

# New triterpenoid acyl derivatives and biological study of *Manilkara zapota* (L.) Van Royen fruits

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## ABSTRACT

$\beta$ -amyrin-3-(3'-dimethyl) butyrate, a new natural compound was isolated from the fruits of *Manilkara zapota* (L.) Van Royen, in addition to lupeol-3-acetate and 4-caffeoylquinic acid (cryptochlorogenic acid). The structures of these compounds were identified using different spectral methods (IR, MS, UV,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and 2D-NMR). The alcoholic and aqueous extracts of the unripe fruits, in addition to their aqueous homogenate exhibited antioxidant, antihyperglycemic and hypocholesterolemic activities.

**Key words:** 4-caffeoylquinic acid, antihyperglycemic, antioxidant,  $\beta$ -amyrin-3-(3'-dimethyl) butyrate, hypocholesterolemic, lupeol-3-acetate, *Manilkara zapota*

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## INTRODUCTION

Different classes of plant constituent were isolated from the different organs of *Manilkara zapota* (L.) Van Royen.<sup>[1]</sup> The fruits, mainly unripe ones, were found to be rich with polyphenolic compounds (tannins and flavonoids).<sup>[2-4]</sup> Also, triterpenes<sup>[5]</sup> were previously isolated from the fruits. They exhibited antioxidant activity mainly due to their polyphenolic content.<sup>[6-8]</sup> In this work, the fruits were subjected to further chemical study. Also, the alcoholic and aqueous extracts and the aqueous homogenate of unripe fruits were tested for antioxidant, antihyperglycemic and hypocholesterolemic effects.

## MATERIALS AND METHODS

### General experimental

IR spectra were run in KBr using Perkin-Elmer infrared spectrophotometer FT-IR 1650. Mass spectrometer, Varian Mat 711 (USA), Finnigan SSQ 7000 was used for EI/MS.  $^1\text{H}$ -(300 MHz) and  $^{13}\text{C}$ -(75 MHz) NMR spectra were recorded on Varian Mercury apparatus at 25°C using TMS as an internal standard and chemical shifts were

given in  $\delta$  (ppm) values. Precoated silica gel plates 60 F 254 (E-Merck) were used for TLC with  $S_1$  (Pet. ether:  $\text{CHCl}_3$  [7:3 v/v]),  $S_2$  (Pet. ether:  $\text{CHCl}_3$  [1:2 v/v]) and  $S_3$  (EtOAc: MeOH: Formic acid [5:1:2 v/v/ drops]) as solvent systems. The chromatograms were visualized under UV light (at  $\lambda_{\text{max}}$  254 and 366 nm) before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde/sulphuric acid spray reagent.

### Plant material

Fresh fruits of *Manilkara zapota* (L.) Van Royan was supplied through the Horticulture Research Centre, Ministry of Agriculture, Giza in January 2007 and kept frozen at -4°C. Identity was verified by Prof. Dr. Mohammed El-Sayed, Vice Head of the same institute. Voucher specimens have been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

### Extraction

The frozen fruits (2 kg) were exhaustively extracted by cold percolation with 70% ethanol. The solvent was then evaporated under reduced pressure, at a temperature not exceeding 50°C, to yield 300 g of a semisolid dark brown residue. An aliquot (100 g) of the ethanol extractive obtained was suspended in distilled water (500 ml) and successively partitioned with petroleum ether (4 × 500 ml), methylene chloride (3 × 500 ml), ethyl acetate (3 × 500 ml) and *n*-butanol (4 × 500 ml).

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The petroleum ether, methylene chloride, ethyl acetate and *n*-butanol extracts were evaporated under reduced pressure to yield 8, 2.1, 10.6 and 10.6 g, respectively, of the corresponding extractives. TLC of both the petroleum ether and methylene chloride fractions showed identical profiles, thus the two fractions were pooled together.

