNJ. *Effects on Liver Cells in Vitro.* Two kinds of liver cells were used: primary rat hepatocytes and H4IIE rat hepatoma cells. Original sterile filtered TNJ was added to the culture medium of liver cells to test the toxicity to these cells. Because the original TNJ contains hydrophilic compounds, such as carbohydrates, which possess osmotic effects on the cells, we also tested organic extracts, which were prepared with ethyl acetate. These extracts contain the lipophilic compounds 50-fold concentrated with respect to the original juices. Three different batches of TNJ were selected for the investigation.

Effects on H4IIE Cells. The influence of the different batches of TNJ, added to the culture medium, on the colony-forming efficiency of the cells was tested. As shown in **Figure 3**, increasing concentrations of TNJ in the medium increased the survival of the cells up to a concentration of 3%. A concentration of 10% TNJ in the medium exerts toxic effects. There was no significant difference between the three batches. After the addition of TNJ extracts to H4IIE cells, toxic effects were

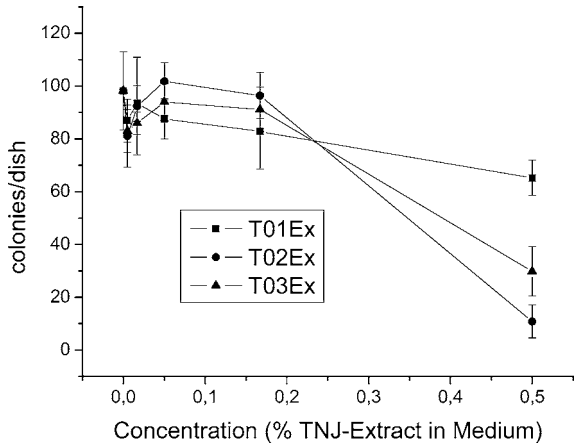

Figure 4. Toxicity of Tahitian noni juice (TNJ) ethyl acetate extracts (50-fold) in H4IIE rat hepatoma cells. The extract dissolved in DMSO was added to the culture medium. Two hundred H4IIE cells per dish were plated in 10 cm^2 dishes with Williams medium E (WME) and 10% FCS. Five dishes were prepared for each test concentration. Colonies were fixed and counted after 1 week.

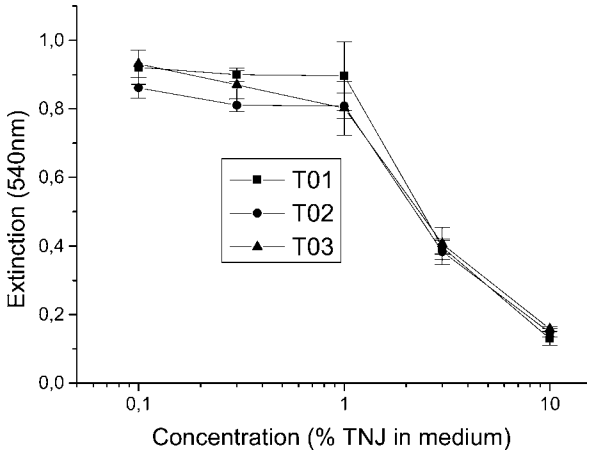

Figure 5. Toxicity of Tahitian noni juice (TNJ) in primary rat hepatocytes. 10^6 cells were plated in 10 cm^2 six-well dishes ($N = 6$). Sterile filtered juice was directly added to the culture medium, and the cells were treated for 4 h. Toxicity was estimated by uptake of the dye neutral red into the cells. Only viable cells accumulate the dye.

observed only with the highest concentration tested, that is, 0.5% (**Figure 4**). This would represent an original juice concentration of up to 25%.

Effects on Primary Rat Hepatocytes. Because these cells do not divide in culture, the toxicity was measured by the Neutral Red method. **Figure 5** demonstrates the toxicity of TNJ to the cells. A concentration of up to 1% TNJ in the medium showed no toxicity. Higher concentrations resulted in a gradual decrease of the Neutral Red accumulation of hepatocytes, indicating toxicity. Again, no difference was observed between the batches of TNJ. **Figure 6** demonstrates the toxicity of the TNJ extract to primary rat hepatocytes. No toxicity was observed in any of the three batches, up to a concentration of 0.1% of the extracts (representing 5% of original juice).

