Nutrition and Cancer

Citrus Limonoids Induce Apoptosis in Human Neuroblastoma Cells and Have Radical Scavenging Activity[1]

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ABSTRACT Citrus limonoid glucosides, a family of fruit bioactive compounds, were postulated to have free radical–scavenging and apoptosis-inducing properties against certain types of cancers. Four highly purified limonoid glucosides, limoin 17β D-glucopypranoside (LG), obacunone 17β D-glucopyranoside (OG), nomilinic acid 17β D-glucopyranoside (NAG), and deacetylnomilinic acid 17β D-glucopyranoside (DNAG) were tested for superoxide radical (O_2^-)-quenching activity and cytotoxic action against undifferentiated human SH-SY5Y neuroblastoma cells in culture. All 4 scavenged O_2^- as measured by inhibition of pyrogallol decomposition in a spectrophotometric assay. Quenching by NAG in particular emulated an equivalent concentration of vitamin C. When added to the medium of SH-SY5Y cells in culture, micromolar amounts of LG and OG, compared with untreated controls, caused a cessation of cell growth and rapid cell death ($P < 0.001$); NAG and DNAG were better tolerated, but nonetheless toxic as well. Cytotoxicity was related to a concentration- and time-dependent increase in caspase 3/7 activity, suggesting that limonoid glucosides were capable of inducing apoptosis. Arrested cell growth and the induction of apoptosis were confirmed by flow cytometry and DNA fragmentation analysis. Importantly, caspase induction at 12 h correlated with cell survival at 24 h ($P = 0.046$), suggesting that apoptosis was the primary cause of cell death. We conclude that citrus limonoid glucosides are toxic to SH-SY5Y cancer cells. Cytotoxicity is exerted through apoptosis by an as yet unknown mechanism of induction. Individual limonoid glucosides differ in efficacy as anticancer agents, and this difference may reside in structural variations in the A ring of the limonoid molecule. J. Nutr. 135: 870–877, 2005.

KEY WORDS: • *bioactive compounds* • *antioxidants* • *apoptosis* • *radical quenching* • *cancer cells*

Citrus fruits possess a wide variety of bioactive compounds with health-promoting, disease-preventing properties that have been shown to be effective against cancer (1). Unique among these and less studied are the limonoid glucosides, a class of furan-containing triterpenes found mainly in the Rutacaeae and Meliacease families of fruits and that differ from flavonoids in chemical structure. To date, 53 limonoids have been identified and characterized. Although limonoid aglycones are water-insoluble compounds that are responsible for a bitter taste in fruits, the limonoid glucosides are water-soluble and tasteless (2,3). Two enzymes, UDPG-limonoid glycosyl transferase and limonoid D-ring lactone hydrolase, perform the interconversion of free to carbohydrate-bearing moieties (4). The transferase gene has been isolated and cloned (5).

Recent studies demonstrated health benefits and chemopreventive action from limonoid ingestion or treatment. For example, limonoid aglycones or glucosides at micromolar concentrations were shown to restrict HIV replication in human mononuclear cells (6,7), act in the capacity of antimalarial and anti-inflammatory agents (8), and inhibit proliferation of breast cancer cells in culture (9). In animal models, these compounds have antineoplastic activity against chemically induced cancers of the colon (10,11), stomach (12), buccal pouch (13–15), and blood (16). Limonoid glucosides taken through the diet reportedly lowered serum cholesterol (17,18). Some, if not all of these properties appear to be associated with the effects on cells and cell growth (19). This report focuses on the mechanism for blocking cancer cell growth.

Reactive oxygen species (ROS)[4] have been implicated in an array of inflammatory diseases, including arthritis, retinitis pigmentosis, coronary artery diseases, and many types of cancers (20,21). Although there is some speculation that citrus limonoids may play a pivotal role in detoxifying ROS or limiting their severity, there is no concrete evidence support-

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[4] Abbreviations used: Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; BAD, background aggregates and debris; CAMP, camptothecin; LG, limonin 17β D-glucopyranoside; OG, obacunone 17β D-glucopyranoside; NAG, nomilinic acid 17β D-glucopyranoside; DNAG, deacetylnomilinic acid 17β D-glucopyranoside; IC_{50}, 50% inhibitory concentration; IL, interleukin-1; ROS, reactive oxygen species.

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ing such a role. Studies of their detoxification properties have focused mainly on the induction of glutathione S-transferase activity (11,22,23) and the inhibition of cytochrome P_{450} activity (24), 2 enzymes that are known to work against redox cycling and assist in detoxification. In this report, we provide evidence that specific limonoid glucosides have the capacity to quench superoxide radicals. We also show that a second facet of their action is to induce apoptosis, as measured by cytotoxic action against the neuroblastoma cell line SH-SY5Y. A surprising finding was an inequality in quenching and apoptotic-inducing properties of individual limonoid glucosides, suggesting that chemopreventive properties depend on the arrangement of specific chemical groups in the molecule's structure.

