# Red Mexican Grapefruit: A Novel Source for Bioactive Limonoids and their Antioxidant Activity

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Citrus limonoids have shown to inhibit the growth of cancer in colon, lung, mouth, stomach and breast in animal and cell culture studies. For the first time in the present study, an attempt has been made to isolate antioxidant fractions and five limonoids from red Mexican grapefruit seeds. Defatted seed powder was successively extracted with hexane, ethyl acetate (EtOAc), acetone, methanol (MeOH) and MeOH/water and the extracts were concentrated under vacuum. Radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and total phenolic content were also measured for comparison with the antioxidant capacity in the phosphomolybdenum method for the above extracts. Acetone and MeOH extracts, respectively, showed the highest (85.7%) and lowest (53.3%) radical scavenging activity, at 500 ppm. The total phenolic contents were found to be highest in the acetone extract (15.94%) followed by the MeOH extract (5.92%), ethyl acetate extract (5.54%) and water extract (5.26%). Antioxidant capacity of the extracts as equivalents to ascorbic acid ($\mu$mol/g of the extract) was in the order, EtOAc extract > acetone extract > water extract > methanol extract. Furthermore, the EtOAC and acetone extracts were loaded onto silica gel columns to obtain four limonoid aglycons. MeOH fraction was loaded onto a dowex-50 and sepabeads resin column to obtain a limonoid glucoside. The purity of the isolated five compounds was analyzed by HPLC using a $C_{18}$ column and UV detection at 210 nm. Finally, the structures of the compounds were identified as obacunone, nomilin, limonin, deacetylnomilin (DAN) and limonin-17-$\beta$-D-glucopyranoside (LG) using $^{1}$H and $^{13}$C NMR studies.

*Key words:* Grapefruit, Limonoids, Antioxidant Activity, Phosphomolybdenum Method

## Introduction

Citrus limonoids are an established group of triterpenoids found in the Rutaceae family. They are present in mature citrus fruit tissue and seeds as aglycon and glucoside derivatives (Hasegawa *et al.,* 1989). Certain limonoid aglycons are bitter (Emerson, 1948) while the limonoid glucosides are non-bitter (Bennet *et al.,* 1989), tasteless, odorless and water-soluble, without any significant toxic effects (Miller *et al.,* 1999). Among the glucosides, limonin glucoside has shown to prevent cancer in hamster buccal pouches by decreasing the average tumor burden by 50–60% (Miller *et al.,* 1992). Our previous studies have shown that certain aglycons such as limonin, nomilin and limonoid glucoside mixtures have shown to inhibit the growth of human breast cancer using cell cultures (Tian *et al.,* 2001). Limonoid aglycons, obacunone and limonin (Fig. 3) were found to inhibit azoxymethane-induced colon cancer in rats (Tanaka *et al.,* 2000). Another of our studies provided *in vivo* evidence for the potential role of grapefruit pulp and its functional components against colon cancer by lowering the proliferative index (Vanamala *et al.,* 2006). The furan moiety attached to the D-ring lactone in the structure of limonoids is believed to be responsible for enhancing the activity of a detoxifying enzyme, glutathione-*S*-transferase (GST) (Lam *et al.,* 1989). This induction inhibits the formation of tumors in laboratory animals (Miller *et al.,* 1989). Recent results from our laboratory demonstrated that certain citrus limonoid glucosides have the ability to induce caspase 3/7 activity, suggesting that limonoid glucosides and

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aglycons were capable of inducing apoptosis (Poulose *et al.,* 2005, 2006, 2007). Yu *et al.* (2005) reported the antioxidant activity of limonoids, flavonoids and coumarins in *in vitro* model systems. However, antioxidant activity of limonoids was found to be weak when compared to flavonoids. There are several reports on the isolation and identification of limonoids and flavonoids from citrus by column chromatography and preparative HPLC (Hasegawa *et al.,* 1989; Emerson, 1948; Bennet *et al.,* 1989*;* Tanaka *et al.,* 2000; Jayaprakasha *et al.,* 2006a, b). Recently, Raman *et al.* (2004, 2005) reported the isolation of limonoids and flavonoids by flash chromatography. However, this method cannot achieve more yields of limonoids and flavonoids.