Results of Salmonella/Microsome Assay. A 100-fold concentrated ethyl acetate extract of TNJ dissolved in DMSO was used for the test. Amounts of 0.2−6 μL of this TNJ extract were tested after dilution with DMSO to a volume of 20 μL per plate. Twenty microliters per plate of the undiluted DMSO extract was toxic to the bacteria. Mutagenic effects were observed in the frame shift mutation strains TA98 and TA1537, but not in the point mutation strain TA100. The effects were more pronounced after addition of the S9 mix (**Table 1**).

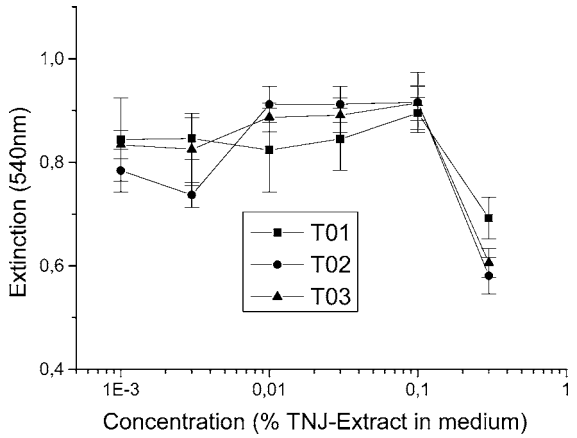

Figure 6. Toxicity of Tahitian noni juice (TNJ) ethyl acetate extracts (50-fold) in primary rat hepatocytes. The extract dissolved in DMSO was added to the culture medium, and the cells were treated for 4 h. Toxicity was estimated by uptake of the dye neutral red into the cells. Only viable cells accumulate the dye.

Table 1. Mutagenicity of TNJ Extracts in *S. typhimurium*[a]

		mutants per plate[b]		quotient[c]	
compound	dose (μL/plate)	+S9	−S9	+S9	−S9
		Strain TA100			
solvent	20 μL	58 ± 13	68 ± 15	1.0	1.0
TNJ extract (100-fold)	0.2	73 ± 13	66 ± 9	1.3	1.0
	0.6	84 ± 11	57 ± 5	1.4	0.8
	2.0	89 ± 7	74 ± 9	1.5	1.1
	6.0	110 ± 30	82 ± 3	1.9	1.2
sodium azide	1 μg/plate	*d*	356 ± 38	*d*	1.5
2-aminoanthracene	1 μg/plate	947 ± 92	98 ± 5	16.3	15.3
		Strain TA98			
solvent	20 μL	22 ± 6	20 ± 0	1.0	1.0
TNJ extract (100-fold)	0.2	27 ± 8	21 ± 3	1.2	1.1
	0.6	43 ± 6	40 ± 3	1.9	2.0
	2.0	82 ± 25	74 ± 13	3.7	3.7
	6.0	164 ± 76	117 ± 9	7.4	5.8
2-nitrofluorene	2 μg/plate	*d*	621 ± 9	*d*	31.0
2-aminoanthracene	1 μg/plate	710 ± 71	75 ± 4	32.0	3.8
		Strain TA1537			
solvent	20 μL	3 ± 2	6 ± 1	1.0	1.0
TNJ extract (100-fold)	0.2	10 ± 2	3 ± 2	3.3	0.5
	0.6	16 ± 7	3 ± 2	5.3	0.6
	2.0	16 ± 1	10 ± 2	5.4	1.7
	6.0	33 ± 5	22 ± 3	11.0	3.8
2-nitrofluorene	2 μg/plate	*d*	212 ± 67	*d*	37.4
2-aminoanthracene	1 μg/plate	287 ± 91	12 ± 4	95.6	2.1

[a] Bacteria were incubated with the test compounds for 30 min prior to inoculation on histidine-free agar plates. Mutation rates are means of two separate experiments. A test is considered to be positive if the mutation rate doubles the background value. [b] ±S9, with(out) S9 mix. [c] Quotient = median of colonies per plate of the test concentration divided by median of colonies of the solvent control. [d] Not tested.