MATERIALS AND METHODS

Chemicals. Pyrogallol, ascorbic acid, camptothecin (CAMP), MTT [bromure of 3(4,5-dimethylthiaszol-2-yl)-2,5-diphenyltetrazolium], propidium iodide were purchased from Sigma Aldrich. Standard rhodamine, caspase-specific substrate Z-DEVD-R110, and caspase inhibitor Z-DEVD-CHO were obtained from Molecular Probes.

Isolation and purification of limonoid glucosides. The extraction of limonoid glucosides from 4.5 kg of orange seeds followed the basic procedure described by Tian et al. (19). The limonoid glucosides were resolved by HPLC using 10–25% acetonitrile with 0.003% (v:v) phosphoric acid in the mobile phase at a flow rate of 75 mL/min and an applied pressure of 0.13/0.17 (radial/back) Pa. Individual limonoids were tested for purity by analytical HPLC and identified by LC-MS and NMR analysis as previously described (25). Purity exceeded 95% for most with the exception of NAG, which was estimated to be 90%. The limonoid glucosides that were used in the study are shown in **Figure 1**. Stock solutions were prepared at 1 mmol/L in distilled, deionized water.

Cell cultures. SH-SY5Y human neuroblastoma cells (CRL-2266), purchased from the American Type Culture Collection, were cultured in 10% (v:v) fetal bovine serum, 1:1 mixture of Eagle's MEM, Ham's F12 as previously described (26). Cells were grown on 25 cm^2 falcon culture flasks at 37°C under 5% CO_2. At 70–80% confluence, they were detached from the flasks with Pucks EDTA solution and quantified in a hemocytometer. A set concentration of 15×10^3 cells was subcultured into 12, 24- and 96-well plates for experiments.

Superoxide radical quenching. Assays of O_2^- radical quenching used a modification of the procedure described by Marklund and Marklund (27). A stock solution of mol/L pyrogallol in 0.5 mol/L HCl was diluted 1:500 with 0.2 mol/L Tris-HCl, pH 7.6; 0.5 mL was used in each assay. Assays were run at room temperature with limonoid glucoside concentrations varying between 0.1 and 10 mmol/L. These adjustments allowed for a more sensitive and biologically relevant measurement of limonoid quenching. Quenchers of O_2^- effectively stop the spontaneous decomposition of pyrogallol, which is seen as a suppressed rate of formation of purpurogallin, an oxidized product. A Hitachi U2001 UV-visible recording spectrophotometer set at 325 nm was used to measure changes in purpurogallin concentration. Rates were determined by the slope of the absorbance curve in the first 2 min of the reaction. Controls containing equal amounts of L-ascorbic acid or 40 U of superoxide dismutase (Sigma) were run concurrently. The 50% inhibitory concentration (IC_{50}) values were computed using the formula described by Stahle et al. (28).

Measurements of cell viability. Cell viability was determined by trypan blue exclusion as measured in a hemocytometer. Cells were seeded in twelve 24-well plates and incubated for 24 h. The media were replaced with media supplemented with 5, 10, or 50 μmol/L of limonoid glucosides, and incubation was continued for up to 48 h. Controls with no limonoids or equivalent amounts of CAMP, a strong inducer of apoptosis, were assayed concurrently. Total cell counts and viability determinations in triplicate were taken at 12, 24, 36, and 48 h of incubation in the limonoid medium. A second study used basically the same protocol with the exception that the MTT reagent (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) was used to assess cell survival (29). Both procedures gave comparable results (**Table 1**).

Assay of caspase 3/7 activity. Upon reaching confluence, SH-SY5Y cells were harvested and cocultured into 24-well culture plates. Individual cultures in triplicate were treated with limonoid glucosides as above. After harvesting, the cells were washed 3 times with PBS buffer and collected by centrifugation (5000 × g, 2 min, 4°C), and the cell pellet was taken up in 200 μL of lysis buffer [0.2 mol/L Tris, pH 7.5, 2 mol/L NaCl, 0.02 mol/L EDTA, 0.02% (v:v) Triton-X100]. Aliquots were analyzed for protein (bicinchoninic acid reagent, Pierce); a lysate volume equivalent to 50 μg of protein was brought to 100 μL with buffer in a 96-well flat bottom black microtiter plate. An equal volume of caspase substrate (Z-DEVD-R110) was added with mixing and the plate was covered with aluminum foil and set aside at 37°C for 30 min. Rhodamine released by caspase action was followed over a period of 2 h using a Perkin-Elmer LS50B luminescence spectrometer set at 499 nm excitation and 521 nm emission and quantified with a known rhodamine standard. Controls with no limonoid glucosides or with CAMP were assayed concurrently. Other controls consisted of limonoid glucosides or CAMP combined with AC-DEVD-CHO, a caspase 3/7 inhibitor. Caspase activity is expressed as pmol rhodamine/(min · μg protein).

