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Antimicrobial constituents of Thompson seedless raisins (*Vitis vinifera*) against selected oral pathogens

J. Fausto Rivero-Cruz [a,1], Min Zhu [a,2], A. Douglas Kinghorn [b,3], Christine D. Wu [a,*]

[a] Department of Pediatric Dentistry, College of Dentistry, University of Illinois at Chicago, 801 S. Paulina, Chicago, IL 60612, United States
[b] College of Pharmacy, University of Illinois at Chicago, 833 South Wood, Chicago, IL 60612, United States

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ABSTRACT

As a part of a project directed toward the discovery of oral antimicrobial compounds from plants, eight known compounds, oleanolic acid (**1**), oleanolic aldehyde (**2**), linoleic acid (**3**), linolenic acid (**4**), betulin (**5**), betulinic acid (**6**), 5-(hydroxymethyl)-2-furfural (**7**), and β-sitosterol were isolated from an hexane-soluble partition of a methanol extract of Thompson seedless raisins (*Vitis vinifera*). From an EtOAc-soluble partition rutin (**8**) and β-sitosterol glycoside were isolated. In an attempt to increase the resultant antimicrobial activity of oleanolic acid (**1**), a series of acylation and etherification reactions were performed on oleanolic acid to obtain derivatives **1a**–**1f**. All the compounds isolated and the derivatives **1a**–**1f** were evaluated for their antimicrobial activity against two oral pathogens, *Streptococcus mutans* and *Porphyromonas gingivalis* associated with caries and periodontal disease, respectively. Compounds **1**, **2**, **7** and **1f** inhibited the growth of the test bacteria with concentrations ranging from 3.9 to 500 μg/mL. Derivative **1f** showed greatly enhanced antimicrobial activity when compared with oleanolic acid (**1**).
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1. Introduction

Previous studies from our laboratory and others have demonstrated that compounds possessing antimicrobial activities against oral pathogens can be isolated from edible plants and plants traditionally used as oral remedies (Cai et al., 2000; Koo et al., 2000; Hwang et al., 2003; He et al., 2006). Raisins are dried grapes, the fruits of *Vitis vinifera* L. (Vitaceae) (Karadeniz et al., 2000). Today most raisins are produced from Thompson seedless grapes, which were introduced to California in 1862 by William Thompson (USDA, 2003). This variety is classified as a raisin-type grape that produces a green, seedless fruit. While dominating raisins production, it is also widely used for fresh consumption and for making juice concentrate and wine as well (Karadeniz et al., 2000; USDA, 2003). Several other raisin grape varieties are used for raisins production including Muscat, Black Corinth, and Sultana. The US *per capita* annual consumption of raisins is approximately 3.26 kg (USDA, 2003). Three types of raisins are economically important in the US. Natural raisins are sun-dried and account for the majority of the raisins produced and consumed. Dipped raisins are dried artificially and have a higher moisture content than natural raisins. Golden raisins are treated with sulfur dioxide to preserve the golden color (Karadeniz et al., 2000).

Besides being a traditional and popular snack food, raisins contain polyphenols, antioxidants, flavonoids and iron that may benefit overall human health. The sweetness of raisins is contributed by mainly glucose and fructose, but not sucrose. It is well documented that sucrose, the main dietary sugar, serves as a substrate for the synthesis of adherent glucans in human dental plaque associated with tooth decay and gum disease (Cury et al., 2000). The various phytochemicals reported in raisins include triterpenes (Zhang et al., 2004), fatty acids (Stafford et al., 1974; Radler, 1965), flavonoids (Liggins et al., 2000), amino acids (Bolin and Petrucci, 1985), hydroxycinnamic acids (Liggins et al., 2000) and 5-hydroxymethyl-2-furaldehyde (Palma and Taylor, 2001). Although various *in vitro* studies have been performed to investigate the mode of actions of these phytochemicals and their effects on bodily functions, much less attention has been paid to their effects on oral health and disease prevention.