Because TNJ contains red grape juice, which is known to be rich in quercetin (a bacterial mutagen), the experiment was repeated with an ethyl acetate extract prepared from pure noni juice concentrate. The mutagenic effects were all much less pronounced compared with the TNJ extract, even though noni fruit is known to also contain quercetin and rutin (another bacterial mutagen) (*26*, *27*). Again, no mutagenicity was observed in strain TA100, and only a marginal mutagenic effect was observable in TA98 with addition of the S9 mix. A 3-fold

Table 2. Mutagenicity of Noni Fruit Syrup Extracts in *S. typhimurium*[a]

		mutants per plate[b]		quotient[c]	
compound	dose (μL/plate)	+S9	−S9	+S9	−S9
		Strain TA100			
solvent	20	138 ± 14	139 ± 3	1.0	1.0
noni fruit syrup extract	0.2	164 ± 6	152 ± 12	1.2	1.1
	0.6	130 ± 7	164 ± 7	0.9	1.2
	2.0	107 ± 16	162 ± 19	0.8	1.2
	6.0	11 ± 18	28 ± 48	0.1	0.2
	20	0 ± 0	0 ± 0	0.0	0.0
sodium azide	1 μg	655 ± 47	*d*	4.8	*d*
2-aminoanthracene	1 μg	169 ± 13	1569 ± 55	1.2	11.3
		Strain TA98			
solvent	20	21 ± 5	24 ± 27	1.0	1.0
noni fruit syrup extract	0.06	20 ± 3	27 ± 11	1.0	1.0
	0.2	21 ± 5	24 ± 2	1.0	0.9
	0.6	25 ± 8	33 ± 3	1.2	1.2
	2.0	30 ± 6	58 ± 5	1.4	2.2
	6.0	0 ± 0	0 ± 0	0.0	0.0
2-nitrofluorene	2 μg	874 ± 28	*d*	41.6	*d*
2-aminoanthracene	1 μg	31 ± 4	1638 ± 80	1.4	61.4
		Strain TA1537			
solvent	20	5 ± 2	6 ± 1	1.0	1.0
noni fruit syrup extract	0.06	4 ± 1	4 ± 1	0.8	0.7
	0.2	8 ± 4	6 ± 1	1.4	1.1
	0.6	7 ± 2	12 ± 3	1.3	2.2
	2.0	8 ± 2	18 ± 2	1.5	3.3
	6.0	4 ± 6	4 ± 4	0.7	0.8
2-nitrofluorene	2 μg	44 ± 4	*d*	8.3	*d*
2-aminoanthracene	1 μg	6 ± 1	180 ± 22	1.2	33.7

[a] Bacteria were incubated with the test compounds 30 min prior to inoculation on agar plates. Mutation rates are means of two separate experiments. A test is considered to be positive if the mutation rate doubles the background value. [b] ±S9, with(out) S9 mix. [c] Quotient = median of colonies per plate of the test concentration divided by median of colonies of the solvent control. [d] Not tested.

increase of the mutation frequency was observed in TA1537 with the addition of S9 mix (**Table 2**).

Results of V79 HPRT Mutagenicity Test. Because a variety of natural compounds in food is mutagenic toward bacteria, such as flavonoids in noni juice, mammalian cells are often used to verify possible risk for humans. The V79 HPRT test in V79 Chinese hamster fibroblasts is widely used for this purpose. Because the cells have only a limited capacity to metabolize xenobiotics, rat liver microsomes (S9 mix) are added to the system. The test results with the TNJ extract are shown in **Table 3**. No mutagenicity of the TNJ extract was observed without the S9 mix.