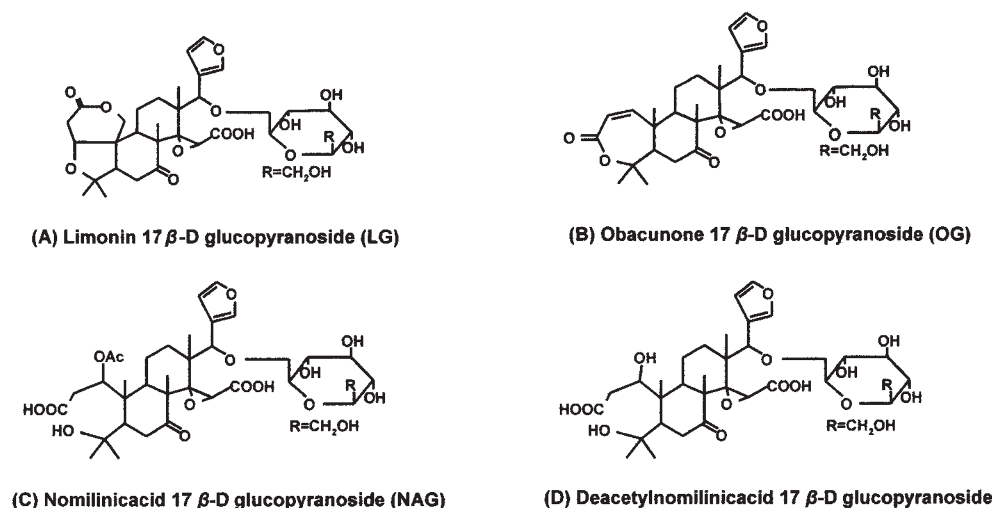

FIGURE 1 Citrus limonoid glucosides showing the A, B, C, and the open D ring.

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TABLE 1

Summary of the activity of citrus limonoid glucosides on pyrogallol autoxidation, caspase 3/7 induction, and human neuroblastoma cell viability[1]

	LG	OG	NAG	DNAG
Inhibition of superoxide radical,[2] *mmol/L*	7.88 ± 0.80*	4.62 ± 0.13*	3.11 ± 0.09	3.53 ± 0.12
Induction of caspase 3/7,[3] *μmol/L*	8.36 ± 1.15	8.17 ± 0.88	14.89 ± 0.513	18.40 ± 0.577
Cell death at 24 h, trypan blue exclusion,[4] *μmol/L*	5.14 ± 0.88	4.197 ± 0.66	28.59 ± 2.02	29.53 ± 0.577
Cell death at 24 h, MTT reduction,[4] *μmol/L*	6.05 ± 0.73	5.54 ± 0.77	22.43 ± 1.57	25.53 ± 1.45
Reduction in S-phase DNA,[5] *% (CV)*	86.67 (4.75)	82.36 (3.70)	57.46 (4.78)	38.35 (4.48)

[1] Values are means ± SEM, $n = 3$.
[2] IC_{50} values were calculated according to Stathle et al. (28) using linear regression. * Different from NAG, $P < 0.005$.
[3] Concentration for 50% of the caspase activity induced by 10 μmol/L camptothecin.
[4] Concentration for 50% cell survival after 24 h compared with untreated.
[5] S-phase DNA was measured using flow cytometry compared with control.

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Flow cytometry analysis. Cells were treated with limonoid glucosides or CAMP and harvested after 24 h. Between 10^6 and 10^7 cells in PBS were centrifuged (200 × g, 6 min, 25°C), and the cell pellet was taken up in 50 μL of PBS and 450 μL 70% ethanol, and stored at −20°C until used. Cells were collected by centrifugation (200 × g, 5 min) decanting the ethanol, and the cell pellet was suspended in 1 mL of 0.1% (v:v) Triton X-100 in PBS, to which was added 200 μg of DNase-free RNase A and 20 μL of propidium iodide. After a 15-min incubation at 37°C to destroy RNA, the suspension was filtered through nylon and collected in tubes that fit a Beckton Dickenson FACS Caliber flow cytometer. DNA fluorescence readings were taken with an excitation blue light set at 488 nm and a detector emission of red fluorescence through a 585-nm filter. Pulse width area signals were used to discriminate between G_2 cells and cell doublets. The data were analyzed using ModFitLT software version 3.1 (PMac). Readouts from the cytometer were analyzed for cells in G_1, S, G_2M phases of the cell cycle. Background aggregates and debris (BAD), a measure of DNA fragmentation associated with apoptosis, was quantified across the different phases.

Statistical analysis. Experimental results represent triplicate determinations for each treatment group and are expressed as means ± SEM. A one-way ANOVA with comparisons based on Fisher's least square differences was used to determine significance at $P < 0.005$. ANOVA for intergroup comparisons was based on Dunnett's multiple comparison analysis. Analyses were performed using SPSS software, version 11.0. The CV in the flow cytometry analysis was < 6%. Reduced χ^2 goodness of fit was assumed to be 0.8–3.0.