Epidemiological studies have indicated that dietary intake of antioxidant substances from plants is inversely associated with mortality from coronary heart disease (Giugliano, 2000). Although most antioxidant intake, such as vitamin E, vitamin C, polyphenols, $\beta$-carotene, lutein, and zeaxanthin, is from familiar plant sources, there are additional plant sources generally less well known. In general, conventional methods such as DPPH and Folin-Ciocalteau methods are used for determining the antioxidant activity of plant extracts and phenolic content, respectively (Parejo *et al.,* 2002). Color development using a Folin-Ciocalteau assay is the generally preferred method for measuring phenolics because most plant-derived antioxidants contain large amounts of polyphenols. Free radical scavenging activity is also used for measuring the antioxidant activity of edible plants with such activity varying according to radical species. Measurement of radical scavenging activity using the discoloration of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH radical scavenging assay) has been widely used due to its stability, simplicity, and reproducibility (Parejo *et al.,* 2002).

Currently the relative nontoxicity and anticarcinogenic properties of citrus limonoids have created a markedly great demand for their isolation and purification. Most of the published literature on citrus bioactive compounds concerns the quantification of limonoids using TLC and HPLC (Fong *et al.,* 1989; Herman *et al.,* 1990; Ozaki *et al.,* 1992*;* Schoch *et al.,* 2002; Ishii *et al.,* 2003). However, this published data suggests that certain citrus limonoids are present in low concentrations and may vary markedly among the different citrus varieties and byproducts.

Red Mexican grapefruit, a seedy red-fleshed, red-rinded type which occurs in variety plantings in Florida and Texas and is listed in the USDA-plant introduction series as number 113535, was imported from Mexico as bud wood in 1936 (Cameron *et al.,* 1964). Tart and tangy with an underlying sweetness, this grapefruit has a juiciness that rivals that of the ever-popular orange and sparkles with many of the same health promoting benefits. Although available throughout the year, red Mexican grapefruits are in season and at their best from winter through early spring (Davies and Albrigo, 1994). Literature survey revealed that there are no reports on the isolation of individual citrus limonoids and their yields from red Mexican grapefruits. For the first time the present study aimed on the isolation of fractions from red Mexican grapefruit seeds, which have antioxidant activity. The active fractions were purified using column chromatography to obtain five limonoids and their structures were characterized using NMR studies. To the best of our knowledge, this is the first report on antioxidant activity and chemical constituents from red Mexican grapefruit.

## Materials and Methods

### *Chemicals*

All solvents/chemicals used were of analytical grade and obtained from EM Science (NJ, USA). Silica gel (200–400 mesh), dowex-50 (50–100 mesh) were purchased from Aldrich (MO, USA). Sepabeads adsorbent resin was purchased from Supelco (PA, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyl-toluene (BHT), Folin-Ciocalteau reagent, ascorbic acid, *para-N,N*-dimethyl amino benzaldehyde and catechin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). TLC plates, silica gel 60 F-254, with thickness 0.20 mm (20 × 20 cm), were obtained from Alltech Associates, Inc. Visible spectra measurements were done using a spectrophotometer (Genesys-20 visible, Milton Roy, NY, USA). $^{1}H$ and $^{13}C$ NMR spectra were recorded at 300 and 75 MHz, respectively, on a JEOL NMR instrument (JEOL USA, Inc., MA, USA). TMS was used as internal standard.

### *Plant material*

Red Mexican grapefruits were harvested in late December 2003 from the orchard of Texas A & M University, Kingsville, Citrus Center, Weslaco,

Texas, USA. The seeds were separated from the fruit manually and dried under shade at 25 °C. The dried seeds were then powdered using a blender.

*Extraction*

Ground red Mexican grapefruit seed powder (4.6 kg) was extracted in a Soxhlet apparatus with hexane for 24 h for the removal of fatty matter. The defatted seeds were extracted in sequence for 8 h each with ethyl acetate (EtOAc), acetone, methanol (MeOH) and water at 60–70 °C. The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) to get a viscous concentrate which was freeze-dried.

*Determination of total phenolics*

The concentration of phenolic compounds in the red Mexican grapefruit extracts was determined according to our method described previously (Negi and Jayaprakasha, 2003) and results were expressed as catechin equivalents. The citrus seed extracts were dissolved in a mixture of MeOH and water. Different concentrations of the standard (+)-catechin and extracts were taken in test tubes and the volume was adjusted to 0.2 ml by addition of distilled water. 1 ml of 10-fold diluted Folin-Ciocalteau reagent and 0.8 ml of 7.5% sodium carbonate solution were added to all the tubes. After the mixture had been allowed to stand for 30 min at room temperature, the absorbance was measured at 765 nm using a Genesys-5 UV-visible spectrophotometer. The estimation of phenolics in all the extracts was carried out in triplicate and the mean results are presented.