In the experiment with the S9 mix, an increase of the mutation frequency was observed; however, this effect was inverse to the dose. The smallest concentration (0.05 μg/mL) exceeded the background mutation frequency by a factor of >2. We therefore repeated the experiment with an overlapping concentration range including smaller concentrations. No mutagenic response was observed in this experiment. We conclude, therefore, that the TNJ extract is not mutagenic to V79 cells.

Results of ex Vivo UDS Assay in Primary Rat Hepatocytes. The results of this test are shown in **Table 4**. To detect early, as well as delayed, effects of the test compound on the liver cells, rats were treated for either 2 or 12 h prior to the isolation of the hepatocytes. Different positive controls, DMN and DMBA, are needed for the short and long treatment times. Both positive controls exerted a clear genotoxic response. No increase of UDS was observed by treatment of the rats with 100 or 1000 mg/kg of noni juice concentrate for 2 or 12 h prior to liver perfusion. This dose represents 10 g of noni juice per kilogram of BW, which is equivalent to about 700 mL for a normal adult human.

Results of DNA Strand-Break Assay (Comet Assay) in Primary Rat Hepatocytes. We utilized the liver cells prepared for the ex vivo UDS assay to perform this test. Because double-strand breaks (DSBs) are short-lived effects, we investigated only the cells isolated after 2 h. The results of this experiment are shown in **Figure 7**. The DNA fragmentation is divided into four classes of degradation: 1 (none), 2 (low), 3 (median), and 4 (high). About 50% of the control cells showed mild degradation of DNA, whereas 28% remained undamaged.

There is a considerable shift toward higher DNA degradation in the cells of rats treated with the carcinogen DMN. Cells of rats treated with the high dose of noni juice showed the same pattern of DSBs as the control, whereas the DNA damage in liver cells of rats treated with the low dose of noni juice was even lower than the control. We thus conclude a possible protective effect of noni juice from endogenous oxidative stress.

DNA Adducts in Rat Hepatocytes in Vivo. Hepatocytes, isolated from rats treated with noni juice concentrate for the determination of UDS, were also investigated for the possible formation of DNA adducts. The results are shown in **Table 5**. Although the positive control DMN causes DNA adducts, as demonstrated by the UDS induction, these adducts are not determined by the ^{32}P postlabeling method, because it can detect only bulky adducts and not methylated DNA bases. Consequently, the concentration of DNA adducts in liver cells of rats increased considerably after treatment of the animals with AAF, which causes bulky adducts. No DNA adduct formation was observed in liver cells after treatment of rats with noni juice concentrate at doses of 100 mg/g or 1000 mg/kg.

Chemical Analysis of TNJ and Noni Roots for Anthraquinones. An overview of the spectrum of anthraquinones occurring in *M. citrifolia* was obtained from a root sample (origin Tahiti), extracted with ethyl acetate. The HPLC chromatogram of this extract is shown in **Figure 8**. Because all HAs with at least one hydroxh group adjacent to the quinone moiety have an absorption maximum between 400 and 450 nm, the detection wavelength was set to 410 nm. The genotoxic HAs lucidin (RT = 16.9 min) and rubiadin (RT = 25.2 min) were detected in the chromatogram. Alizarin, which often occurs in Rubiaceae plants, was not detected; however, the glycoside lucidin primveroside (RT = 9.15) was present in the noni extract. Two more HAs with unknown structures were also observed, with RT values of 15.2 and 24.9 min, respectively, together with a compound at RT = 19.4. The corresponding absorption spectra of the HAs are all very similar with maxima near 410 nm (data not shown). The compound with RT = 19.4 min, and an absorption maximum at 370 nm, is most probably not an anthraquinone.

To test for possible HAs occurring in the TNJ samples from Austria and Germany, extracts, concentrated 50-fold, were prepared. These extracts were analyzed by HPLC. Additionally, three HAs (lucidin, alizarin, and rubiadin) were added to the juices before extraction in concentrations of 1 ppm each.

