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Limonin Methoxylation Influences the Induction of Glutathione *S*-Transferase and Quinone Reductase

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Previous studies have indicated the chemopreventive potential of citrus limonoids due to the induction of phase II detoxifying enzymes. In the present study, three citrus limonoids were purified and identified from sour orange seeds as limonin, limonin glucoside (LG), and deacetylnomilinic acid glucoside (DNAG). In addition, limonin was modified to defuran limonin and limonin 7-methoxime. The structures of these compounds were confirmed by NMR studies. These five compounds were used to investigate the influence of phase II enzymes in female A/J mice. Our results indicated the highest induction of glutathione *S*-transferase (GST) activity against 1-chloro-2,4-dinitrobenzene (CDNB) by DNAG (67%) in lung homogenates followed by limonin-7-methoxime (32%) in treated liver homogenates. Interestingly, limonin-7-methoxime showed the highest GST activity (270%) in liver against 4-nitroquinoline 1-oxide (4NQO), while the same compound in the stomach induced GST by 51% compared to the control. The DNAG treated group induced 55% in stomach homogenates. Another phase II enzyme, quinone reductase (QR), was significantly induced by limonin-7-methoxime by 65 and 32% in liver and lung homogenates, respectively. Defuran limonin induced QR in lung homogenates by 45%. Our results indicated that modification of limonin has differential induction of phase II enzymes. These findings are indicative of a possible mechanism for the prevention of cancer by aiding in the detoxification of xenobiotics.

KEYWORDS: Citrus; mice; chemoprevention; bioactive compounds; xenobiotics

1. INTRODUCTION

Health maintaining properties of citrus fruits have recently been promoted due to their potential benefits on cancer prevention based on cell culture and animal studies (*1*, *2*). Current research has transitioned from the study of classical vitamin deficiency related diseases such as scurvy to the study of thousands of bioactive compounds that may have important roles in the prevention of several diseases such as cancer, heart disease, and Alzheimer's. Initial research on citrus limonoids was initiated to ameliorate the bitterness problem in citrus juice due to the bitter limonoid aglycones, limonin and nomilin (*3*).

Limonoids are a group of structurally related triterpene derivatives found in plant families such as Rutaceae and Meliaceae (*4*). Citrus limonoids are composed of two main nucleus structures. The structure of limonin exemplifies the first general nucleus, which consists of five rings designated as A, A′, B, C, and D. The second limonoid structure, nomilin, consists of four rings designated as A, B, C, and D.

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The role of limonoids on human health and their biological activities have been demonstrated in our lab (*2*, *5*−*7*) and elsewhere (*8*−*10*). Citrus limonoids have been attributed to lowering cholesterol levels by reducing the production of medium apo B in cultured human liver cells, HepG2 (*9*). Furthermore, a recent report provided evidence that limonin and nomilin have the ability to inhibit HIV-1 replication (*10*). During the past decade, the chemopreventive properties of citrus limonoids have been explored using animal models. In two Japanese studies, it was reported that orange juice and citrus limonoids, obacunone and limonin, played an important role in the inhibition of azoxymethane-induced colon cancer (*8*). Our recent study provided supporting evidence that bioactive compounds found in grapefruit, one of them being limonin, protect against azoxymethane induced aberrant crypt foci (*11*). Inhibition of 7,12-dimethylbenz[*a*]anthracene induced oral tumors by citrus limonoids has also been reported (*3*, *12*). Additionally, benzo[*a*]-pyrene induced forestomach neoplasia in mice was inhibited by citrus limonoids (*13*).

In the present study, we have analyzed the induction of phase II detoxification enzymes, GST and QR, by three limonoids purified from *Citrus aurantium*. In addition, limonin was modified to defuran limonin and limonin 7-methoxime, and used for structure−activity relationship studies of phase II enzymatic activity.