* Corresponding author. Tel.: +1 312 355 1990; fax: +1 312 996 1981.
E-mail address: chriswu@uic.edu (C.D. Wu).
[1] Present address: Departamento de Farmacia, Facultad de Química, Universidad Nacional, Autónoma de México, Ciudad Universitaria, México, D.F., Mexico.
[2] Present address: Dows Institute of Dental Research, University of Iowa College of Dentistry, Iowa City, IA 52242, United States.
[3] Present address: Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, United States.

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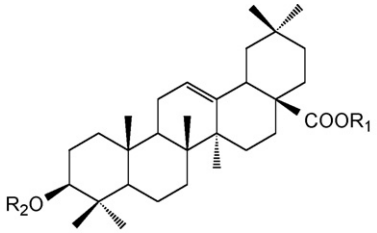


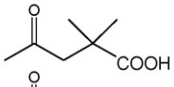
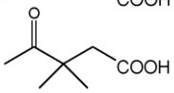
	R_1	R_2
1	H	H
1a	H	CH_3CO
1b	CH_3	CH_3CO
1c	CH_3	H
1d	H	
1e	H	
1f	Na^+	H

Fig. 1. Structures of oleanolic acid and its derivatives.

In an ongoing program directed toward the discovery of novel oral antimicrobial agents from plants (Li et al., 1998; Cai et al., 2000; Hwang et al., 2003), Thompson seedless raisins were chosen for further detailed investigation because the hexane-soluble fraction of the crude methanol extract demonstrated antimicrobial activity when evaluated against two oral pathogens, *S. mutans*, and *P. gingivalis*. We hypothesized that raisins contain antimicrobial phytochemicals that suppress the growth of oral pathogens thereby benefiting oral health. In this study, bioassay-guided fractionation of hexane- and ethyl acetate-soluble partitions of *V. vinifera* was performed to isolate and identify antimicrobial compounds **1**, **2**, **7**, and **8** possessing growth inhibitory effects against oral pathogens. Selected analogs (**1a**–**1f**) of oleanolic acid (**1**) were also prepared in an effort to afford derivatives with enhanced antimicrobial activity against test bacteria.

2. Results and discussion

All of the compounds isolated from *V. vinifera* in the present investigation were previously reported from species in the family Vitaceae. The substances, namely, oleanolic acid (**1**) (Seebacher et al., 2003), oleanolic aldehyde (**2**) (Mahato and Kundu, 1994), linoleic acid (**3**) (Su et al., 2002), linolenic acid (**4**) (Su et al., 2002), betulin (**5**) (Peng et al., 1998), betulinic acid (**6**) (Sholichin et al., 1980), 5-(hydroxymethyl)-2-furfural (**7**) (Hearn, 1976), rutin (**8**) (Lallemand and Duteil, 1977), β-sitosterol (Slomp and MacKellar, 1962) and β-sitosterol glucoside (Tiwari and Choudhary, 1979) were identified by comparing their physical and spectroscopic data with those of published values. The results in the present study were in general in agreement with the expected chemotaxonomic pattern for a member of Vitaceae. After their purification, the triterpenoids (**1**–**2** and **5**–**6**), linoleic acid (**3**), linolenic acid (**4**), 5-(hydroxymethyl)-2-furfural (**7**), rutin (**8**) and the derivatives (**1a**–**1f**) were tested for antimicrobial activity against *S. mutans* and *P. gingivalis*. Six previously described semi-synthetic derivatives of oleanolic acid (**1a**–**1f**) were also prepared, in an effort to enhance the oral microbial activity of the parent triterpenoid (**1**) (Fig. 1).

Table 1
Antimicrobial activity (MIC values) of test compounds against *Streptococcus mutans* and *Porphyromonas gingivalis*

Compound	Minimum inhibitory concentration (μg/mL)[a]	
	Streptococcus mutans	*Porphyromonas gingivalis*
1	625	488
2	488	250
3	>1000	>1000
4	>1000	>1000
5	1000	1000
6	1000	1000
7	31	16
8	250	>1000
1a	79	1000
1b	781	>1000
1c	1000	1000
1d	>1000	156
1e	500	98
1f	7.8	3.9
CHX[b]	1.2	0.3

[a] Values represent the average obtained from a minimum of three experiments.
[b]CHX: chlorhexidine gluconate.