In **Figure 9**, an overlay of the chromatograms with and without addition of HAs of a TNJ batch from Austria (T02) is shown. The three HAs are clearly detectable in the spiked, but not in the original, juice. No other compound with an absorption spectrum typical for anthraquinones was observed in the original juice. Two other batches (T02 and T03) gave similar results (data not shown). These findings confirm the absence of anthraquinones in concentrations of 1 ppm or higher.

Table 3. Mammalian Mutagenicity Test (V79-HPRT Test) with TNJ Ethyl Acetate Extract[a]

treatment	concn (μL/mL)	colonies per dish			mutants per 10^5 surviving cells
		CFE1	CFE2	mutants	
		Test without S9 Mix			
control		99.0 ± 7.0	103.0 ± 13.4	11.0 ± 2.5	4.27
MNNG	1 μg/mL	31.0 ± 7.0	87.3 ± 7.2	88.2 ± 4.8	40.4
TNJ extract	0.05	99.5 ± 19.0	126.0 ± 6.0	7.8 ± 2.5	2.48
	0.15	116.5 ± 0.7	118.0 ± 1.2	14.8 ± 4.4	5.02
	0.3	65.0 ± 4.2	122.7 ± 12.5	19.0 ± 5.4	6.19
	0.5	61.5 ± 3.5	95.3 ± 12.1	7.2 ± 3.0	3.02
	1.5	2.5 ± 0.7	87.3 ± 15.3	4.0 ± 2.2	1.83
	3	0.0 ± 0.0	toxic		
		Test with S9 Mix			
control		07.0 ± 22.6	147.7 ± 13.6	10.8 ± 4.0	2.92
DMBA	10 μg/mL	88.5 ± 5.0	147.0 ± 24.9	86.6 ± 11.0	23.56
TNJ extract	0.05	115.0 ± 15.0	150.3 ± 11.5	31.0 ± 6.6	8.27
	0.15	108.5 ± 13.4	86.3 ± 9.5	10.8 ± 4.8	5.01
	0.3	104.0 ± 10.0	111.7 ± 6.5	12.8 ± 2.3	4.85
	0.5	103.5 ± 3.5	54.3 ± 7.0	3.0 ± 1.4	2.21
	1.5	91.5 ± 2.1	115.7 ± 8.7	7.0 ± 2.4	2.42
	3.0	68.5 ± 7.8	81.7 ± 8.5	. 6 ± 1.5	0.78
		Repeated Test with S9 Mix			
neg control		86.0 ± 0.0	77.3 ± 8.6	15.0 ± 5.2	7.76
DMBA	10 μg/mL	37.5 ± 2.1	43.0 ± 12.1	44.0 ± 8.5	40.93
TNJ extract	0.003	81.5 ± 9.2	87.3 ± 4.5	7.6 ± 2.0	3.48
	0.01	86.5 ± 0.7	129.0 ± 23.8	10.6 ± 5.2	3.29
	0.03	80.5 ± 13.4	157.3 ± 27.2	24.8 ± 4.4	6.31
	0.06	82.0 ± 2.8	108.7 ± 21.1	21.2 ± 7.4	7.80
	0.1	86.5 ± 2.1	178.3 ± 35.0	12.0 ± 3.5	2.69
	0.3	93.5 ± 2.1	124.3 ± 25.1	11.0 ± 3.4	3.54

[a] MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (positive control without S9 mix); DMBA, 7,12-dimethylbenz[*a*]anthracene (positive control with S9 mix); CFE1, colony-forming efficiency after first plating (toxicity), $N = 3$; CFE2, colony-forming efficiency after second plating (cell viability before mutant selection), $N = 3$; mutants, HPRT-negative colonies, $N = 5$.

DISCUSSION

Juices prepared from the fruits of *M. citrifolia*, known as noni, are consumed worldwide by millions of humans daily. No severe side effects have been reported after almost 10 years of usage. Recently, liver damage in three patients was associated with the consumption of noni juice (*4*, *5*). The authors stated that HAs present in noni juice could be responsible for the toxic effects on the liver by formation of reactive radicals. Although the cited publication used to confirm the presence of HAs in noni juice did not contain such data, several anthraquinones were detected later in noni fruit extracts, although in very small concentrations (*6*, *7*). To confirm the possible risk of drinking noni juice, we performed extensive toxicological investigations with special attention to the liver and the possible presence of HAs.