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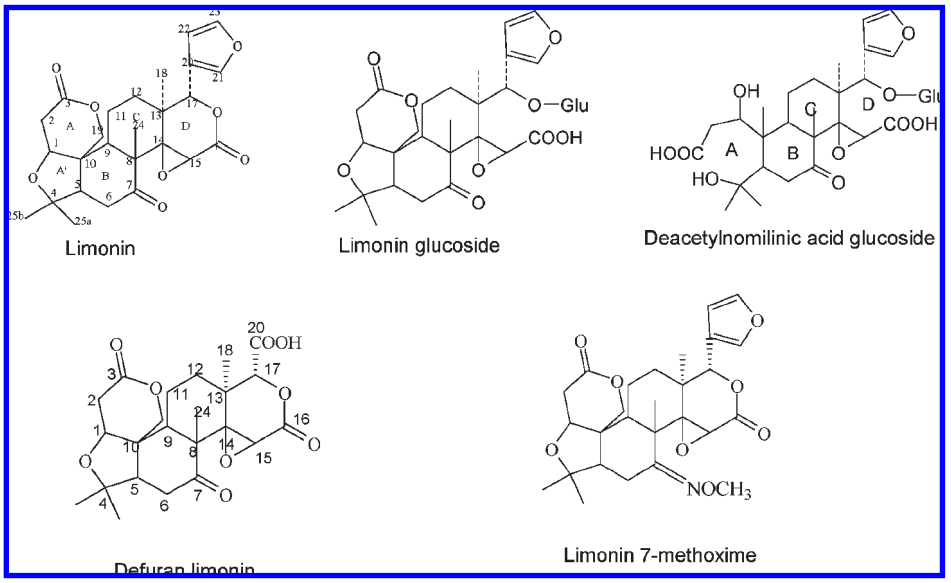

Figure 1. Structure of purified limonoids from citrus and modified compounds.

2. MATERIALS AND METHODS

2.1. Materials. β-Nicotanamide adenine dinucleotide phosphate reduced tetrasodium salt, 2,6-dichlorophenol-indophenol, 1-chloro-2,4-dinitrobenzene, 4-nitroquinoline 1-oxide, glutathione reduced, flavin adenine dinucleotide disodium salt, and all solvents were purchased from Sigma-Aldrich Company (St. Louis, MO). Bovine serum albumin was obtained from Intergen (Purchase, NY). ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded at 400 and 100 MHz, respectively, on a JEOL NMR instrument (JEOL USA, Inc., MA, USA). Tetramethylsilane (TMS) was used as the internal standard.

2.2. Purification of Limonoids. Citrus seeds (*Citrus aurantium* L.) were collected from the Texas A&M University Kingsville Citrus Center Orchard, Weslaco, TX. Dried seeds were powdered (2.2 kg) and extracted with hexane (4 L) for the removal of fatty acids. Then, the defatted powder was separately extracted with ethyl acetate (EtOAc) (4 L) and methanol (4 L) for 8 h. The extract was filtered and concentrated under vacuum to obtain a viscous liquid. Dried EtOAc extract was impregnated with silica gel and loaded onto a silica gel column for chromatography. The silica column was eluted with different concentrations of mobile phase (chloroform and acetone) to obtain limonin. A MeOH extract was fractionated on an Amberlite XAD-2 column, and elution was carried out with increasing concentrations of MeOH in water to obtain LG and DNAG (**Figure 1**) as per our earlier publication (*14*).

2.3. Preparation of Limonin-7-methoxime. Two grams of limonin was dissolved in pyridine (42 mL), and methylhydoxylamine hydrochloride (2.45 g) was dissolved in absolute ethanol (42 mL). Both solutions were then transferred to the round-bottom flask, and the reaction mixture was refluxed for 6 h at 75 °C−80 °C and cooled to room temperature. The completion of the reaction was determined by the disappearance of the limonin spot on TLC using chloroform/acetone (9:1) as the mobile phase. Saturated solution of sodium chloride was added to the cold reaction mixture until the complete precipitation of pyridinium chloride, which was removed by filtration. The filtrate was extracted using ethyl acetate (4× 50 mL). The pooled ethyl acetate layer was washed with 2 N HCl (4× 50 mL), followed by washing with sodium bicarbonate (2× 50 mL), and finally water. The organic phase was concentrated under vacuum to obtain the precipitate. Further purification was performed by silica gel column chromatography. The column was eluted with hexane and ethyl acetate as the mobile phase. Limonin-7-methoxime was eluted at 30% ethyl acetate in hexane.

2.4. Preparation of Defuran Limonin. Limonin (1.5 g) acetonitrile (23 mL), carbon tetrachloride (23 mL), and water (33 mL) were mixed in a 100 mL round-bottom flask, and the reaction mixture was kept stirring at room temperature for 10 min. Then, sodium periodate (9 g) and ruthenium trichloride (220 mg) were added, and stirring was continued until the completion of the reaction (~40 h). The reaction was monitored by TLC. The reaction mixture was extracted with ethyl acetate in a separating funnel, decolorized using activated charcoal, filtered, and precipitated using hexane to obtain defuran limonin.

2.5. HPLC Analysis. The purity of the isolated compounds were analyzed by high performance liquid chromatography using Agilent 1200 Series (Foster City, CA, USA) consists of degasser, quaternary pump, auto sampler, column oven, and diode array detector as per our published method (*15*). The compounds were eluted using a C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 μm particle size, (250 × 4.6 mm) with a flow rate of 1.0 mL/min. The column temperature was set at 27 °C. The elution was carried out with gradient mobile phase (A) 3 mM phosphoric acid and (B) acetonitrile, starting at 85% of solvent A, reduced to 77% in 5 min, 74% after 25 min, which was further reduced to 60% at 30 min and completing the gradient at 54% at the end of 60 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before the next run. The purity of the compounds was calculated using calibration curves as per our method (*15*).