As shown in Table 1, antimicrobial screening against two oral pathogens revealed that compounds **1**, **2**, **7**, **1d**, **1e,** and **1f** were inhibitory to the growth of *P. gingivalis,* the Gram-negative anaerobic oral bacterium most commonly associated with gum diseases, with MIC values ranging from 3.5 to 488 μg/mL (Table 1). Compounds **1**, **2**, **7**, **8**, **1a**, **1e**, and **1f** were active against *S. mutans* (7.8–625 μg/mL), a Gram-positive facultative anaerobic coccus that is the etiologic agent of human dental caries (Table 1). Among these, compounds **1**, **2**, **7**, and **8**, were either equally or more potent than their respective crude extract of origin. The hexane and EtOAc extracts were more potent than the $CHCl_3$, and methanol and 1-butanol extracts. The differential antimicrobial activity observed against *P. gingivalis* suggests that compounds **2**, **7**, **1d**, **1e**, and **1f** may benefit periodontal health. Earlier *in vitro* studies have shown that oleanolic acid (**1**) inhibited insoluble glucan synthesis (ISG) of mutans streptococci in the oral cavity (Kozai et al., 1999; Sasazuka et al., 1995; Herrera et al., 2006). Several pharmacological properties of oleanolic acid have been demonstrated: anti-inflammatory, antitumor, hepatoprotective, cytotoxic, anti-diabetogenic, antibacterial and anti-HIV activities (Sasazuka et al., 1995). In the present study, the activity of oleanolic acid sodium salt (**1f**) against *P. gingivalis* was about 100 times more potent (3.9 μg/mL) than oleanolic acid (**1**), thereby demonstrating an enhancement of activity as a result of the substitution of a more polar functionality in **1f** when compared with the parent triterpene acid. The enhanced antibacterial activity of **1f** could be a surfactant effect, which is currently under investigation. Although chlorhexidine has a considerably lower MIC than the active compounds in this study (1.25 and 0.312 μg/mL for *S. mutans* and *P. gingivalis*, respectively, Table 1), its side effects such as tooth staining and taste alterations have been well documented (Quirynen et al., 2000). During the search for potential anti-plaque agents from natural sources, several compounds of plant origin have shown growth inhibitory activity against oral pathogens, e.g., the naphthalene glucosides, diospyrosides A–D, and some flavonoids, including kaempferol, myricetin, and rhamnocitrin (Li et al., 1998; Cowan, 2003). We believe that plant-derived antimicrobial compounds may serve as alternatives to the commonly used chemicals for dental plaque and oral disease control. Raisins may benefit oral health since they possess the antimicrobial phytochemicals that suppress growth of oral bacteria associated with dental diseases.

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3. Experimental

3.1. General

Melting points of the isolates were determined using a Fisher-Johns melting point apparatus, and are uncorrected. Optical rotations were obtained on a PerkinElmer mode 241 polarimeter. UV spectra were measured on a Beckman DU-7 spectrometer. IR spectra were taken on a Genesis Series FT IR ATI Mattson spectrometer. ^{1}H NMR, ^{13}C NMR (including DEPT), HMQC, HMBC and ^{1}H–^{1}H COSY spectra were measured on Bruker DRX-500 and -300 instruments. Compounds were analysed in $CDCl_3$, with tetramethylsilane (TMS) as internal standard. ^{13}C NMR multiplicities were determined using APT and DEPT experiments. EIMS were recorded on a Finnigan MAT-90/95 sector field mass spectrometer. A YMC-pack ODC-AQ column (5 μm, 15 cm × 2 cm i.d., YMC Co., Wilmington, NC) and YMC-guard pack ODC-AQ guard column (5 μm, 15 cm × 2 cm i.d.) were used for preparative HPLC, along with two Waters 515 HPLC pumps and a Waters 996 photo-diode array detector (Waters, Millford, MA).

