KASHIF GHAFOOR
Department of Food Science and Nutrition,
College of Food and Agricultural Sciences, King Saud University,
PO Box 2460, Riyadh 11451, Saudi Arabia


Kashif Ghafoor

PEER REVIEWED

Antioxidant properties
of oleanolic acid from grape peel

KEYWORDS: Grape peel, Vitis labrusca, oleanolic acid, ferric reducing power, lipid peroxidation inhibition, free radical scavenging

Abstract The consumption of grape (Vitis labrusca B.) is associated with various health benefits. It is considered as good source of health benefiting phytochemicals such as phenolic compounds, having antioxidant properties. In this study 429 mg of oleanolic acid was obtained from 50 g of dried grape peel by using silica gel column chromatography. Methanol, ethyl acetate and n-hexane were used as solvents. 1H-NMR, 13C-NMR and EI-MS spectra were used for structure elucidation and identification of purified compound. Oleanolic acid from Vitis labrusca B. peel showed good antioxidant properties such as ferric acid reducing (0.77 FRAP value), lipid peroxidation inhibition (24.66%) and DPPH radical scavenging (85.3%). The antioxidant properties of oleanolic acid were comparable with those of commercial antioxidants.

INTRODUCTION

Due to increasing health consciousness and more demand for natural products, bioactive compounds from plant sources can be incorporated in the diet or can be applied as natural therapeutic alternatives. In this context, terpenoids can be regarded as one of the most effective compounds because they possess strong pharmacological properties. Oleanolic acid is one type of triterpenoid from natural sources. It can be categorized as aglycone consisting of various saponins. Chemically it is described as 3β-hydroxy-olea-12-en-28-oic acid. Figure 1 shows the chemical structure of oleanolic acid. It is present in different types of foods such as vegetable oils, winter savory (1), and in the roots and leaves of *Olea europaea*, *Viscum album* L., *Aralia chinensis* L. It is estimated that this compound in present in plant species of 120 types (2-4). It is attributed to possess different pharmacological properties that include anti-inflammatory (5), anti-hyperlipidemic (6), antiulcer (7), antioxidant activity (8), hepatoprotective (9), anti-HIV (10), antimicrobial (11); anti-tumor (12) and hypoglycemic (13) properties, all of which are important for human health.

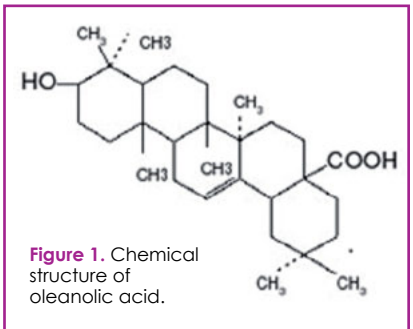

Figure 1. Chemical structure of oleanolic acid.

Use of methanol for the recovery of such compounds from various plant species (14) and other organic solvent systems (15), has been reported. In general, conventional techniques that may include silica gel, polyamide and preparative reversed-phase liquid chromatography are routinely applied for the isolation of natural products from medicinal plants (16). Oleanolic acid has been isolated and characterized from different plant sources and different derivatives of this compound have also been synthesized due to its pharmacological significance (17). Grape peel, an important source of different bioactive compounds, had not been utilized before for the isolation of oleanolic acid. The objective of our study was to isolate, purify and indentify oleanolic acid from of *Vitis labrusca* B. grape peel. We also aimed to evaluate the antioxidant properties of oleanolic acid using different methods in comparison to other synthetic antioxidants.

MATERIALS AND METHODS

Plant material and chemicals

Grapes (*Vitis labrusca* B.) cultivar selected for this research was identified as 'Campbell Early'; peel was removed manually using knife and dried in an oven at 50°C for around 48 h until the moisture level was constant (5.8% w/w). Grape peel powder was obtained by drying the dried peel in grinder and passed through 0.3 mm sieve. Methanol, ethyl acetate and n-hexane were purchased from VWR international, Arlington Heights, IL, USA. Antioxidant standard compounds and chemicals used in determination of antioxidant properties were purchased from Sigma-Aldrich Corporation, St. Louis, ME, USA.