2.6. Identification. The structures of the purified limonoids and modified compounds were elucidated by ^{1}H, ^{13}C NMR, and DEPT studies using a JEOL ECS-400 spectrometer at 298 K using a 5 mm broadband probe equipped with a shielded z-gradient and Delta software version 4.3.6 using TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequence. One-dimensional ^{13}C spectra using distortionless enhancement of polarization transfer (DEPT-135) using a 135 degree decoupler pulse was also performed to aid the structure. Mass spectrometric analyses were performed using a ThermoFinnigan LCQ-DECA instrument (Thermo, San Jose, CA, USA).

2.7. Animal Studies. The animal studies model described by Lam et al. (*13*) was adapted to investigate the phase II enzyme induction in mice. Female A/JOlaHsd 8−9 week old mice were purchased from Harlan Sprague−Dawley Laboratory (Indianapolis, IN). The mice were kept on an AIN-76 semipurified custom diet without vitamin E obtained from MPBiomedical (Solon, OH) and tap water ad libitum. Vitamin E was excluded from the diet because of its ability to induce phase II enzymatic activity. The mice were housed in plastic cages in an environmentally controlled room on a 12 h light/12 h dark cycle.

The mice were divided ($n = 4$) into five experimental groups and one control group. The treatments consisted of limonin, LG, DNAG, limonin-7-methoxime, and defuran limonin. Each compound (20 mg) was suspended in dimethyl sufoxide (DMSO)/corn oil (1:1) (v/v) and administered by oral gavages every two days. A total of five treatments were administered in 10 days. The control group was given the equivalent amount of DMSO/corn oil (1:1) without compounds. After 48 h of the last treatment, the mice were sacrificed by cervical dislocation. The lung, intestine, stomach, and liver were harvested and washed with cold phosphate buffer solution (PBS). A portion of the tissue was stored for future use, while the remaining portion was weighed and

homogenized using a Pro200 homogenizer with PBS (10 mM, pH 7.0), containing β-mercaptoethanol (1.4 mM) to obtain 10% (w/v) homogenate. The homogenate was centrifuged at 22,000*g* for 45 min in a Beckman Avanti 30 centrifuge at 4 °C, and the supernatant was carefully removed and stored at −20 °C until further use. Each organ within a group was homogenized under the same conditions, and the homogenates were used for bioassays.

2.8. Determination of Enzyme Activities and Protein Concentration. GST activity against CDNB was determined as described by Habig et al. (*16*) with slight modifications. One milliliter of enzyme assay consisted of 100 mM phosphate assay buffer, (pH 6.5), 20 mM CDNB, and 10 mM GSH, and 50 μL of tissue homogenate sample. The absorbance was measured at 340 nm using a Beckman DU 640 UV/visible spectrophotometer against a blank. The blank consisted of assay buffer, GSH, and CDNB, without the tissue homogenate in a total volume of 1 mL.

GST activity against 4NQO was performed using a modified method developed by Stanley et al. (*17*). In this reaction, the glutathione replaces the nitro group of 4NQO. The 1 mL enzyme assay consisted of 100 mM phosphate assay buffer at pH 6.5, 5 mM 4NQO, 10 mM GSH, and 20 μL of tissue homogenate. The absorbance was measured at 350 nm in a Beckman DU 640 UV/ visible spectrophotometer against an appropriate blank consisting of assay buffer, 4NQO, and GSH without the tissue homogenate in a total volume of 1 mL.

The QR activity was determined by slight modification of the method reported by Wang et al. (*18*). The 1 mL enzyme assay system consisted of 25 mM Tris/HCl at pH 7.5; 0.18 mg/mL BSA; 5 μM FAD; 0.2 mM NAD(P)H; 40 μM DCPIP; and 20 μL of tissue homogenate. The blank contained all of the above except the tissue homogenate and 0.2 mM NAD(P)H, while the control contained all except the enzyme sample. The absorbance was measured at 600 nm.

The spectrophotometer was equipped with enzyme kinetic software and programmed to calculate enzyme units. The amount of enzyme used, 1 μmole of substrate per min at 25 °C, is equivalent to one unit of enzyme activity. The protein content of the samples were quantified by Bradford's method (*19*). The absorbance was read at 595 nm. Bovine serum albumin was used as a standard.