We used liver cells in vitro (primary rat hepatocytes and H4IIE rat hepatoma cells) as a model for possible hepatotoxic effects of noni juice on the liver. Treatment of the cells with a common noni juice (TNJ) from the market, either directly or after preparation of an organic extract, showed no extraordinary toxic effects. The cells tolerated concentrations of about 1% of the juice, which is the maximum nontoxic dose when osmotic effects are measured. Up to 0.2% of organic extracts, 50-fold concentrated with respect to the original juice, was tolerated by the cells. Although liver cells in vitro do not fully represent the situation in a complex liver in vivo, the results can be used to demonstrate the absence of a special acute toxic effect of noni juice to the liver.

We used two different strategies to clarify a possible influence of HAs present in noni juice on the liver or other organs: chemical analysis and a set of biological assays. Kilogram amounts of noni fruit powder were extracted by Pawlus et al. (*6*) and Kamiya et al. (*7*); however, only milligram amounts of anthraquinones were isolated. Because noni juice contains only 9−11% dry fruit material, we estimated this would produce anthraquinone concentrations below 1 ppm. To verify, we analyzed a common noni fruit juice from the market (TNJ) using a sensitive HPLC method for the detection of HAs. To get an overview of HAs in the plant, we first analyzed a root sample, because the roots of Rubiaceae plants are known to contain numerous HAs (*8*). We were able to identify the highly genotoxic HAs lucidin and rubiadin, together with some others. These HAs, however, were not detectable in the noni juice. To verify the detection limit of at least 1 ppm, we spiked the juice with known quantities of HAs, demonstrating that a possible natural occurrence is lower than 1 ppm.

Only HAs with at least two hydroxh groups next to the quinone moiety can be metabolized to form reactive semiquinone radicals that lead to the formation of reactive oxygen species (*25*). These HAs do not occur in TNJ. Thus, no radical formation can be expected from the HAs reported in refs *6* and *7*. Nevertheless, we used a DNA strand-break assay (comet assay), known to be most sensitive for monitoring DNA damage by free radicals. No DNA strand breaks were observed in liver cells of rats treated with a noni juice concentrate up to a single dose of 1 g/kg, which represents about 700 mL of juice for an adult human. Even if a radical formation not detected by our method did occur, it would be without effect because the human body has the ability to rapidly eliminate such radicals. No damage will occur unless the capacity of the antioxidative defense system is overloaded, which would require large amounts of compounds capable of producing free radicals.

In addition to acute toxic effects, which were not demonstrated with noni juice, genotoxicity has the potential to induce

Table 4. Ex Vivo UDS Test with Noni Juice Concentrates (NJC) in Wistar Rats[a]

treatment	dose (mg/kg)	net silver grains/ nucleus	% positive nuclei	*p* value
		2 h Post-treatment		
control (saline)	2.5	3.5 ± 4.4	3.0	
		–5.7 ± 6.1	4.0	
		–5.4 ± 5.0	2.0	
DMN	10.0	50.3 ± 22.1	99.0	<0.001
		50.3 ± 23.8	100	<0.001
		37.5 ± 19.5	100	<0.001
NJC	1000	–5.3 ± 6.4	3.0	>0.5
		–3.8 ± 5.7	6.0	>0.5
		–4.1 ± 5.3	2.0	>0.5
NJC	100	–5.4 ± 7.2	4.0	>0.5
		–8.3 ± 7.8	1.0	>0.5
		–6.5 ± 7.2	2.0	>0.5
		12 h Post-treatment		
AAF	50	17.4 ± 13.7	85	<0.001
		20.8 ± 18.1	86	<0.001
		17.4 ± 13.9	89	<0.001
NJC	1000	–3.7 ± 6.0	6.0	0.421
		–6.5 ± 8.6	6.0	>0.500
		–3.3 ± 8.7	10.0	0.309
NJC	100	–2.8 ± 6.0	6.0	0.136
		–5.2 ± 8.0	8.0	>0.500
		–5.6 ± 7.6	8.0	>0.500