RESULTS

Superoxide quenching activity. The potential for each purified limonoid glucoside to quench free radicals was tested against pyrogallol in a slightly alkaline (pH 7.6) solution. At 5 mmol/L, clearly discernible differences were evident in quenching efficacies (**Fig. 2***A*). LG was the weakest and NAG the strongest O_2^- quencher. NAG showed a quenching activity that approached L-ascorbic acid (vitamin C) and was nearly as effective as superoxide dismutase in slowing the decomposition rate over the 5-min assay period. Focusing on NAG alone, sustained inhibition, i.e., preventing the decomposition rate (slope) from attaining the control rate in 5 min, required a concentration of at least 1 mmol/L (Fig. 2*B*). Lower concentrations (0.1–0.5 mmol/L) were effective only in the first 1–3 min of the reaction. Neither a change in the pH nor pyrogallol concentration affected the profiles for the different limonoid glucosides.

Cell viability. Each limonoid glucoside was tested for its effect on SH-SY5Y cell survival. Viability was measured at 12-h intervals. The limonoid glucosides were present at a final concentration of 5, 10, or 50 μmol/L. Depending on concentration, growth inhibition and cytotoxicity became apparent as early as 12 h after exposure and intensified for up to 48 h (**Fig. 3**). The lethal potential was not uniform, however, among the different limonoid glucosides. LG and OG, for example, were more effective ($P < 0.001$) in stopping cell growth and initiating killing than an equivalent concentration of NAG or DNAG. Untreated cells grew steadily and had >60% survivability after 36 h and ~50% at 48 h, whereas survival for cells treated with 50 μmol/L of any of the limonoid glucosides was <5% at 48 h.

Using MTT reduction to gauge cell survival, it was apparent that viability changed very little over the first 12 h, and there was little change in cell numbers from 12 to 24 h (**Fig. 4**). Cellular DNA content, however, dropped sharply between 24 and 36 h after exposure to the limonoids. As little as 1 μmol/L OG or NAG gave a maximal survival time of 36 h. In contrast, 1 μmol/L DNAG changed cell numbers

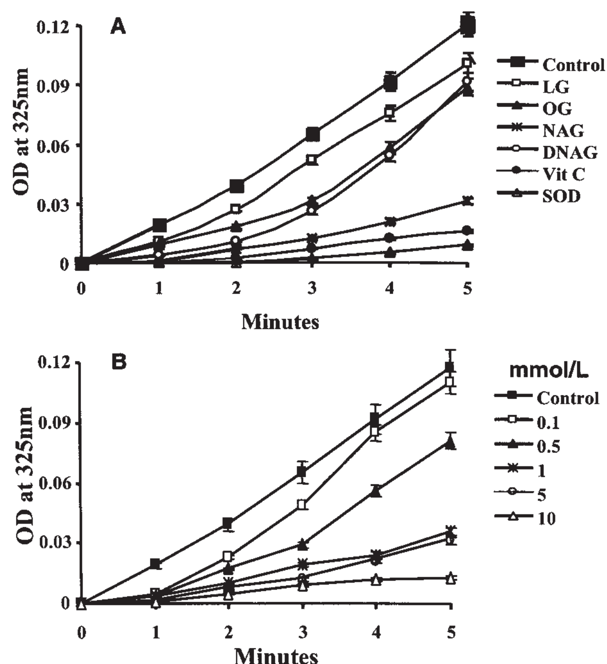

FIGURE 2 Superoxide radical quenching potential of citrus limonoid glucosides in vitro. (*A*) Test of limonoids at 5 mmol/L; vitamin C and superoxide dismutase (40 U) were positive controls. (*B*) Quenching by NAG alone. Values are means ± SEM, $n = 3$.