2.9. Statistical Analysis. Each treatment group consisted of four mice from which the lungs, intestine, stomach, and liver were extracted. Each organ homogenate from each mouse represented one sample; all assays were performed in triplicate. The results were expressed as the average of three replications. Student's *t*-test was used to asses the significance of the data.

3. RESULTS

The ethyl acetate extract of *Citrus aurantium L.* provided three pure compounds. The purity of the isolated limonoids were analyzed by HPLC, and the chromatograms are presented in Figure S1 (Supporting Information). Along with three natural limonoids, limonin was modified to defuran limonin, and limonin methoxime was used to study the GST activity. The purity of the modified compounds were analyzed by HPLC using calibration graphs similar to those in our published paper (*15*). The resulting chromatograms are presented in Figure S2 (Supporting Information). The structures of the purified limonoids and modified compounds have been elucidated by MS, 1H, ^{13}C NMR, and DEPT studies. The proton and ^{13}C spectra of five compounds with assignments of various signals are shown in **Figures 2** and **3**, respectively. On the basis of the NMR results, the structures of the isolated compounds have been identified as limonin, limonin glucoside, and deacetyl nomilinic acid glucoside. In addition, mass spectra of the modified limonoids reported the molecular ion peak at m/z 506.2294 $[M + Li]^+$ and 447.1145 $[M - H]^-$ for limonin-7-methoxime and defuran limonin, respectively. Modified compounds were also confirmed as defuran limonin and limonin methoxime by ^{1}H and ^{13}C NMR spectra (**Figures 2** and **3**). Moreover, chemical shifts of isolated compounds were compared with reported values (*20*, *21*).

Three purified citrus limonoids and two modified limonoids (**Figure 1**) were tested for induction of phase II enzymatic activity. The GST activities were assayed using CDNB and 4NQO as substrates. Induction of QR was also evaluated. Mice were treated with the five limonoids in order to evaluate the induction of GST activity against CDNB. In the lung, DNAG was the only limonoid showing significant induction (67%) of GST activity compared to that in the control. Interestingly, in the liver, modified methoxylated limonin-7-methoxime showed significant induction of GST activity, while LG and DNAG showed a decrease in activity (**Table 1**).

GST activity was also measured against 4NQO. In stomach homogenates, DNAG (55%) showed the highest induction of GST activity, followed by limonin-7-methoxime (51%). In liver homogenates, limonin-7-methoxime induced GST activity was three times higher (270%) than that of the control. In intestine homogenates, defuran limonin and DNAG decreased GST activity, while no activity change was observed in lung homogenates (**Figure 4**).

Quinone reductase activity was measured in the lung, intestine, liver, and stomach homogenates. In the liver and lung, limonin-7-methoxime showed significant induction of QR at 65% and 32%, respectively, compared to that in the control. In the intestine and stomach homogenates, limonin-7-methoxime increased QR activity, but induction was not statistically significant. In lung homogenates, defuran limonin showed the highest induction (45%) activity followed by limonin methoxime. Interestingly, DNAG decreased QR activity in the lung. No significant change in activity was observed in stomach homogenates (**Table 2**).

4. DISCUSSION

Some of the reported compounds in the present study are not available commercially. Hence, we have isolated and identified them by spectroscopic studies as per our published paper (*14*). Moreover, we have quantified limonoid concentration using the HPLC method (*15*), and the purity of the compounds were found to be more than 95%. Limonoid glucosides are tasteless water-soluble compounds formed in fruits during maturation from their corresponding aglycones. However, certain aglycones are bitter compounds with low water solubility and are principally found in seeds (*22*). Naturally occurring citrus limonoids contain a furan ring attached to the D ring at the 3-position. The overall focus of the study was on the furan ring and changing the ketone group at the seventh position to methoxime. Interestingly, modified compounds showed very significant GST activity in the liver against 4NQO. Defuran limonin is similar in structure to limonin except for the absence of the furan ring. Limonin glucoside differs from limonin only by the presence of a glucose moiety at the C-17 position. Deacetyl nomilinic acid glucoside differs from limonin glucoside in the A ring.

The presence of the furan moiety is thought to be responsible for the induction of the phase II detoxifying enzyme glutathione *S*-transferase (GST) activity. In addition to GST, another important phase II enzyme that protects against toxic and neoplastic effects of xenobiotics is NAD(P)H:quinone reductase (QR). QR protects against cytotoxicity, and the increased levels are positively correlated with chemoprevention (*23*). Recent findings suggest that the consumption of citrus fruits, specifically grapefruit and oroblanco, modulates both phase I and phase II metabolizing enzymes in rats (*24*).