### Fractionation and isolation

The combined petroleum ether and methylene chloride fractions of the alcoholic extract of the fruits (10.1 g) was chromatographed on a silica gel 60 column (2.4 × 20 cm). Gradient elution was carried out starting with petroleum ether and increasing the polarity by 5% stepwise addition of methylene chloride till 100% methylene chloride then increasing the polarity by 5% stepwise addition of ethyl acetate till 100% ethyl acetate. Fraction 200 ml each were collected to obtain 41 fractions. Similar fractions were combined together. Fractions (15-25) and (31-38) after evaporation under reduced pressure and recrystallization from chloroform afforded compounds 1 (50 mg) and 2 (30 mg), respectively.

The *n*-butanol fraction (12 g), was chromatographed on a column of sephadex LH-20 (2.5 × 20 cm). Elution was carried out with water, followed by 10% stepwise addition of methanol till 100% methanol to give 11 fractions, each of 100 ml. Fraction (1) upon evaporation under reduced pressure yielded compound 3 (90 mg).

#### Compound 1

White powder, soluble in chloroform,  $R_f = 0.38$  ( $S_1$ ). IR  $\nu_{\max}$  (KBr)  $\text{cm}^{-1}$  2933 (-C-H stretching vibration), 1630 (C = O), 1446 and 1375 (C-CH<sub>3</sub> bending vibration) and 1018 (C = C). EI/MS  $m/z$  (70 eV) 524.52 ( $M^+$ ), 218.17 (100%), 203.14, 189.13, 135.20 and 99.10. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta_H$  5.13 (1 H, br.s, H-12), 4.52-4.54 (1H, m, H-3), 2.30 (2 H, br.s, H-32), 1.08 (3 H, s, H-27), 1.02 (3 H, s, H-25), 0.99 (3 H, s, H-26), 0.92 (3 H, s, H-23), 0.90 (3 H, s, H-29), 0.90 (3 H, s, H-24), 0.88 (9 H, s, H-34, H-35 and H-36), 0.81 (3 H, s, H-28), 0.81 (3 H, s, H-30). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta_C$  173.53 (O-C = O, C-31), 139.57 (C-13), 124.29 (C-12), 80.55 (C-3), 59.06 (C-18), 55.26 (C-5), 47.62 (C-9), 42.06 (C-14), 41.52 (C-22), 40.03 (C-8), 39.64 (C-19), 39.59 (C-20), 38.44 (C-1), 37.72 (C-4), 36.78 (C-10), 33.72 (C-17), 32.87 (C-7), 32.4 (C-33), 31.32 (C-21 and C-32), 28.72 (C-15), 28.09 (C-28), 28.06 (C-34), 26.60 (C-16 and C-2), 23.62 (C-24), 23.36 (C-27), 23.22 (C-11), 22.28 (C-35 and C-36), 21.35 (C-23 and C-29), 18.24 (C-6), 17.47 (C-30), 16.85 (C-26), 15.69 (C-25).

#### Compound 2

White crystals, soluble in chloroform, m.p. 215-218°C,

$R_f = 0.80$  ( $S_2$ ). IR: 2949 and 2845  $\text{m}^{-1}$  (-C-H stretching vibration), 1642  $\text{cm}^{-1}$  (C = O), 1447 and 1385  $\text{cm}^{-1}$  (C-CH<sub>3</sub> bending vibration) and 1020  $\text{cm}^{-1}$  (C = C). EI/MS  $m/z$ : 468.84 ( $M^+$ ), 453.33 ( $M\text{-CH}_3^+$ ), 218.15, 189.02, 161.02, 135.18, 107.09 (100%), 81.10 and 55.26. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta_H$  4.70 (1 H, s, H-29b), 4.58 (1 H, s, H-29a), 4.45-4.51 (1H, m, H-3), 2.05 (3 H, s, CH<sub>3</sub> of acetate group), 1.70 (3 H, s, H-30), six methyl groups at 1.04 (3 H, s), 0.99 (3 H, s), 0.95 (3 H, s), 0.88 (3 H, s), 0.86 (3 H, s), 0.79 (3 H, s). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta_C$  170.93 (O-C = O, C-31), 150.91 (C-20), 109.33 (C-29), 80.98 (C-3), 55.42 (C-5), 50.38 (C-9), 48.33 (C-18), 48.02 (C-19), 43.01 (C-17), 42.86 (C-14), 40.89 (C-8), 40.02 (C-22), 38.43 (C-1), 38.08 (C-4), 37.82 (C-10), 37.12 (C-13), 35.60 (C-16), 34.26 (C-7), 29.87 (C-21), 27.97 (C-23), 27.47 (C-15), 25.15 (C-12), 21.13 (C-2), 20.98 (C-11), 19.31 (C-30), 18.24 (C-6), 18.02 (C-28), 16.51 (C-24), 16.19 (C-25), 16.01 (C-26), 14.53 (C-27).