Extraction procedure

Extraction was carried out by using methanol and ethyl acetate (18). 50 g sample was extracted in a flask with 500mL of methanol in sonication water bath set at 40°C for 50 min. The working frequency and sonication power were fixed at 40 kHz and 250 W, respectively (19). The solution was vacuum dried at 40°C to get syrup using rotary evaporator (Unimax 1020, Heidolph

Instruments, Schwabach, Germany). To the syrup 200 mL of ethyl acetate was added and the contents were mixed on a magnetic stirrer. Extraction was again carried out in sonication water bath for 50min at 40°C. Solution was filtered using filer paper # 5A (Whatman, Kent, UK). From the filtered solution solvent was evaporated at room temperature and the extract was dried overnight at 50°C in an oven. A crude extract of approximately 15g was obtained which was further worked up by using 10 g $NaHCO_3$ and 200 mL 95% ethyl acetate in a separatory funnel. Organic phase was collected and washed with water. The process was repeated 4 to 5 times by using vigorous shaking in separatory funnel. All the organic phase was evaporated in rotary evaporator at 40°C under reduced pressure and the extract was collected for silica gel column chromatography.

Purification by silica gel column chromatography

Silica gel column chromatography was accomplished using silica gel and see sand packed in a glass column. Dried extract from previous step was kept inside the column and eluted with ethyl acetate (95 %) and n-hexane (95%). Thin layer chromatography was applied for the assessment of possible number of compounds in the purified extract. The process of purification was continued as explained before until a single spot compound was obtained. Finally the compound was collected by evaporation of solvent at 40°C under reduced pressure using rotary evaporator and kept at -20°C before further analysis.

Nuclear magnetic resonance

^{1}H and ^{13}C NMR spectra were recorded on Brucker Advance Digital 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz respectively. Chemical shifts were given in δ (ppm) from tetra methyl silane (TMS).

The NMR results of the sample are summarized as follows: ^{1}H-NMR (400 MHz, $CDCl_3$); δ 0.74, 0.79, 0.89, 0.91, 0.923, 0.98 and 1.12 (each 3H, s, CH_3), 2.83 (1H, br. Dd, *J*=4.0, 14.0 Hz, H-18), 3.22 (1H, br. dd, *J*=4.0, 9.5 Hz, H-3) and 5.28 (each 1H, m, H-12). ^{13}C NMR (100 MHz, $CDCl_3$) δ 79.02, 77.34, 77.02, 76.70, 58.49, 55.18, 47.60, 46.51, 45.85, 41.56, 40.92, 39.29, 38.74, 38.36, 37.07, 33.77, 33.07, 32.57, 32.42, 30.67, 29.71, 28.09, 27.66, 27.15, 25.94, 23.57, 23.38, 22.88, 18.42, 18.27, 17.13, 15.54.

EI-MS analysis

An Agilent (Wallborn, Germany) GC (HP 6890N) connected to MS detector (HP 5973, EI, 70 eV), split– splitless injector and auto sampler (HP 7683) was used for the oleanolic acid analysis. An aliquot (1 µL) of silylated oleanolic acid (at a split ratio 1:20) was injected into GC. Sample separation was accomplished using capillary column (HP-5 MS, 5% phenyl–95% methyl siloxane, 30 m x 0.25 mm x 250 µm). The carrier gas was helium with a a flow rate of 0.6 mL/min. The transfer and injector line temperatures were set at 300 and 280°C, respectively. The oven temperature program was: initial temperature 50°C for 5 min, 300°C and held for 2 min and then 400°C and held for 2 minutes. The detection of target compound and/or terpenic acids was done using a selective ion monitoring (SIM) GC/ MS method. Detection of was based on the ±0.05 RT presence of target and qualifier ions of the standard at the predetermined ratios. Target and qualifier ions (T, Q1 and Q2) were quantified for oleanolic acid as following: 203, 248 (2).

The chromatographic peaks were identified through the comparison of time the peak was obtained and the fragment ions ratios with those of the reference compound, while quantization was accomplished using 3-(4-hydroxyphenyl)-1-propanol being the internal standard or reference at target ion m/z 206 and qualifiers 191 and 179 (20). Internal standard quantization was based on standard mixture of oleanolic acid containing the same quantity of internal standard as that of sample. A good linearity was obtained in the range of quantization limit (0.051-0.058 µg) and up to 1000-fold concentration for oleanolic acid.

Ferric reducing antioxidant power (FRAP)

The FRAP was based on the modified method of Benzie and Strain (21). Stock solutions containing 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution, were prepared. The preparation of working solution was done each time by mixing 25 ml acetate buffer, 2.5 ml $FeCl_3 \times 6H_2O$ solution, and 2.5 ml TPTZ solution, followed by incubation at 37°C before every use. Oleanolic acid and commercial antioxidants (BHT, ascorbic acid and gallic acid) solutions (0.5 mg/mL dimethyl sulfoxide or DMSO) were made to react for 30 min with 2.80 ml of the FRAP solution under dark. The absorbance values of colored complex, ferrous tripyridyltriazine, formed after this reaction were recorded at 593 nm. The FRAP value represented the ratio between the slope of the linear plot for reducing Fe^{3+}- TPTZ reagent by the antioxidant compared to the slope of this plot for $FeCl_3$ (expressed in ΔA per mM). ΔA presents the absorbance values of each solution.