A study conducted in The Netherlands, reported that the habitual consumption of fruits and vegetables was positively correlated with human rectal GST activity (*25*). Recent research on citrus limonoids shows that both limonin and nomilin could inhibit certain chemically induced carcinogenesis in different

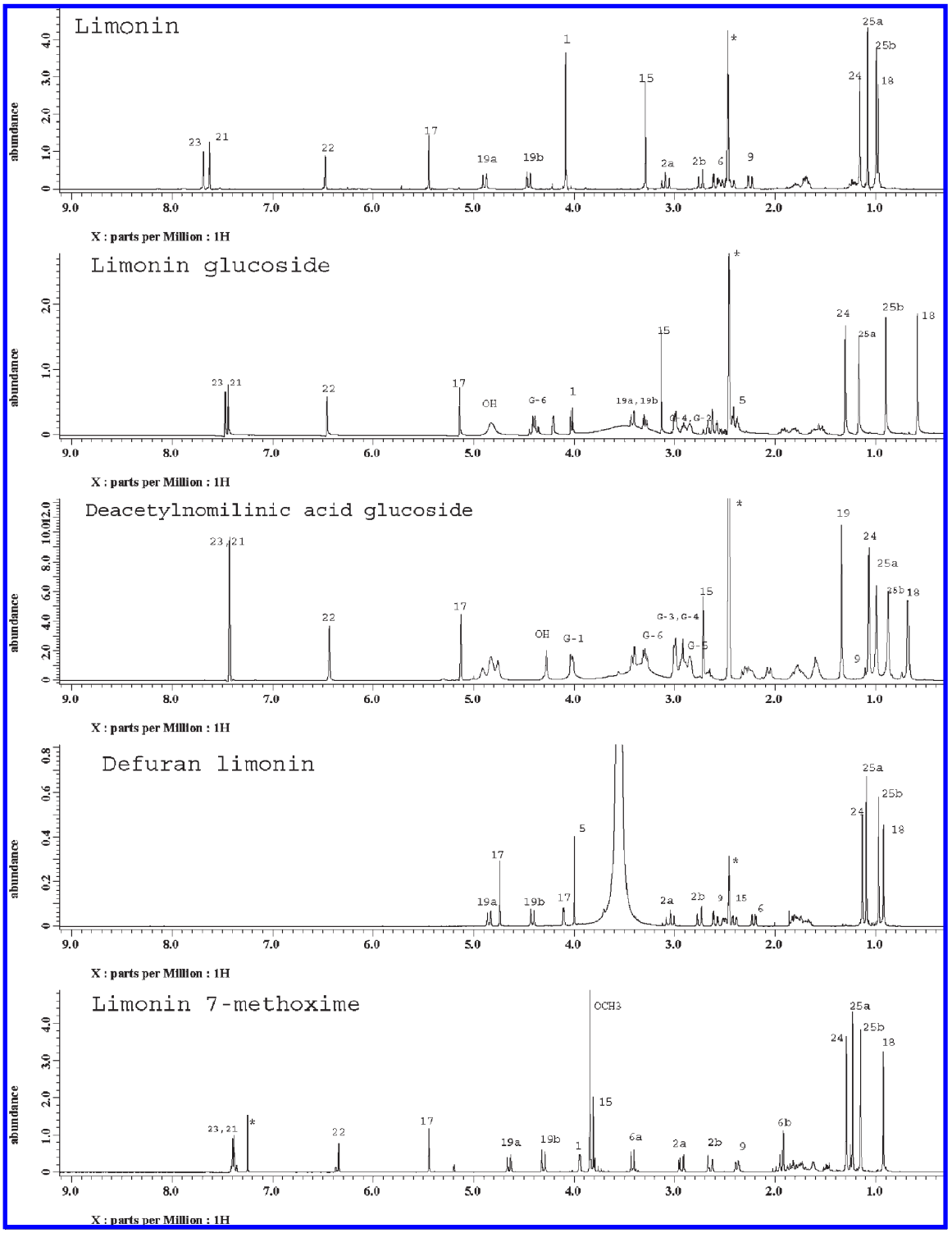

Figure 2. [1]H NMR spectra of purified compounds from *Citrus aurantium* and modified compounds recorded on a JEOL ECS-400 MHz spectrometer. Assignments of various protons are made on the respective spectrum for each compound. G-1 to G-6 indicate the glucose signals. Peaks from DMSO-d_6 and $CDCl_3$ are marked with asterisks.

animal models (*13*, *26*). Additionally, Lam et al. (*13*) also evaluated different concentrations of limonoid treatment in mice. In a two stage model for skin carcinogenesis, it was shown that nomilin was more effective as an inhibitor during the initiation stage of carcinogenesis, while limonin was more active during the promotional phase of carcinogenesis (*27*). Structurally, limonin has an A and A′ ring, while nomilin has only a seven-membered A ring. Furthermore, it was suggested that there is a possible induction of GST activity in mice by citrus limonoids (*13*).