#### Compound 3

Yellow powder, soluble in methanol,  $R_f = 0.56$  ( $S_3$ ). Showed blue fluorescence in UV at  $\lambda_{365\text{ nm}}$  before and after exposure to NH<sub>3</sub> vapour. <sup>1</sup>H-NMR (DMSO): Caffeic acid:  $\delta_H$  7.39 (1H, d,  $J = 16$  Hz, H-7'), 7.04 (1, br.s, H-2'), 6.97 (1 H, d,  $J = 7.5$  Hz, H-6'), 6.75 (1 H, d,  $J = 7.5$  Hz, H-5'), 6.16 (1 H, d,  $J = 16$  Hz, H-8'). Quinic acid:  $\delta_H$  5.08 (1 H, dd, H-4), 4.33 (1 H, ddd, H-3), 4.00 (1 H, ddd, H-5), 2.50 (1 H, dd, H-6 eq), 2.41 (1 H, dd, H-2 ax), 1.95 (1 H, dd, H-2 eq), 1.80 (1 H, dd, H-6 ax). <sup>13</sup>C-NMR (DMSO):  $\delta_H$  175.5 4 (C-7), 166.10 (C-9'), 148.51 (C-4'), 145.73 (C-7'), 144.69 (C-3'), 125.76 (C-1'), 126.60 (C-6'), 115.97 (C-5'), 114.82 (C-8'), 114.52 (C-2'), 79.14 (C-1), 79.61 (C-4), 68.55 (C-5), 62.94 (C-3), 40.35 (C-2), 37.44 (C-6).

### Biological study

#### Plant extracts

The alcoholic and aqueous extracts of the unripe fruits were prepared by percolating 200 g of fresh material with 70% ethanol and distilled water, separately, the resulting extracts evaporated under reduced pressure. Aqueous homogenates were prepared by mixing sliced fruits (100 g), with 200 ml distilled water, in a homogenizer and then kept frozen.

#### Experimental animals

Male Wistar strain rats weighing 150-160 g and aged three months, supplied from the Research Institute of Ophthalmology were used. They were housed in separate cages under standardized temperature (25-28°C), humidity (50-60%) and light (12 hours' light/dark cycles) conditions. They were fed the standard laboratory diet consisting of vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), starch (54.3%) and casein (10.5%).

### Drugs and chemicals

Alloxan: Sigma Co, Germany, dissolved in normal saline.  
Cholesterol and bile salts powders: Lab Plus, UK.

Metformin (Cidophage<sup>®</sup>): Chemical Industries Development Co (CID Co.), Giza, Egypt.

Vitamin C (Vitacid C<sup>®</sup>): Chemical Industries Development Co (CID Co.), Giza, Egypt.

Atorvastatin (Lipitor<sup>®</sup>): Pfizer Company, Cairo, Egypt.

### Kits

Cholesterol and glucose kits: Biocon, Germany.

Total antioxidant kits: Biodiagnostic, Cairo, Egypt.

### Model induction

Hypercholesterolemia and diabetes were induced by feeding the male Wistar rats with standard laboratory diet mixed with 1% cholesterol and 0.25% bile salts powders from the diet weight<sup>[9]</sup> and intraperitoneal injection with a single dose of alloxan (100 mg/kg b.wt),<sup>[10]</sup> respectively.

### Animal grouping

The animals were divided into nine groups, each of 10 animals, as follows: from the beginning of line. Untreated control group: Served as the negative control group and received daily an equivalent volume of distilled water orally (0.2 ml distilled water) beside standard laboratory diet.