*Radical scavenging activity using the DPPH method*

Various quantities (70, 175 and 350 $\mu$l equivalent to 100, 250 and 500 ppm) of ethyl acetate, acetone, MeOH, water extracts and ascorbic acid were taken in different test tubes. The volume of the samples and ascorbic acid was adjusted to 500 $\mu$l by adding MeOH. 3 ml of a methanolic 100 $\mu$m DPPH solution were added and shaken vigorously and the tubes were allowed to stand at 27 °C for 20 min (Singh *et al.*, 2002). The control was prepared as explained above without samples/standards and MeOH was used for the baseline correction. The changes in the absorbance of all the samples and standards were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage; it was calculated using the following formula: % radical scavenging activity = (control optical density – sample optical density/control optical density) × 100.

*Evaluation of antioxidant capacity by the phosphomolybdenum method*

Antioxidant capacity of extracts of ethyl acetate, acetone, MeOH, water and BHT were evaluated by the method of Prieto *et al.* (1999). An aliquot of 0.1 ml of sample solution and BHT (equivalent to 200 and 400 ppm) was combined with 1 ml of reagent solution (0.6 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of the blank, 0.1 ml of MeOH was used in place of the sample. The tubes were incubated in a boiling water bath at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance of each aqueous solution was measured at 695 nm against the blank in the spectrophotometer. For samples of unknown composition, water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid ($\mu$mol/g).

*Purification of ethyl acetate and acetone extract*

Freeze-dried ethyl acetate and acetone extracts were loaded onto a silica gel column (100 cm × 35 mm). The column was washed thoroughly with hexane and eluted with a linear gradient solvent of 1% ethyl acetate in hexane to 85% ethyl acetate in hexane. Fractions (500 ml) collected at a flow rate of 10 ml/min were then concentrated under vacuum (Büchi). Compounds **1**, **2**, **3** and **4** were eluted with hexane/EtOAc (60:40), (55:45), (50:50) and (45:55), respectively, and the yields obtained were 392.5, 1895.0, 41.2 and 305.0 mg.

*Purification of MeOH and MeOH/water extract*

Freeze-dried MeOH and water extracts were mixed and loaded onto an activated dowex [$H^+$] resin column. The column was washed thoroughly with excess water. Elute from the dowex column was passed through a sepabeads resin (120 cm × 10 cm) which was then eluted with a linear gradient solvent of 1% MeOH in water to 15% MeOH in water. Fractions (each 1000 ml) were collected at a flow rate of 30 ml/min. All the fractions were analyzed by HPLC. Fractions containing similar peaks were pooled and concentrated under vacuum. The concentrated fractions were stored for crystallization at 3–4 °C. Fraction eluted with

7.5% MeOH in water gave compound **5**. The compound was collected by filtration and was dried in vacuum desiccators to obtain pure compound **5** with a yield of 4.932 g.

*TLC analysis*

Purified compounds **1**–**5** were spotted on silica gel 60 F-254 plates. The plates were developed using dichloromethane/acetone (98:2) and EtOAc/methyl ethyl ketone/water/formic acid (5:3:1:1) for aglycons and glucosides, respectively. The plates were sprayed with Ehrlich's reagent (2% *N*,*N*-dimethyl amino benzaldehyde in EtOH) and developed in a HCl gas chamber. Typical pink/reddish-colored spots were obtained for limonoids (Maier and Edward, 1970). In addition, the plates were sprayed with 10% sulfuric acid in MeOH followed by heating at 100 °C for 10 min.