The differential induction potential of certain citrus limonoids to induce GST activity was further attributed to different structural components of the limonoid nucleus. It was suggested that an intact A ring is required for antineoplastic effects, such as those present in nomilin (*28*). It is possible that modification to the B ring of the limonoid nucleus may also alter the induction of GST activity.

The D-ring of the limonoid nucleus has a furan ring attached to its third position. Several studies have been conducted on the importance of the furan ring in the induction of GST activity.

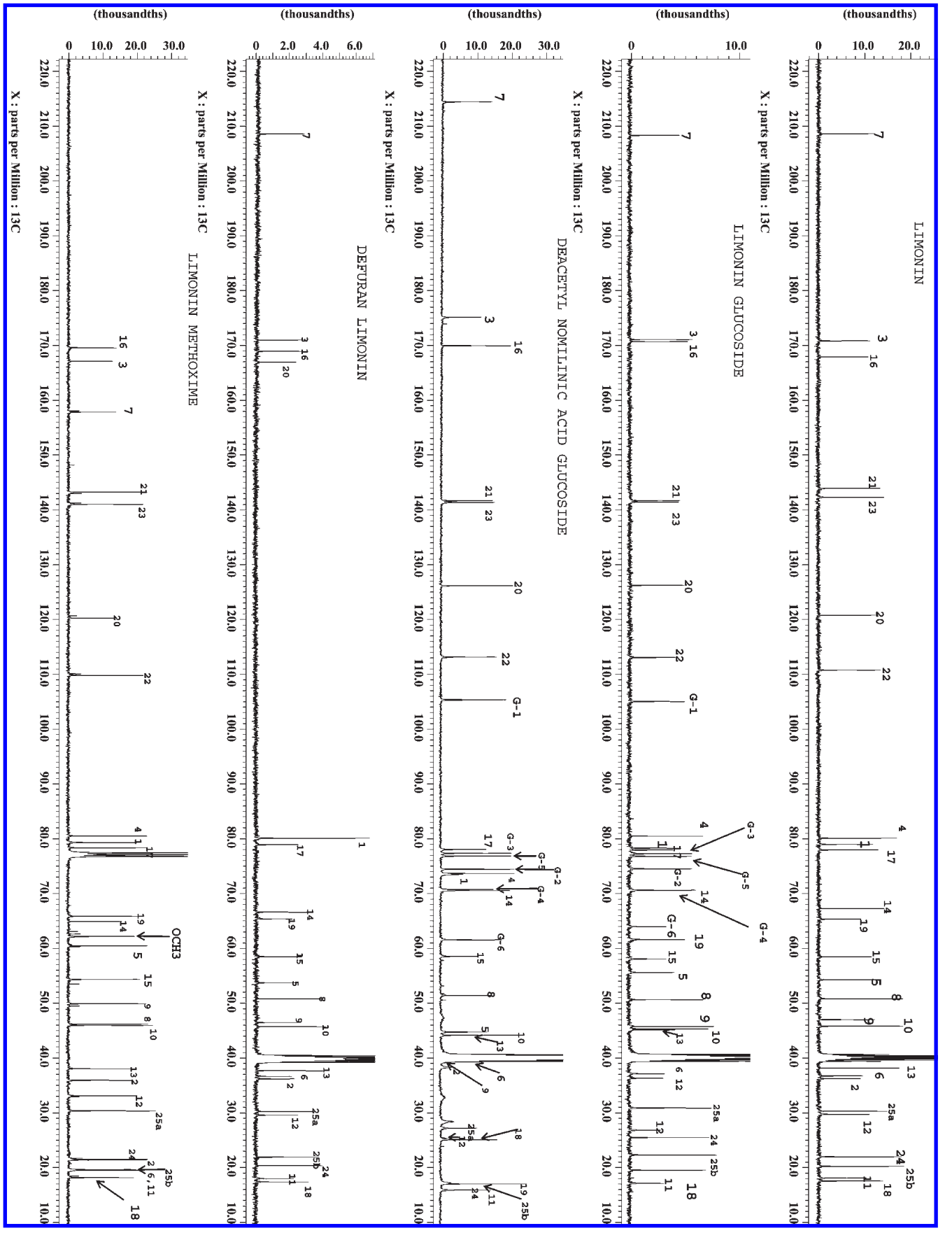

Figure 3. ^{13}C NMR spectra of purified compounds from *Citrus aurantium* and modified compounds recorded on a JEOL ECS spectrometer at 100 MHz. Peaks from DMSO-d_6 and $CDCl_3$ are marked with asterisks. Assignments of various carbons are made on the respective spectrum for each compound. G-1 to G-6 indicate the glucose signals.