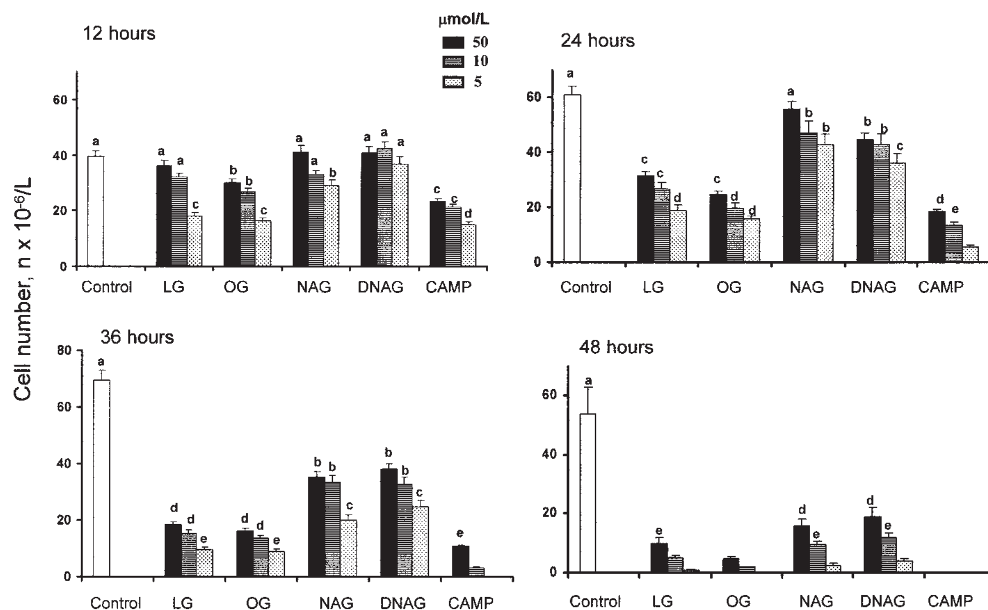

FIGURE 3 Viability of human neuroblastoma cells treated with limonoid glucosides or CAMP at 12, 24, 36, and 48 h. Viability was determined by trypan blue exclusion Values are mean ± SEM, $n = 3$. Means in a panel without a common letter differ, $P < 0.001$.

only slightly between 24 and 36 h. Higher concentrations of DNAG never reached the potency of OG or NAG. Cells treated with CAMP underwent a rapid decline in cellular DNA before 12 h, but at 36 h, there was no difference between cells surviving from CAMP or 1 μmol/L LG or OG ($P < 0.001$). A striking observation, however, was in cells that had been treated with Ac-DVED-CHO before the addition of LG (**Fig. 5**). Even at 50 μmol/L LG, cell death was markedly suppressed for up to 48 h. Because Ac-DVED-CHO is a potent inhibitor of caspase activity, there was a clear indication that toxic action of limonoid glucosides was being exerted through an apoptosis-inducing mechanism.

Test of apoptosis. If apoptosis caused cell death, it should be possible to observe apoptotic changes in the cells before a rapid death rate sets in. Cells were tested for caspase 3/7 activity and DNA fragmentation at or before 24 h. Cytosolic extracts from cells treated for 12 h, matched in protein content, were assayed for caspase 3/7 activity. At 10 μmol/L, all of the limonoid glucosides induced caspase 3/7 activity (**Fig. 6***A*). Induction was seen as early as 6 h and, for LG and OG, peaked at 12 h. LG and OG, however, had less caspase activity at 24 h than at 12 h. In contrast, the caspase activity in cells treated with NAG and DNAG, although lower than LG and OG at 12 h, continue to increase beyond 24 h. Untreated cells showed very little change.

Camptothecin, however, caused a strong rise in caspase activity at 12 h, but like LG and OG, showed a decline at 24 h. Induction of caspase activity was a function of the concentration of limonoid glucosides in the medium (Fig. 6*B*). As little

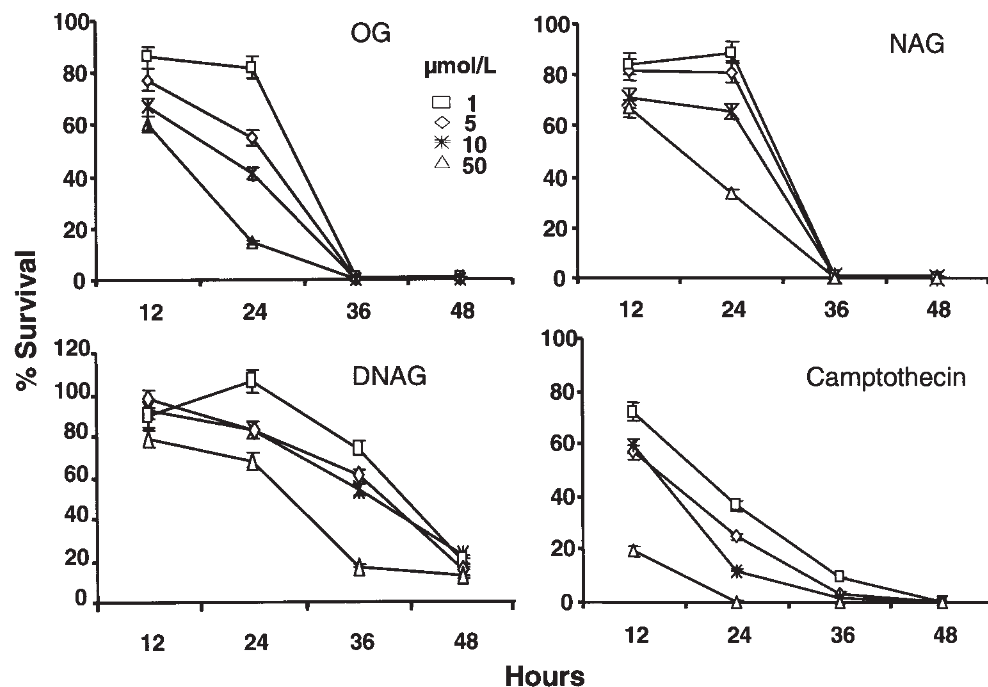

FIGURE 4 Viability of human neuroblastoma cells treated with limonoid glucosides or CAMP. Viability as determined by MTT reduction. Untreated cells were used as a control. Values are mean ± SEM, $n = 3$.