*High performance liquid chromatography (HPLC) analysis*

The HPLC system used for analysis of compounds in the present study consisted of a Spectra System Model P-4000 instrument (Thermo Separation Products, San Jose, CA,USA). It was equipped with a quaternary HPLC pump, fitted with a Waters Prevail C18 analytical column (15 cm × 4.6 mm I. D., 5 microns particle size; Alltech, Deerfield, IL, USA). The auto-injection system used was a 20 $\mu$l sample loop (Spectra System AS 3000, Thermo Separation Products). Detection was done by a UV 6000 LP wavelength detector at the wavelength of 210 nm. All the compounds **1**–**5** were quantified using ChromQuest software. All the column fractions and compounds **1**–**5** were filtered through a 0.45 $\mu$m filter and then subjected to HPLC analysis. The gradient mobile phase used for compound **5** consisted of (A) 10% acetonitrile in water and (B) 24% acetonitrile in water at a flow rate of 1.0 ml/min. Compounds **1**–**4** were eluted using an isocratic mobile phase, *i.e.* 40% acetonitrile in water for 30 min, with a flow rate of 1 ml/min. Compounds were detected at 210 nm. The elution program involved a linear gradient from 0 to 100% of solvent A to B within 0 to 30 min, 30 to 35 min 100 to 0% B to A and an isocratic run from 35 to 40 min followed by 5 min of equilibrium with 100% A.

*Nuclear magnetic resonance (NMR)*

The structures of the isolated compounds **1**–**5** were characterized and identified as obacunone, limonin, nomilin, deacetylnomilin and limonin glucoside, respectively, using $^{1}H$ and $^{13}C$ NMR data, and the chemical shifts were compared with reported values (Tables II and III).

## Results and Discussion

Defatted citrus seed powder was extracted in a Soxhlet extractor with ethyl acetate, acetone, MeOH and water to obtain crude fractions. Table I shows the yield and phenolics present in different red Mexican grapefruit seeds. Extraction with MeOH and EtOAc gave a maximum and minimum yield, respectively, whereas acetone extract and water extract showed maximum and minimum phenolics, respectively. Generally hexane was used for the extraction of non-polar compounds like fatty materials, while EtOAc extracted some of the medium polar compounds. The acetone, MeOH and MeOH/water (80:20) mixture solutions were used for the extraction of medium polar and polar compounds like aglycons and glucosides of flavonoids and limonoids depending upon their polarity.

Free radical scavenging potentials of red Mexican grapefruit extracts and ascorbic acid at 100, 250 and 500 ppm were tested by the DPPH method and the results are presented in Fig. 1. In this method, antioxidants react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and is converted to 1,1-diphenyl-2-picrylhydrazine due to the hydrogen donating ability of antioxidants at a very rapid rate (Bondet *et al.,* 1997). The level of discoloration indicates the scavenging potentials of the extracts. At 500 ppm, acetone extract and ascorbic acid showed 85.7 and 95% free radical scavenging activity, respectively. The different antioxidant activity of the fractions was due to their hydrogen do-

Table I. Percentage of yield and phenolics in red Mexican grapefruit seeds.

| Solvent | Yield [g/100 g of seeds] | Phenolics [g/100 g of extract] |
|---|---|---|
| EtOAc | 1.27 | 5.54 ± 0.42 |
| Acetone | 2.06 | 15.94 ± 1.55 |
| MeOH | 6.00 | 5.92 ± 1.01 |
| Water | 1.46 | 5.26 ± 0.34 |

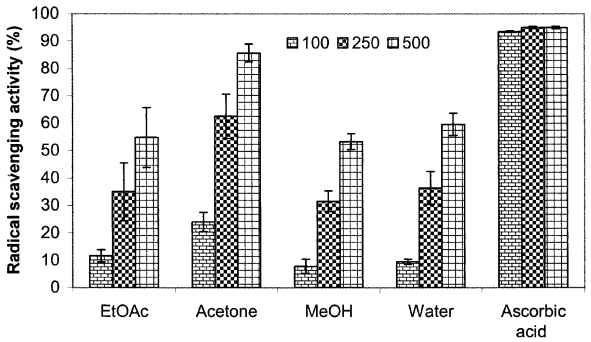


Fig. 1. Radical scavenging activity of red Mexican seed extracts at different contents (ppm).

nating ability. Antiradical antioxidants act by donating hydrogen atoms to lipid radicals. Radicals obtained from antioxidants with molecular structures such as phenols, limonoids, carotenoids, ascorbic acid are stable species and will stop the oxidation chain reaction (Pedrielli *et al.,* 2001). Furthermore, there is now growing evidence that polyphenols may also possess inhibitory effects on cancer (Mukai *et al.,* 2000). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of lipids (Sherwin, 1978; Jayaprakasha *et al.,* 2004; Jayaprakasha and Patil, 2007). The data obtained revealed that the red Mexican grapefruit seed extracts are free radical scavengers and primary antioxidants that react with DPPH radicals, which may be attributed to their proton donating ability.