Lipid peroxidation inhibition by ATC assay

The ammonium thiocyanate (ATC) assay (22), was applied for determining the lipid peroxidation inhibition ability of oleanolic acid in comparison to synthetic antioxidants. The reaction solution contained 0.2 ml of oleanolic acid or standard compounds (0.5 mg/mL dimethyl sulfoxide or DMSO), 0.2 ml of linoleic acid emulsion (25 mg/ml in 99% ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4). This solution was kept under dark conditions for incubation at 40 °C. A 0.1 ml of this solution was mixed to 3 ml of 70% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. To this reaction mixture was kept exactly for 3 min after addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid followed by measuring the absorbance (500 nm) values of the resulting red color complex. Aliquots from this complex were evaluated for their absorbance values every 24 h till the absorbance of the control solution reached maximum value. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated as follows:

Inhibition (%) = $[(A_o - A_1)/A_o] \times 100$

where A_o was the absorbance of the control reaction and A_1 the absorbance in the presence of the oleanolic acid or standard compounds.

DPPH radical scavenging assay

Briefly, 1 mL solution of antioxidants (0.5 mg/mL DMSO) was mixed with 2 mL of 10 mg/L methanolic solution 1, 1-diphenyl-2-picrylhydrazyl or DPPH (23). This solution was then mixed thoroughly and kept at ambient temperature for 5 min followed by absorbance (DA) measurement at 517 nm. The control solution for this test was made by mixing reagent solution with methanol and it did not contain oleanolic acid. It was prepared by using procedure as explained earlier. The DPPH antiradical activity or radical scavenging activity (*SA*) was obtained using following formula:

$$SA\ [\%] = \frac{\left(\Delta A_{517\ control} - \Delta A_{517\ sample}\right)}{\Delta A_{517\ control}} \times 100$$

Statistical analysis

The analytical measurements were done in triplicates followed by expressing the obtained results as means ± standard deviation. Statistical analysis was performed by using the Sigma Plot (12.3.0, Systat Software Inc. Chicago, IL, USA). The analysis of variance was used for analyzing the data and the significance was assigned when the probability values was less than 0.05 ($P < 0.05$).

RESULTS AND DISCUSSIONS

Compound was obtained as white powder with slight green pigmentation. Total yield was 429 mg from 50 g or 8.58 mg/g of dried peel of *Vitis labrusca* grapes. A comparison of this yield obtained from grape peel with that obtained from different plant materials is given in Table 1. These studies also showed that when the extraction method was changed the yield of oleanolic acid also varied. The maximum approximate quantities of oleanolic acid using optimized methods in each study are presented in Table 1. The structure

No.	Source	Quantity of oleanolic acids	Reference
1	Grape peel	8.58 mg/g	Present study
2	Pomegranate flower	9.72 mg/g	Fu et al. (24)
3	White nettle	104.2 µg/g	Wojciak-Kosior et al. (25)
4	Sage leaves	7.2 mg/g	Kontogianni et al. (26)
5	Olive leaves	8.3 mg/g	Kontogianni et al., (26)
8	Thymus eaves	2.5 mg/g	Kontogianni et al., (26)
9	Marjoram leaves	1.9 mg/g	Kontogianni et al., (26)

Table 1. Comparison of oleanolic acid recovery from different plant sources.

and identification of purified compound from grape peel was performed by using spectroscopic procuders (^{13}C-NMR, ^{1}H-NMR, EI-MS). In the ^{1}H-NMR spectrum, multiplets were detected for seven methyl group in the range of δ 0.74~1.12. A singlet peak at δ 7.48 was due to the hydroxyl and carboxyl groups whereas methine showed a peak at δ 5.48. ^{13}C-NMR spectrum showed the presence of 30 carbons, suggesting that it might be a triterpene. The resonance at δ 183.62 indicated the presence of a carbonyl carbon. Two sp^2 carbons were appeared at δ 143.59 and 122.61, indicating presence of one double bond. The signal at δ 55.18 was the evidence for an oxygenated carbon. From these data, the structure of this compound was postulated as oleanolic acid ($C_{30}H_{48}O_3$) and verified through comparison of its NMR data with other reports (27,28). For further verification, EI-MS spectrum was obtained which showed a molecular ion peak at *m*/z 456 (M^+) that has baseline peaks at *m*/z 248 and *m*/z 203. The EI-MS spectrum of oleanolic acid (A) and its reference from library record (B) is presented in Figure 2. Chromatogram of oleanolic acid isolated from grape peel also conformed to that of reference compound.