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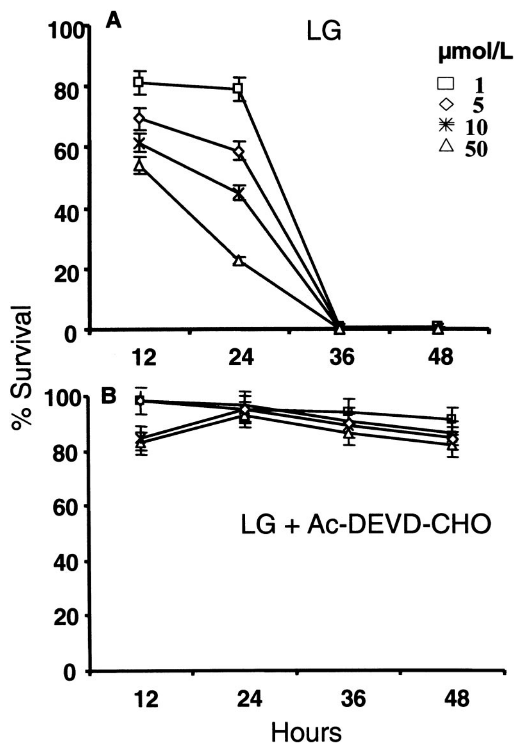

FIGURE 5 Survivability of human neuroblastoma cells treated with LG alone or LG + Ac-DEVD-CHO, a caspase inhibitor. Values, based on MTT reduction, are means ± SEM, $n = 3$.

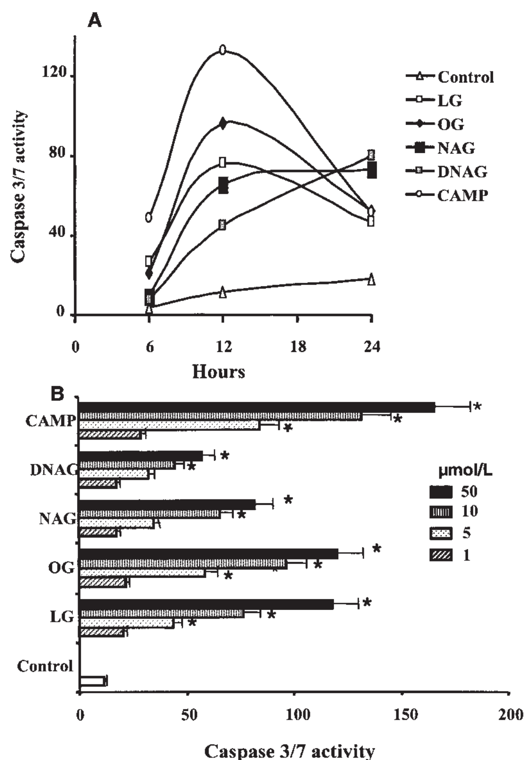

FIGURE 6 Induction of caspase activity in human neuroblastoma cells by limonoid glucosides. (*A*) Time-dependent change in caspase 3/7 activity in cells treated with 10 μmol/L limonoid glucosides or CAMP. (*B*) Limonoid concentration-dependent change in caspase 3/7 activity at 12 h. Values are mean ± SEM, $n = 3$. *Different from control, $P < 0.001$.

as 1 μmol/L brought about a noticeable increment over basal caspase activity in 12 h; 5 μmol/L brought about a significant difference ($P < 0.001$). It is apparent that LG and OG, the most effective suppressors of cell growth, were also the strongest inducers of caspase 3/7 activity. In fact, taking the values for cells treated with 10 μmol/L of individual limonoid glucosides, the 12-h caspase activity correlated with 24-h cell survival numbers ($r = -0.954$, $P = 0.046$). When the limonoid glucoside concentrations were 50 μmol/L, the correlation was stronger ($r = -0.998$, $P = 0.002$). The data, therefore, confirmed the earlier study, which suggested that lethal effects were manifested through apoptosis.

Flow cytometry. To gain additional insights into the effects of limonoid glucosides, treated human neuroblastoma cells were analyzed by flow cytometry (**Fig. 7**). The analysis revealed that both treated and control cells were mainly in the G_1 and G_2M phases with lesser numbers in the S phase subpopulation. Limonoid glucosides LG and OG treatment lowered the population of S phase cells by >80% after 24 h compared with 57 and 38% for NAG and DNAG, respectively (Table 1). The position marked BAD in Fig. 7 shows univariate DNA, a marker of DNA degradation. BAD was higher for cells treated with LG and CAMP than for control or NAG-treated cells. The concentration of BAD between the G_1 and G_2 phases was determined to be 35.4% for CAMP, 31.3% for LG, and 30.9% for OG, compared with 8.6% for control, 17.5% for NAG, and 11.1% for DNAG. Cell numbers at the G_0/G_1 phase for all treatment groups were the same. Overall, the results provided supporting evidence that limonoid glucosides are able to arrest cells at the G_1/S phase of the cell cycle, which is consistent with an interruption in cell division and a predilection toward apoptotic change.