The phosphomolybdenum method is based on the reduction of molybdenum(VI) to molybdenum(V) by the antioxidant compounds and the formation of a green molybdenum(V) complex, which has a maximal absorption at 695 nm. The different red Mexican grapefruit seed extracts exhibited various degrees of antioxidant capacity. It is difficult to ascertain an order of antioxidant capacities of different extracts because of the different responses. Maximum antioxidant capacities were observed in the EtOAc extract. The order of antioxidant capacity (equivalent to trolox) of EtOAc, acetone, water, MeOH extracts were found to be (7754.5 ± 219), (7172 ± 135), (5929 ± 152), (4130.5 ± 220), respectively, whereas BHT showed (6482.4 ± 148) at the same tested concentration. Variations in antioxidant capacity of different extracts may be attributed to the differences in their chemical composition such as phenolics, limonoids, ascorbic acid and carotenoids. Our recent results also indicated that certain citrus limonoids were found to possess good antioxidant activity (Patil *et al.,* 2004). Gorinstein *et al.* (2004) reported the antioxidant activity of the citrus fruit extracts and correlated the activity to the presence of flavonoids, carotenoids and ascorbic acid. More recently, Sun *et al.* (2005) reported antioxidant capacities of limonin and nomilin in four mature fruit tissues, determined by the $\beta$-carotene bleaching method. The antioxidant capacities of limonin and nomilin varied in different tissues and cultivars. In the three tissues other than albedo, the antioxidant capacities of limonin and nomilin were higher (2.9–8.3 times) than that of ascorbic acid. On the basis of these considerations, evaluation of antioxidant activity of different solvent extracts and their compounds from red Mexican grapefruit was undertaken.

In order to explore potential bioactive components responsible for the antioxidant activity, the individual fractions were analyzed by TLC and HPLC for their composition and were subjected to column chromatography. The ethyl acetate and acetone extracts showed 4–5 spots over TLC with different concentrations. Hence, both the extracts were mixed and loaded for column chromatography over silica gel. The column was eluted with hexane, mixtures of hexane/EtOAc and EtOAc to obtain four limonoids. Compounds **1**, **2**, **3** and **4** were eluted with hexane/EtOAc (60:40), (55:45), (50:50) and (45:55 v/v), respectively, and yielded 392.5 mg, 1895.0 mg, 41.2 mg and 305.0 mg. Similarly, HPLC analysis of MeOH and water extracts showed 5–6 peaks with different concentrations. Hence, they were mixed and loaded onto dowex-50 [$H^+$] resin and the column was eluted with excess water. The water fraction was concentrated and loaded to the synthetic adsorbent divinyl benzene. Furthermore, the column was eluted with water, mixtures of MeOH/water (80:20) and MeOH. All the fractions were analyzed by TLC and HPLC for their compositions. Fractions containing similar compositions were pooled, concentrated under vacuum and stored for crystallization at 3–4 °C. Fractions eluted with MeOH/water (80:20) yielded compound **5** with a yield of 4932.0 mg. Finally, purity of all the compounds was analyzed by TLC and HPLC. TLC analysis of compounds **1**, **2**, **3**, **4** and **5** gave the mobilities

0.74, 0.46, 0.58, 0.28 and 0.32, respectively, which matched to reported $R_f$ values (Hasegawa and Berhow, 2000). No additional spots were visualized on both plates with either Ehrlich reagent or methanolic sulfuric acid followed by heating at 100 °C for 10 min, thus confirming the purity of the isolated compounds. Furthermore, the purity of the isolated compounds was confirmed by HPLC using isocratic and gradient systems for aglycons and glucosides, respectively. Fig. 2 depicts HPLC chromatograms of limonoid aglycones and glucoside such as **1**–**4** and **5**, respectively. The identities of obacunone, limonin, nomlin, deacetylnomilin and limonin glucoside were confirmed by relative retention times of authentic standards. Further the structures of the isolated compounds have been elucidated and confirmed by NMR