Table 1. GST Activity against 1-Chloro-2,4-dinitrobenzene[a]

sample	stomach	intestine	liver	lung
control	0.88 ± 0.07	0.86 ± 0.09	1.78 ± 0.02	0.36 ± 0.08
limonin	0.83 ± 0.13	0.63 ± 0.01	1.63 ± 0.27	0.34 ± 0.05
limonin-7-methoxime	0.89 ± 0.18	1.34 ± 0.47	2.36 ± 0.05**	0.36 ± 0.03
defuran limonin	0.86 ± 0.12	0.79 ± 0.09	1.45 ± 0.54	0.34 ± 0.08
LG	0.86 ± 0.13	0.75 ± 0.06	1.41 ± 0.08*	0.44 ± 0.09
DNAG	1.15 ± 0.38	0.70 ± 0.13	1.35 ± 0.20*	0.60 ± 0.12**

[a] Specific activity (units/mg protein). The results are the means ± SD (n = 4). *indicates statistically significant ($p < 0.05$) decrease of activity using Student's t-test. ** indicates statistically significant ($p < 0.05$) induction of activity using Student's t-test.

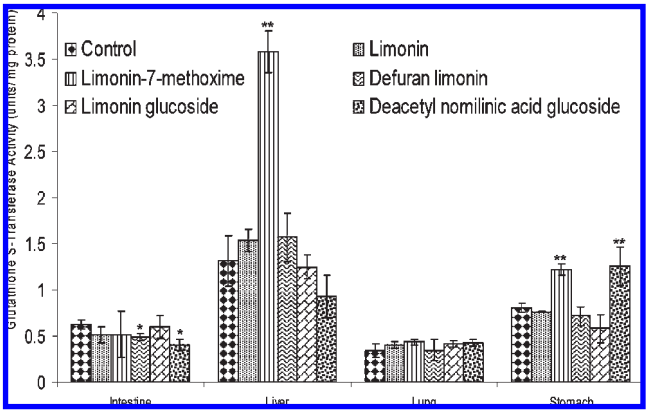

Figure 4. GST activity in different organ homogenates against 4-nitroquinoline 1-oxide, a potent xenobiotic tumorigenic to the lung, esophagus, forestomach, glandular stomach, skin, and other organs. The bars indicate the mean ± SD (n = 4). * indicates statistically significant ($p < 0.05$) decrease in activity. ** indicates statistically significant ($p < 0.05$) induction using Student's t-test.

Table 2. Quinone Reductase Activity[a]

sample	stomach	intestine	liver	lung
control	8.30 ± 1.73	2.33 ± 0.27	0.52 ± 0.04	0.26 ± 0.05
limonin	8.56 ± 1.58	2.47 ± 0.72	0.38 ± 0.10	0.29 ± 0.04
limonin-7-methoxime	10.45 ± 1.12	4.01 ± 1.15	0.85 ± 0.11**	0.34 ± 0.01**
defuran limonin	8.98 ± 0.79	2.00 ± 0.24	0.41 ± 0.02	0.38 ± 0.05**
LG	8.12 ± 2.38	2.45 ± 0.14	0.47 ± 0.06	0.31 ± 0.05
DNAG	9.58 ± 4.07	2.29 ± 0.50	0.23 ± 0.02*	0.28 ± 0.01

[a] Specific activity (units/mg protein). The results are the means ± SD (n = 4). * indicates statistically significant ($p < 0.05$) decrease of activity using Student's t-test. ** indicates statistically significant ($p < 0.05$) induction of activity using Student's t-test.

Kahweol and cafestrol, furan containing diterpenes, are reported inducers of GST activity (*28*). All of the naturally occurring citrus limonoids contain a furan moiety. Furthermore, all of the citrus limonoids tested in this study have the furan moiety present except the modified, defuran limonin. Previous studies have shown that the furan moiety plays a role in the induction of GST activity (*13*). In the current study, defuran limonin exhibited no induction in any of the GST assays. Interestingly, some induction of QR activity due to defuran limonin was observed from lung homogenates. It seems that the furan moiety may be important for the induction of phase II enzymes but not essential to chemopreventive activity.