[a] Male Wistar rats (3 per dose) were treated with saline (negative control) or test compounds via gavage. Dimethylnitrosamine (DMN) and acetylaminofluorene (AAF) served as positive controls. Five slides per liver were plated with 106 hepatocytes. Silver grains over 100 nucleic areas and 300 adjacent cytoplasmic areas were counted per slide for calculation of net silver grains per nucleus. Student's *t* test was performed for calculation of *p* value. A positive result requires three conditions: (1) mean of net silver grains per nucleus >5.0 (i.e., positive nucleus); (2) percentage of positive cells per test concentration >50%; (3) *p* value <0.001.

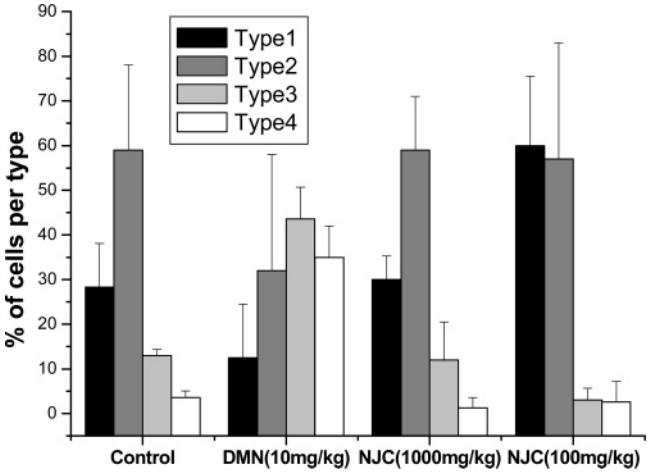

Figure 7. Determination of DNA strand breaks (comet assay) with liver cells derived from rats treated with noni juice concentrate (NJC) for 2 h. Three slides per concentration with 100 cells per slide were scored with respect to DNA damage into type 1–4 degradation. Definition of degradation scores is as follows: type 1, none; type 2, mild; type 3, considerable; type 4, severe.

Table 5. Formation of DNA Adducts after Treatment of Wistar Rats with Noni Juice Concentrates (NJC)[a]

treatment	dose (mg/kg)	DNA adducts/10^9 DNA bases
	2 h Post-treatment	
saline	1000	19.8 ± 3.5
DMN	10	7.4 ± 4.8
NJC	100	16.5 ± 8.4
NJC	1000	15.8 ± 2.0
	12 h Post-treatment	
saline	1000	10.9 ± 5.8
AAF	50	60.8 ± 17.3
NJC	1000	13.3 ± 3.9
NJC	1000	9.7 ± 5.1

[a] The ^{32}P-DNA postlabeling assay was used for the determination of DNA adducts in the livers of rats treated with NJC. The livers were the same as used for the ex vivo UDS test (**Table 4**). Triplicate batches of 10^6 cells each per rat from three rats per dose were labeled with ^{32}P and DNA adducts per 10^9 bases were calculated ($N = 9$). DMN, dimethylnitrosamine (positive control for 2 h treatment); AAF, 2-acetylaminofluorene (positive control for 12 h treatment); NJC, 10-fold concentrate of pure noni juice from French Polynesia.

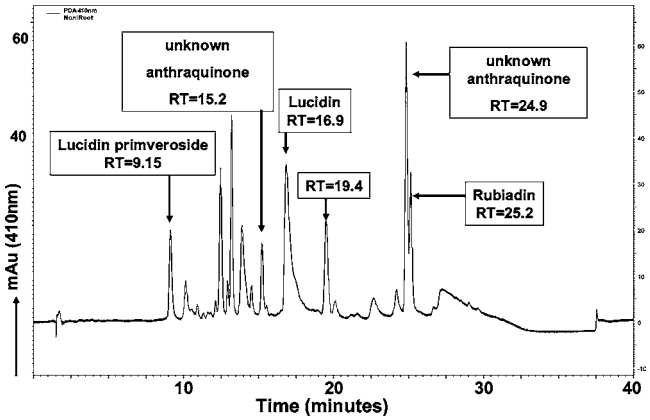

Figure 8. HPLC chromatogram of an ethyl acetate extract of noni roots. Preparation of the extract and chromatographic conditions are explained under Materials and Methods.