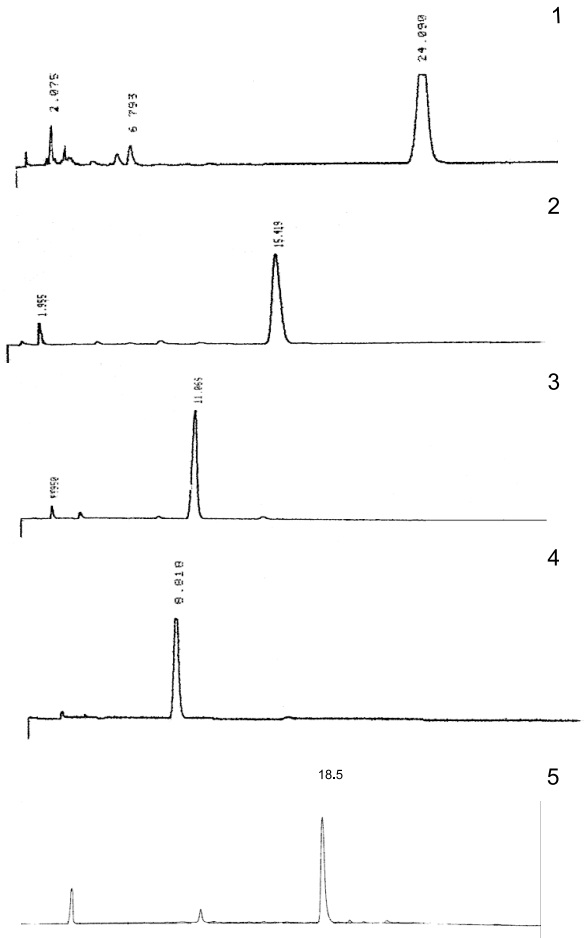


Fig. 2. HPLC separation of compounds **1**–**4** using an isocratic mobile phase and of compound **5** using a gradient mobile phase.

Table II. $^{13}$C NMR data of compounds **1**, **2**, **3**, **4** and **5**.

| C | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| 1 | 156.9 | 78.8 | 70.8 | 68.9 | 80.5 |
| 2 | 122.9 | 36.1 | 35.3 | 39.5 | 36.9 |
| 3 | 166.8 | 170.3 | 169.2 | 171.4 | 171.1 |
| 4 | 84.1 | 79.5 | 84.5 | 84.4 | 78.3 |
| 5 | 57.3 | 58.4 | 51.1 | 49.9 | 55.8 |
| 6 | 39.9 | 36.6 | 38.9 | 39.2 | 36.8 |
| 7 | 207.6 | 208.1 | 206.9 | 209.3 | 208.1 |
| 8 | 53.0 | 50.3 | 52.9 | 52.6 | 50.6 |
| 9 | 49.3 | 46.9 | 44.2 | 44.3 | 45.3 |
| 10 | 43.2 | 45.3 | 44.4 | 44.8 | 45.7 |
| 11 | 19.5 | 19.7 | 16.7 | 17.3 | 17.0 |
| 12 | 32.8 | 29.3 | 32.2 | 31.9 | 26.8 |
| 13 | 37.5 | 37.7 | 37.5 | 36.8 | 45.2 |
| 14 | 65.2 | 64.9 | 65.5 | 66.3 | 70.9 |
| 15 | 53.3 | 54.1 | 53.4 | 53.2 | 57.6 |
| 16 | 167.1 | 167.4 | 166.8 | 167.8 | 170.5 |
| 17 | 78.1 | 77.9 | 78.1 | 78.1 | 77.2 |
| 18 | 21.2 | 17.6 | 17.1 | 20.8 | 23.9 |
| 19 | 17.1 | 66.7 | 17.2 | 16.7 | |
| 20 | 120.1 | 120.3 | 120.1 | 16.5 | 126.1 |
| 21 | 143.2 | 143.4 | 143.3 | 120.8 | 141.7 |
| 22 | 109.8 | 110.2 | 109.7 | 143.9 | 113.1 |
| 23 | 141.1 | 141.7 | 141.1 | 110.8 | 141.1 |
| $CH_3$ | 32.1 | 17.1 | 20.8 | 142.2 | 30.9 |
| $CH_3$ | 26.9 | 29.8 | 33.5 | 20.1 | 25.6 |
| $CH_3$ | | 21.4 | 23.4 | 33.6 | 22.2 |
| $CH_3$-CO- | | | 169.2 | 23.6 | 19.5 |
| $CH_3$ | | | 20.8 | | 17.0 |
| Glu-1 | | | | | 105.1 |
| Glu-6 | | | | | 61.5 |
| Glu-2 | | | | | 78.1 |
| Glu-3 | | | | | 76.9 |
| Glu-4 | | | | | 74.4 |
| Glu-5 | | | | | 70.5 |

studies. The $^1$H and $^{13}$C NMR chemical shifts (Tables II and III) were matched against reported values (Manners *et al.,* 2000).