3.2. Plant material

The Thompson seedless raisins (*V. vinifera* L., henceforth referred to as "raisins") were provided by the California Raisin Marketing Board (Fresno, CA 93726, USA). A voucher specimen representing the sample investigated is maintained at the College of Dentistry, University of Illinois at Chicago (CDW001).

3.3. Antibacterial activity assay

Growth inhibitory effects of the isolated compounds, fractions and extracts were tested against *Streptococcus mutans* (ATCC 10449) and *Porphyromonas gingivalis* (ATCC 33277), oral bacteria frequently associated with dental caries and periodontal disease. The procedures employed were as described by Cai et al., 2000. Sterile 96-well microtiter plates were used. Each well in the microtiter plate contained *S. mutans* (final concentration of 5×10^5 colony forming units (CFU)/mL or *P. gingivalis* of 5×10^6 CFU/mL), serially diluted test compound, and the appropriate growth medium. Triplicate samples were performed for each test concentration. The controls included inoculated growth medium without test compounds. Sample blanks contained uninoculated growth medium only. All plates were incubated at 37 °C under appropriate atmospheric conditions with growth estimated spectrophotometrically ($A_{660\ nm}$) after 24 and 48 h using a microtiter plate reader. The minimum inhibitory concentration (MIC) was defined as the minimum concentration of test compound limiting growth to OD < 0.05 $A_{660\ nm}$. Chlorhexidine gluconate (CHX, Sigma) was used as the antimicrobial positive control.

3.4. Activity-guided compound isolation

The dried and ground raisins (5.0 kg) were extracted with 10 L of MeOH three times by maceration, for up to 3 days each. The resultant extracts were combined and concentrated *in vacuo* at 40 °C. The concentrated extract was suspended in 90% MeOH (2.5 L), and then partitioned with 2.5 L of hexane three times to afford a hexane-soluble syrup (D001) on drying. Next, the aqueous methanol extract was partitioned with 2.5 L of $CHCl_3$ three times to give a $CHCl_3$-soluble extract (D002). Finally, the aqueous extract was partitioned again with 2.5 L of EtOAc three times to give an ethyl acetate-soluble extract (D003) and an aqueous residue (D004). The MIC values against *S. mutans* for D001, D002, D003, and D004 were 0.5, 1.0, >1.0, and >1.0 mg/mL, respectively. The hexane-soluble extract (68.4 g; MIC 0.5 mg/mL, *S. mutans*) was subjected to silica gel (1.0 kg) column chromatography and eluted with a gradient mixture of $CHCl_3$–MeOH (1:0 → 0:1) to give nine pooled fractions. Fractions 3, 4 and 5 were active when tested against *S. mutans* (MIC 1.0 mg/mL, respectively). Fraction 3, eluted with $CHCl_3$, was chromatographed over a silica gel VLC column (2.5 cm × 15 cm) using a gradient mixture of hexane–isopropyl alcohol (98:2 → 50:50), to afford compounds **1** (340 mg) and **2** (35 mg). Fraction 4, eluted with $CHCl_3$–MeOH (95:5), was chromatographed over a silica gel column (2.8 cm × 35 cm), using a gradient mixture of hexane–EtOAc–MeOH (1:1:0.1 → 1:1:0.5) as solvent system, to give **1** (125 mg), **2** (32 mg), **5** (89 mg), **7** (245 mg), and β-sitosterol (128 mg). Fraction 5, eluted with $CHCl_3$–MeOH (9:1), was chromatographed over a silica gel column (5.6 cm × 35 cm), eluted with hexane–AcOEt (30:1 → 2:1), and afforded **6** (76 mg) and **7** (34 mg).

The EtOAc-soluble extract [86.2 g; MIC 1.0 mg/mL, *S. mutans*] was subjected to Diaion HP-20 column chromatography and eluted with a gradient mixture of H_2O–MeOH (1:0 → 0:1, 500 mL per fraction) to give four pooled fractions. Fraction 3, was chromatographed over a silica gel column (5.6 cm × 20 cm), using a gradient mixture of $CHCl_3$–MeOH (1:0 → 0:1) as solvent systems, to give β-sitosterol glycoside (78 mg). Fraction 4, eluted with MeOH, was chromatographed over a Sephadex LH-20 column (2.8 cm × 35 cm) using MeOH as a solvent and afforded rutin (**8**, 234 mg).