DNA fragmentation. As a final analysis, the potential for limonoid glucosides to induce DNA breakage as a sequel to or concomitant with the induction of apoptosis was determined.

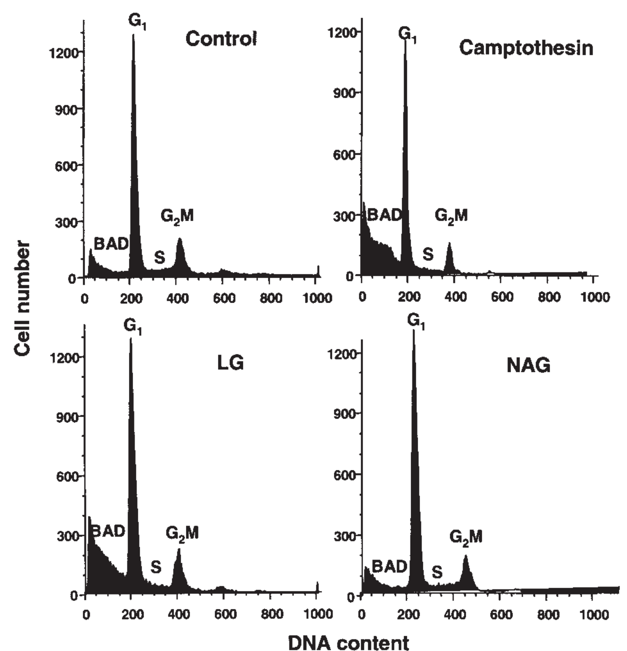

FIGURE 7 Flow cytometry analysis of human neuroblastoma cells treated with 10 μmol/L limonoids for 24 h.

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An equal amount of cellular DNA isolated for measurements of apoptosis using the method of Herrmann et al. (30) was applied to the lanes of a 1% agarose electrophoresis gel. As expected, untreated cells had high-molecular-weight DNA, most of which was intact as shown by heavier ethidium bromide staining of slower moving components (**Fig. 8**). In contrast, cells treated with LG, OG, or CAMP had little high-molecular-weight DNA, but instead a spectrum of fragments that were present in a familiar ladder pattern typical of endonuclease cleaved DNA. Fragmentation of DNA was less evident in cells treated with NAG and DNAG. These data support the flow cytometry results and provide direct evidence for DNA degradation as a consequence of limonoid action on the cells.

DISCUSSION

Controlled diets and epidemiologic studies have supported the conclusion that a high intake of fruit and vegetables lowers the risk of many degenerative diseases including cancer (31). In citrus fruits, the bioactive compounds implicated include carotenoids, vitamin C, folic acid, flavonoids, and the limonoid glucosides whose bioactivity is believed to reside in antioxidant and apoptosis-inducing properties. Concrete evidence supporting those conclusions for limonoid glucosides, however, is lacking.

Two important facets of limonoid glucoside action tested in this study were the ability to quench O_2^- radicals and to induce apoptotic changes in neuroblastoma cells in culture. Quenching, as detected by an in vitro spectrophotometric assay, was evident when millimolar levels of the limonoid glucosides were present. Based on the IC_{50} values reported in Table 1, NAG was 1.5–2.5 times more effective in quenching efficacy than LG or OG. The assay performed with these compounds was designed to measure the scavenging of superoxide anions generated de novo. Action against the hydroxyl radical, the more reactive of the oxygen-centered radicals (20), was not evaluated. Quenching was seen only in the early stages of the reaction; for LG, OG and DNAG, it did not persist beyond the first 2 or 3 min. NAG, however, showed sustained activity over the 5-min period and was gauged to be nearly as effective as an equivalent concentration of vitamin C. What can be deduced from the in vitro assay is that limonoid glucosides have the capacity to interact with O_2^-, but that their action in situ may depend on a reducing agent to recycle inactive to active forms of the molecule.

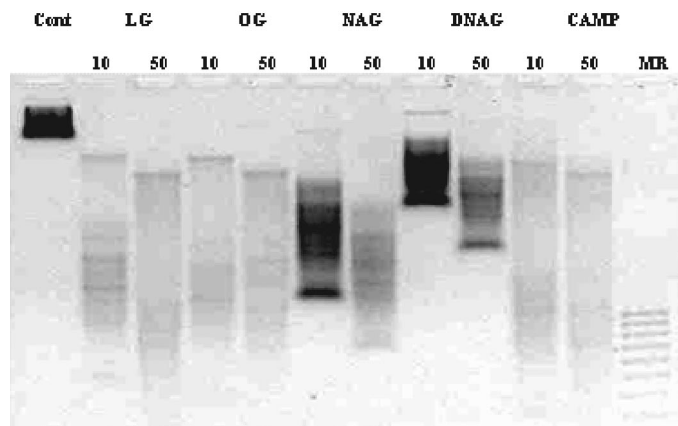

FIGURE 8 Fragmentation analysis of human neuroblastoma cell DNA after 24 h of treatment with limonoid glucosides. A 1% agarose gel was used with detection based on ethidium bromide staining. MR, 100–1000 bp molecular weight ladder.