In order to understand structural differences of the A and A′ rings of limonoids and their influence on biological activity, the induction potential of citrus limonoids with an open A ring (DNAG) and intact A and A′ rings (limonin, defuran limonin, LG, and limonin-7-methoxime) was analyzed. Previous work has indicated that modifications to the A and A′ rings produce significant differences in the ability to induce GST activity (*13*). Authors demonstrated that citrus limonoids with intact A and A′ rings (limonin, limonol, and deoxylimonin) are not active GST inducers, while ichangin, with an open A′ ring, showed induction of GST. It is clear from the previous study that citrus limonoids with only an intact A ring were responsible for most of the induction activity. Interestingly, our current results showed that limonin-7-methoxime, with intact A and A′ rings, had significant GST induction in the liver against CDNB and in the liver and stomach against 4NQO. In QR assays, limonin-7-methoxime showed induction in lung and liver homogenates and defuran limonin showed induction in the lung only. DNAG, with an open A ring also showed induction of GST activity in the lung against CDNB and in the stomach against 4NQO.

Considering modifications to the B-ring, all of the tested limonoids, except for limonin-7-methoxime, contain a ketone at the B ring. In limonin-7-methoxime, the ketone was substituted by a methoxime functional group; the rest of the limonoid structure was identical to that of limonin. While limonin did not show any induction activity in any of the enzymatic assays performed, limonin-7-methoxime showed the induction of phase II enzymes in several organ homogenates assayed. Furthermore, limonin-7-methoxime showed the induction of GST activity in the liver homogenate against CDNB. In GST against 4NQO, induction was seen in liver and stomach homogenates. Additionally, limonin-7-methoxime showed QR induction in liver and lung homogenates. Our results indicate that modification to the B ring with a methoxy group plays a very important role in the induction of phase II detoxifying enzymes, as compared to the inactive limonin. LG did not show any induction of activity in any of the assays performed.

Previous findings have reported the induction of phase II enzymes by a few citrus limonoids (*13*, *29*−*31*). Dosage studies by Lam (*13*) and Kelley (*30*) have used 1−20 mg of limonoids for the animal studies. The concentration of limonoids in the citrus fruit depends upon the variety of fruit. According to literature, limonin glucoside and deacetyl nomilin glucoside are present to the extent of 500 and 200 ppm, respectively (*15*, *32*). Hence, we have used 20 mg treatments for our studies.

In these studies, GST activity was tested against 1-chloro-2,4-dinitrobenzene (CDNB), which is a commonly used substrate for a variety of GST isozymes (*33*). One study suggests that the μ GST isozyme optimally conjugates 4-nitroquinoline 1-oxide (4NQO) (*34*). Most of the GST isozymes found in mammalian tissues are grouped under four major classes, namely, α, μ, π, and θ (*35*, *36*). Because of the variability of these enzymes in different mammalian tissues, it is important to explore how citrus limonoids affect the activity of GST enzymes.

The induction of phase II enzymes by citrus limonoids can potentially inhibit carcinogenesis by conjugating harmful substances into more water-soluble forms. The increase in polarity of these electrophilic substances facilitates their excretion from the body. Hence, by enhancing GST activity, more of the potentially carcinogenic xenobiotics can be expelled from the body through urine. Furthermore, it has been reported that plant-derived phase II enzyme inducers may be potentially important in the incidence of age-related macular degeneration (*37*). Phase II enzymes are important in the prevention of age-related degenerative conditions. Induction of phase II enzymes helps in the elimination of reactive oxygen species, which accumulate with age. Therefore, citrus limonoids could be considered as a potential antiaging agent by inducing the activity of GST and QR. Even though many studies have been performed on the health benefits of citrus limonoids, few studies have focused on the bioavailability of these valuable compounds in humans (*38*). Further studies are needed

to determine the fate of the limonoid metabolite to elucidate their precise role in human health.

In conclusion, citrus limonoids with different structural characteristics were evaluated to understand their detoxification potential. To the best of our knowledge, this is the first study to report on the induction of GST using 4NQO as a substrate and QR. Additionally, defuran limonin and limonin methoxime were also examined for the induction of the phase II enzymes GST and QR. The ability of these compounds to induce the activity of detoxifying phase II enzymes makes them valuable bioactive compounds in the quest to prevent cancer, antiaging, and oxidative related diseases and deserves more in-depth research in order to improve human health.

ABBREVIATIONS USED

BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; DCPIP, 2,6-dichlorophenol-indophenol; DMSO, dimethyl sufoxide; DNAG, deacetylnomilinic acid glucoside; EtOAc, ethyl acetate; FAD, flavin adenine dinucleotide; GSH, glutathione reduced; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; LG, limonin glucoside; 4NQO, 4-nitroquinoline 1-oxide; PBS, phosphate buffer solution; QR, quinone reductase; NADPH, nicotanamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; MeOH, methanol; TLC, thin layer chromatography; TMS, tetramethylsilane.

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Supporting Information Available: HPLC chromatograms of purified citrus limonoids and modified compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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