Treated control groups: Three groups, they were administered 0.2 ml of 4% solution in distilled water of each of the tested extracts, as a single daily oral dose for six weeks.

Untreated model group: Served as the negative model group and received daily 0.2 ml of distilled water orally beside standard laboratory diet.

Treated model groups: three groups, administered 0.2 ml of 4% solution in distilled water of the tested extracts, as a single oral dose for six weeks.

Model group treated with reference drugs: Vitamine C (100 mg/kg body weight), metformin (20 mg/kg body weight) and atorvastatin (5 mg/kg body weight).

### Determination of glucose and cholesterol levels and total antioxidant<sup>[11,12]</sup>

Blood samples were taken at the 6<sup>th</sup> week of experiment and then centrifuged at 4000 rpm for 10 minutes. The supernatant (serum) was separated and divided into three portions. Serum glucose,<sup>[13]</sup> cholesterol

levels<sup>[14]</sup> and antioxidant capacity<sup>[15]</sup> were determined by measurement of the color developed with specific kits in each case. The color intensity was measured using Perkin Elmer 3300 spectrophotometer (Germany) at  $\lambda_{\max}$  500-520 nm.

### Statistical analysis

Results were expressed as mean  $\pm$  SD [Table 1]. Comparison between groups was statistically analyzed by one way ANOVA followed by *post hoc* tests and student *t* tests.<sup>[16]</sup> All the above results were considered significant if  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Chemical study

Three compounds were isolated from the alcoholic extract of *M. Zapota* fruits.

#### Compound 1

Spectral data of this compound revealed the presence of  $\beta$ -amyryn skeleton,<sup>[17]</sup> additional signal at  $\delta_C$  173.53 indicates the presence of an acyl group. <sup>1</sup>H-NMR showed a singlet at  $\delta_H$  0.88 integrated to nine protons revealed the presence of three equivalent methyl groups. Mass spectrum showed molecular ion peak at 524.52 ( $M_+$ ) corresponding to  $\beta$ -amyryn-3-(3'-dimethyl) butyrate structure [Figure 1]. The identity of this compound was confirmed by HMBC spectral data. This is the first report on the isolation of this compound in nature.

#### Compound 2

By comparing its spectral data with the published data,<sup>[17]</sup> compound **2** was identified as lupeol-3-acetate [Figure 2]. The identification was also confirmed by melting point and cochromatography with an authentic sample. This compound was previously isolated from the leaves of the same plant.<sup>[1]</sup>

#### Compound 3

NMR spectrum displayed the characteristic signals of a caffeic acid and quinic acid moieties.<sup>[18]</sup> The downfield shift of H-4 ( $\delta_H$  5.08) indicates acylation of the quinic acid at C-4 hydroxyl group. This was confirmed by the downfield shift of C-4 to  $\delta_C$  79.61. By comparing the spectral data of this compound with the published data,<sup>[18]</sup> compound **3** was identified as 4-caffeoylquinic acid (cryptochlorogenic acid) [Figure 3]. This compound was isolated for the first time from the plant.