Purified limonoid glucosides tested in this study appeared to be both cytostatic and cytotoxic to undifferentiated human neuroblastoma cells in culture. Cytostasis was inferred on the basis of flow cytometry evidence, which showed a strong reduction in the number of S phase cells, suggesting an arrest of cell division at the G_1/S interface. All of these events occurred at micromolar concentrations, showing that neuroblastoma cells are highly sensitive to their action. Tian et al. (19) showed earlier that OG was superior to NAG in causing the death of MCF-7 breast cancer cells in culture. Moreover, the aglycones of NAG and LG were as effective as the glucosides in the killing action, suggesting that the glucoside appendage on the molecule is not important for toxic effects. The previous study, however, ruled out apoptosis as a primary mechanism of action because of the relatively high amounts (100 mg/L) that were required to induce an apoptotic response (19). In the present study, we demonstrated that limonoid glucosides in amounts as low as 1–5 μmol/L (0.6–3.0 mg/L) induced caspase activity and fragmented DNA in SH-SY5Y cells. Why MCF-7 cells do not show a similar heightened sensitivity against limonoid glucosides is not clear at this time.

Apoptosis can be induced in SH-SY5Y cells by a number of external factors and conditions such as tumor necrosis factor-α (32), amyloid-B-peptides (33), UV radiation (34), and ceramide (35). In contrast, retinoic acid, prostaglandin E_2, leptin, guanosine, interleukin (IL)-1α, and IL-6 appear to protect SH-SY5Y cells against oxidants and neurotoxins (36–39), as do flavones such as lutoelin, apigenin, and epigallocatechin (40–43). The antioxidant and protective properties of limonoid glucosides appear similar to those of flavonoids. In terms of their cancer killing potential, it is important to heed the observation that limonoid glucosides have little effect on cancer cell lines HL-60, NCI-SNU-1, HeLa, SCOV-3, and HepG2 (19). Further, we found that Chinese hamster ovary cells remain fully viable under conditions in which SH-SY5Y cells succumbed to the limonoid treatment (Shibu et al., unpublished observations). The data, therefore, suggest that induction of apoptosis by limonoid glucosides may depend on cell type or differentiation state and that neuroblastoma cells meet these criteria for reasons not yet clarified.

The difference in the efficacy of the different types of limonoid glucosides was seen in a number of experiments reported here. The data that show these differences are summarized in Table 1. It is clear that LG and OG induced cell death at one-fourth the level of NAG and DNAG. Caspase induction was also achieved at a lower level of LG and OG than NAG and DNAG. Quenching, however, favored NAG. A careful inspection of the structures (Fig. 1) shows that OG and LG share the same structural features as NAG and DNAG with the exception of sealed A ring with no carboxyl group in the molecules of LG and OG. Miller et al. (14) speculated earlier that the A ring as opposed to the D, is more decisive in the biological activity of limonoid glucosides. Our data extend that suggestion by showing that superior quenching and apoptosis-inducing potential may also be associated with the A-ring configuration.

Future research endeavors can now turn to the mechanism of induction of apoptosis to better explain the underlying mechanism of the action of limonoid glucosides on SH-SY5Y

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cells. By activating 2 proteases, mu-calpain and caspase 12, Ca^{2+} was strongly implicated in the induction of apoptosis and the regulation of the apoptotic signaling pathway (44). Mitochondria regulate apoptosis in vertebrates by releasing cytochrome c, an inducer of caspase 3; Bcl-2 regulates cytochrome c release (45). The activation of the c-jun N-terminal kinase signaling pathway was also shown to be an early event in the induction of apoptosis in neuronal cells (34). c-Jun and Bcl-2, are targets of the phosphorylation reaction that modulates an apoptotic response. No data are available that connect limonoid glucosides with these internal events. Another consideration is the synergistic action by which one limonoid promotes the action of another. Tian et al. (19) found that mixtures of limonoid glucosides had greater killing and apoptosis-inducing potential than any pure compound tested singularly. Moreover, Mertens-Talcott et al. (46) recently showed that ellagic acid significantly potentiated the action of quercetin in stimulating caspase activity in MOLT-4 human leukemia cells in culture. The 2 fruit polyphenols in combination had stronger anticarcinogenic effects than either one alone. These important observations suggest that limonoid glucosides could act in tandem or work with flavones to promote a more powerful action. Synergism rather than individual action may better explain the health-promoting effects in nutrition.

In conclusion, the data in this report clearly support limonoid glucosides at micromolar concentrations as being lethal to neuroblastoma cells in culture. Because not all cancer cells succumb to their effects, limonoid glucoside may target specific cell types, which makes it imperative to identify the vulnerable site(s) in cells that underlie their mechanism of action.

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