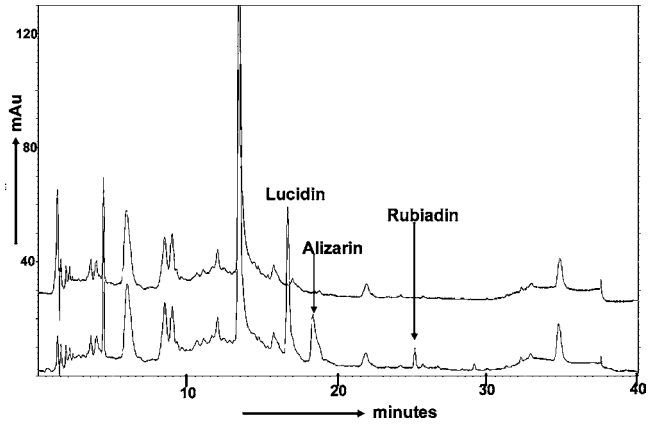

Figure 9. HPLC chromatogram overlay of an ethyl acetate extract from Tahitian noni juice (TNJ), batch T02 (from Austria): (top) original extract from the TNJ; (bottom) extract from TNJ after addition of 1 ppm of lucidin, alizarin, and rubiadin. Preparation of extracts and chromatographic conditions are explained under Materials and Methods.

mutations leading to malignant tumors that can be fatal to the host. With respect to genotoxicity we must distinguish between DNA damage requiring fast or slow repair. DNA damage caused by radicals involves rapid repair, because such damage commonly occurs in the cells of our bodies. Investigations with a variety of HAs published in two doctoral theses (*28*, *29*) confirm this hypothesis. Of concern are compounds producing bulky DNA adducts, such as lucidin. These adducts persist long enough to remain in cells during their replication cycle, which will cause mutations. No DNA adduct formation can be predicted from the structures of HAs published to occur in Noni

fruits (*6*, *7*). Our investigations, performed with TNJ (**Table 5**), support this assertion.

The possible genotoxicity of TNJ, or organic extracts prepared from noni juice, was investigated using a battery of short-term tests, including bacterial and mammalian systems. Slight mutagenic effects were demonstrated in the *Salmonella*/microsome test. This effect was more pronounced with TNJ when red grape juice was added. Quercetin occurs in red grape juice, and in noni to a lesser extent, and is identified as a bacterial mutagen (*30*). This compound, however, is not mutagenic in mammalian systems and is widely accepted as not harmful for human consumption (*31*, *32*). We used two mammalian systems widely used for risk assessment studies, the V79-HPRT assay in Chinese hamster fibroblasts and the ex vivo UDS assay in rats, to investigate the possible genotoxicity of noni juice. V79 cells are often used to verify a mutagenic effect observed in bacteria. Because the cells have only a limited capacity to metabolize xenobiotics, rat liver microsomes are added to the cells during exposure to the test compounds. The ex vivo UDS test detects DNA damage in liver cells after treatment of rats with the test compound. Pharmacokinetic aspects are, therefore, recognized by this test. No indication of genotoxicity was observed in either assay, although extremely high concentrations were tested.

In summary, following a battery of in vitro tests, we conclude there are no indications of hepatotoxic or genotoxic effects from noni juice. Furthermore, the concentration of HAs in the juice is below 1 ppm. Thus, even if 1 L of juice is consumed per day, the total amount of HAs would be <1 mg. No data are available to demonstrate toxic effects of HAs at such low concentrations. Together with data about the absence of toxic effects in laboratory animals and humans, which will be published separately (*33*), we consider noni juice to be safe for human consumption, as long as the juice is produced under controlled conditions and does not contain additives not indicated on the label.

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