Recent results from our group and others indicated that some of the citrus limonoids are found to possess good antioxidant activity (Yu *et al.*, 2005; Patil *et al.,* 2004; Sun *et al.,* 2005). Hence, in the present study the antioxidant activity have not been studied for the isolated compounds **1**–**5**. A typical limonoid structure as illustrated consists of five rings termed as A, A′, B, C and D (Fig. 3). Nomilin structure differs from limonin in the absence of ring A′. Obacunone and deacetylnomilin are similar in structure to nomilin except changes in the A ring at C-1 and C-2. Maturation of fruit triggers glucosidation of the D ring in limonin resulting in the formation of LG (Hasegawa and Berhow, 2000). Previous studies proved that the intact A ring in limonin, limonin glucoside, nomi-

Table III. $^1$H NMR data of compounds **1**, **2**, **3**, **4** and **5**.

| H | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| 1 | 6.52 (d, 1H) | 4.10 (br.s, 1H) | 4.07 (dd) | 3.63 (t) | 3.47 (d, 1H) |
| 2 | 5.95 (d, 1H) | 2.26 (dd, 14.8; 3.2 Hz)<br>2.65 (dd, 14.8; 3.2 Hz) | 3.16 (dd) | 2.98 (t, 2.0 Hz);<br>3.30 (dd, 14.0; 2.0 Hz) | |
| 5 | 2.58 (dd, 1H) | 2.47 (dd, 15.0; 2.8 Hz) | 2.42 (dd) | 2.42 (dd, 15.0; 2.8 Hz) | |
| 6 | 2.25 (dd, 1H);<br>2.96 (t, 1H) | 2.75 (dd, 15.0; 2.8 Hz);<br>3.10 (t, 15.0 Hz) | 2.75 (dd) | 2.28 (dd, 15.0; 2.8 Hz);<br>2.65 (t, 15.0 Hz) | |
| 15 | 3.64 (s, 1H) | 4.09 (s) | 3.76 (s, 1H) | 3.75 (s) | 4.85 (d, 1H, 10.7 Hz) |
| 17 | 5.43 (s, 1H) | 5.45 (s) | 5.41 (s, 1H) | 5.35 (s) | 5.18 (s, 1H) |
| 18 | 1.47 (s, 3H) | 1.09 (s, 3H) | 1.98 (s, 3H) | 1.98 (s, 3H) | |
| 19 | 1.42 (s, 3H) | 4.46 (d, 13.0 Hz);<br>4.90 (d, 13.0 Hz) | 1.14 (s, 3H) | 1.12 (s, 3H) | 1.34 (s, 3H) |
| 21 | 7.39 (dd, 1H) | 7.63 (br.s, 1H) | 7.37 (br.s, 1H) | 7.62 (br.s, 1H) | 7.50 (d, 7.4 Hz) |
| 22 | 6.33 (d, 1H) | 6.48 (br.s, 1H) | 6.30 (br. s, 1H) | 6.65 (br.s, 1H) | 6.51 (s, 1H) |
| 23 | 7.39 (dd, 1H) | 7.69 (br.s, 1H) | 7.37 (br.s, 1H) | 7.71 (br.s, 1H) | 7.50 (d, 7.4 Hz) |
| 24 | 1.21 (s, 3H) | 0.98 (s) | 1.15 (s, 3H) | 1.09 (s) | 0.94 (s, 3H) |
| 25 | 1.47 (s, 3H) | 1.17 (s, 3H) | 1.43 (s, 3H) | 1.26 (s, 3H) | 1.21 (s, 3H) |
| 26 | 1.09 (s, 3H) | 1.01 (s, 3H) | 1.52 (s, 3H) | 1.44 (s, 3H) | 0.63 (s, 3H) |
| Me-CO | | | 2.01 (s, 3H) | | |
| Sugar<br>H-1 | | | | | 4.85 (br.d, 2H) |
| | | | | | 2.66 (1H)<br>2.45 (1H)<br>3.34 (d, 1H)<br>3.35 (s, 1H)<br>2.65 (d, 1H)<br>1.61 (t, 1H) |