3.5. Derivatization of oleanolic acid

3.5.1. 3-Acetyloleanolic acid (**1a**)

To a stirred solution of **1** (20 mg) in pyridine (1.5 mL), 10 equiv. of acetic anhydride and *N*,*N*-dimethyl-4-aminopyridine (0.5 mg) were added. When TLC indicated complete consumption of starting material, the reaction solution was diluted with CH_2Cl_2 (10 mL), washed with 1N HCl (3× 10 mL), saturated $NaHCO_3$ solution (3× 10 mL), and water (3× 10 mL), then dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The resulting mixture was purified by preparative TLC on silica gel, eluting with hexane–ethyl acetate (10:3), to yield an acetyl derivative (**1a**, 18.9 mg, R_f 0.64). Compound **1a** exhibited closely comparable spectroscopic data (^{1}H NMR ^{13}C NMR and EIMS) to published values (Zhu et al., 2001).

3.5.2. Methyl 3-acetyloleanolic acid (**1b**)

Oleanolic acid was acetylated with 10 equiv. of acetic anhydride in anhydrous pyridine to give 3-acetoxy-oleanolic acid (**1b**) and then treated with ethereal diazomethane to yield methyl 3-acetyloleanolic acid. The resulting mixture was purified by preparative TLC on silica gel, eluting with hexane-ethyl acetate (10:1), to yield an acetyl derivative (**1b**, 18.9 mg, R_f 0.43). Compound **1b** exhibited closely comparable spectroscopic data (^{1}H NMR ^{13}C NMR and EIMS) to published values (Hichri et al., 2003; Kashiwada et al., 1996, 2000).

3.5.3. Methyl oleanolic acid (**1c**)

Oleanolic acid (20.0 mg) was treated with ethereal diazomethane to yield methyl oleanolic acid. The resulting mixture was purified by preparative TLC on silica gel, eluting with hexane–ethyl acetate (10:2), to yield an acetyl derivative (**1c**, 19.5 mg, R_f 0.54). Compound **1c** exhibited closely comparable spectroscopic data (^{1}H NMR ^{13}C NMR and EIMS) to published values (Zhu et al., 2001).

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3.5.4. 3-O-Acyl-oleanolic acid derivatives (1d and 1e)

3-*O*-Acyl-oleanolic acid derivatives were prepared by reacting oleanolic acid (50 mg/5 mL pyridine) with 2,2-dimethylsuccinic anhydride (1.5 equiv.) in the presence of *N*,*N*-dimethyl-4-amino-pyridine (0.5 mg) at reflux. The reaction with 2,2-dimethylsuccinic anhydride gave a mixture of 3-*O*-(2′,2′-dimethylsuccinyl)- and 3-*O*-(3′,3′-dimethylsuccinyl)-oleanolic acids (**1d** and **1e**, respectively). The mixture was separated by semipreparative-scale HPLC (CH_3CN–H_2O = 50:50, 7 mL/min), and the structures of these isomers were assigned by NMR spectroscopy. Compounds **1d** (t_R 18.9) and **1e** (t_R 21.3) exhibited closely comparable spectroscopic data (^1H NMR ^{13}C NMR and EIMS) to published values (Zhu et al., 2001).

3.5.5. Sodium salt of oleanolic acid (1f)

A solution of oleanolic acid (50 mg) was treated with 1N NaOH in $(CH_3)_2CO$–H_2O (1:1) at room temperature for 30 min. After removal of $(CH_3)_2CO$ by evaporation the resulting solution was passed through a MCI-gel CHP20P (Sulpelco, Bellefone, PA, USA) column, and washed with water to remove the excess of NaOH. Subsequent elution with methanol gave the sodium oleanoate (**1f**) as a white powder. Compound **1f** exhibited closely comparable spectroscopic data (^1H NMR ^{13}C NMR and EIMS) to published values (Zhu et al., 2001).

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