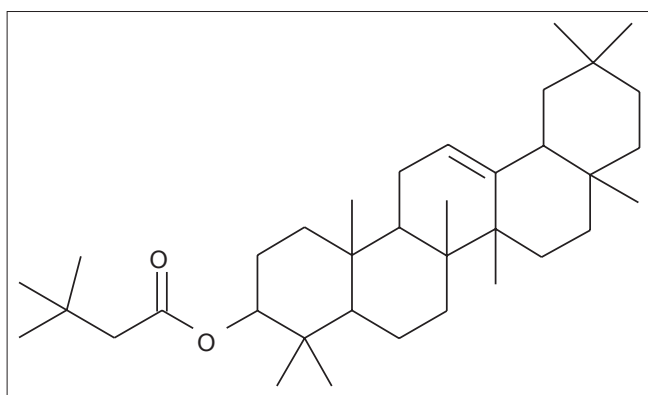
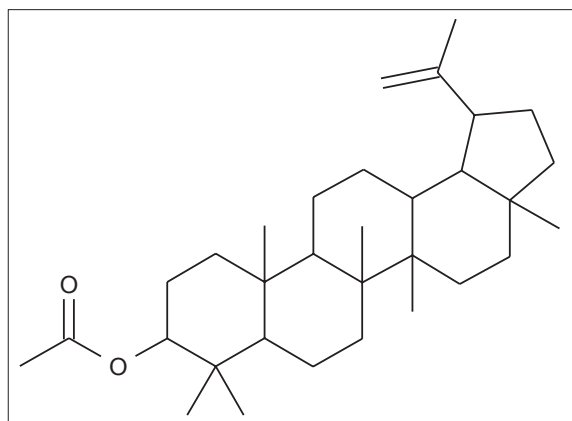
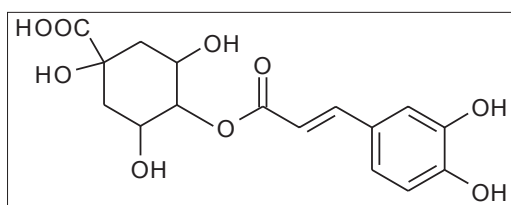
### Biological study

The aqueous extract of the fruits showed significant antihyperglycemic, hypocholesterolemic and a potent antioxidant effect in the model group. Water soluble polyphenolic compounds mainly tannins and flavonoids,

**Table 1: Antioxidant, antidiabetic and hypocholesterolemic activities of *M. zapota* fruits**

Groups	TAO (mM/L) (Mean±SD)	Glucose level (mg/dl) (Mean±SD)	Cholesterol level (mg/dl) (Mean±SD)
Negative control	0.309±0.033	81.5±13.13	64.1±4.99
Model of diabetic and hypercholesterolemic non treated rats	0.127±0.011	151.0±13.6	90.5±6.02
Model treated with reference drugs	0.601±0.020 <sup>a</sup>	86.8±15.2 <sup>a</sup>	65±5.2 <sup>a</sup>
Model treated with alcoholic extract	0.201±0.020	136.0±20.15	55.5±3.24 <sup>a</sup>
Control group treated with alcoholic extract	0.287±0.088	80.0±7.07	53.4±4.40
Model treated with aqueous extract	0.699±0.028 <sup>a</sup>	90.1±12.6 <sup>a</sup>	88.0±20.7 <sup>a</sup>
Control group treated with aqueous extract	0.764±0.269 <sup>b</sup>	74.6±3.65	64.9±3.24
Model treated with aqueous homogenate	0.332±0.059 <sup>a</sup>	95.3±4.97 <sup>a</sup>	82.3±10.8
Control group treated with aqueous homogenate	0.447±0.032	74.5±3.77	65.8±2.93

<sup>a</sup>Value is significant difference when compared to the untreated model group at  $P < 0.05$ ; <sup>b</sup>value is significant difference when compared to the negative control group at  $P < 0.05$

**Figure 1:**  $\beta$ - amyrin - 3-(3' - dimethyl) butyrate (Compound 1)**Figure 2:** Lupeol-3-acetate (Compound 2)**Figure 3:** 4-Caffeoylquinic acid (Cryptochlorogenic acid) (Compound 3)

previously isolated from the fruits,<sup>[2-4]</sup> could be responsible for the observed bioactivities of the aqueous extract of the fruits.<sup>[6-8,19-22]</sup>

The model group treated with the alcohol extract of the fruits showed marked decrease of cholesterol level, minimal improvement of glucose level, while no significant change in the TAO level was observed.

A minimal improvement in the cholesterol level with no significant difference was observed in the model group treated with the aqueous homogenate of the fruits, while significant antihyperglycemic and antioxidant activities, lower than that of the aqueous extract, were observed. The high fibre content in the aqueous homogenate of the fruits (12.28%),<sup>[23]</sup> may contribute to its slow digestion and absorption, this could explain the decrease in the level of its bioactivities compared to the aqueous extract of the fruits.<sup>[24]</sup>

The tested extracts showed no significant change in the blood glucose level of the normal rats, the same as the antihyperglycemic drugs biguanides e.g., metformin.<sup>[25]</sup>

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