lin and obacunone (Fig. 3) inhibited the activity of average tumor burden in 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch tumors (Miller *et al.,* 1989). Nomilin, obacunone and limonin, in decreasing order, have shown to be potent inducers of a detoxifying enzyme system, glutathione-*S*-transferase, leading to the inhibition of chemically induced carcinogenesis (Lam *et al.,* 1989), because of the presence of a furan moiety at 3$^{rd}$ position of the D ring lactone combined with the differences in the A and A′ ring of their chemical structures. Another study reports obacunone and limonin to be active as inhibitors of preneoplastic lesions in the rat colon, with an induction of liver GST (Tanaka *et al.,* 2000). In addition to the human health benefits, citrus limonoids have also shown to possess certain chemical activity in the control of specific insect pests. Obacunone and nomilin due to the presence of a 7-membered lactone ring(A ring) have shown to inhibit moulting in mosquito (*Culex quinquefasciatus*), when compared to limonin suggesting their potentiality in the formulation of eco-friendly pesticides (Jayaprakasha *et al.,* 1997). Another study reported high significant antifeedent activity of limonin followed by obacunone in a feeding study conducted with *Spodoptera frugiperda* larvae, which may be attributed to the chemistry of the C-7 carbonyl and furan ring present in their structures (Ruberto *et al.,* 2002).

There is a markedly growing demand for bioactive citrus limonoids due to their significant potential of health promoting properties and structure-activity relationship. Because of difficulties involved in scaling up of techniques for the isolation of limonoids in sufficient quantities needed for certain biological studies the task still remains a big challenge. In this context, studies exploring new sources and methods of isolation of the citrus limonoids are very critical. Very few reports are available on the yields of limonoids from citrus seeds. In the present study, our results provide isolation techniques for five limonoids with their respective yields. Furthermore the present results suggest that red Mexican grapefruit is a valid and good source of limonin and limonin glucoside in addition to obacunone, nomilin and deacetylnomilin. To our knowledge, this is the first report on the isolation of limonoids from red Mexican grapefruit seeds.

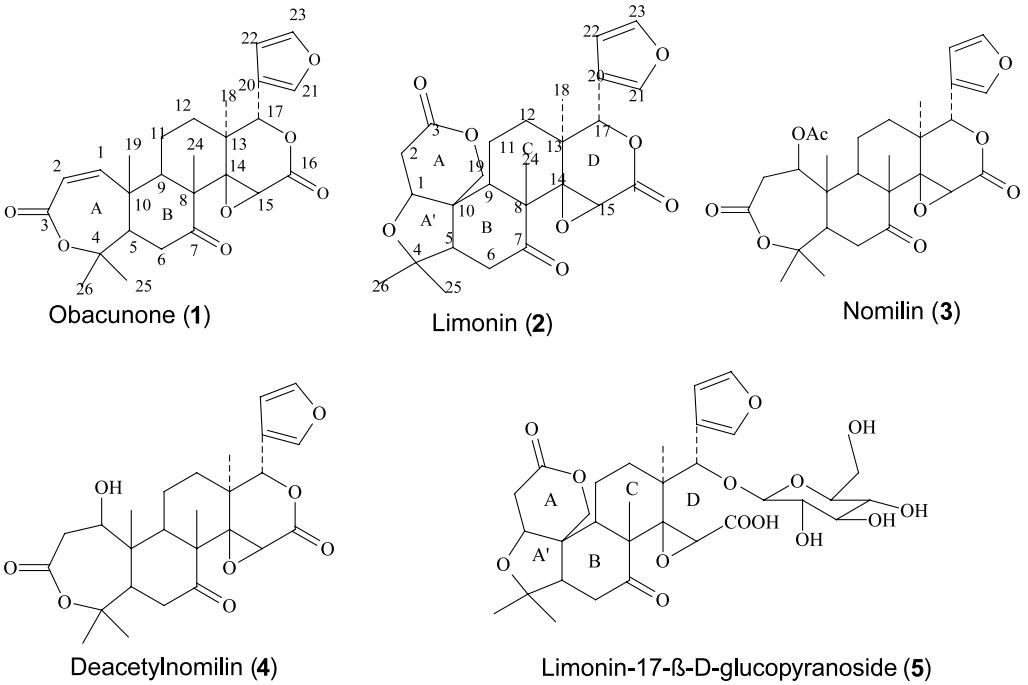


Fig. 3. Structures of limonoids isolated from red Mexican grapefruit seeds.

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