Antioxidant activities of oleanolic acid from grape peel

The ferric reducing antioxidant power (FRAP) method is used to quantify the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants, which are reductants with half-reaction reduction potentials above Fe^{3+}/Fe^{2+} (29). The FRAP value of oleanolic acid was 0.77±0.08 which was slightly higher than that of gallic acid (0.75±0.09) (Figure 3A). It shows that oleanolic acid purified from grape peel can be effectively

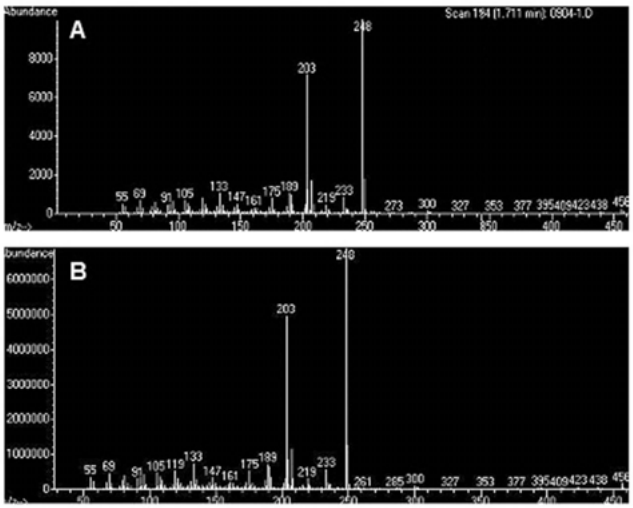

Figure 2. The EI-MS spectrum of oleanolic acid from grape peel (A)) and its reference from library record (B).

used to reduce ferric ions. Antioxidants tested had FRAP value in an order of ascorbic acid (0.94±0.05) > BHT (0.85±0.06) > oleanolic acid (0.77±0.08) > gallic acid (0.75±0.09). Xu et al. (30) isolated flavones and triterpenoids including oleanolic acid from *Tridax procumbens* and tested their bioactivity through DPPH and FRAP methods.

The results of lipid peroxidation by oleanolic acid and other antioxidants is shown in Figure 3B which shows that oleanolic acid from grape peel had 24.66**±1.45**% lipid peroxidation inhibition property which was lower than that of BHT (31.21**±1.43%)** and ascorbic acid (30.25**±0.88%)** but higher than that of gallic acid (21.28**±1.25%)**. Wang et al. (3) also observed the lipid peroxidation inhibitory effects of oleanolic acid in rats liver microsomes after incubating them with vitamin C/Fe^{2+}, CHP or CCl_4/NADPH at pH 7.4. Lipids can easily oxidize due the presence of polyunsaturated fatty acids and their ability to associate with enzymes-assisted and other non-enzyme systems in cellular membrane, which result in the generation of free radical species. Results of present study suggest that oleanolic acid may also act as lipid peroxidation inhibitor. Figure 3C show the abilities of different antioxidants to scavenge free DPPH radicals. It is noticeable that oleanolic acid had 85.30±1.84% DPPH-scavenging activity, which was comparable with that of ascorbic acid (90.11±2.14%), BHT (88.20±2.33%) and gallic acid (83.85±1.32%).

Oxidative stress is important factor in the pathogenesis of different diseases such as liver disorders, inflammation, tumor and diabetes, in which oleanolic acid treatment has been observed to be effective (3,31). Reactive oxygen species (ROS) continue to form it human body due to its routine oxygen utilization such as for respiration and during some cell-mediated immune functions. ROS are also referred as free radicals, including superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), peroxynitrite ($\bullet ONOO^-$) and hydroxyl ($\bullet OH^-$) (3,32). ROS, at physiological concentrations, may be needed in normal cell function. However, if not effectively eradicated by cellular constituents, excess ROS can react with many bio-molecules such as lipids, DNA and proteins. This can initiate membrane lipids peroxidation that leads to accumulation of lipid peroxides and DNA and protein damage that finally lead to onset of diseases. In fact, ROS have been associated for the onset of more than 100 diseases (33). Wang et al. (3)

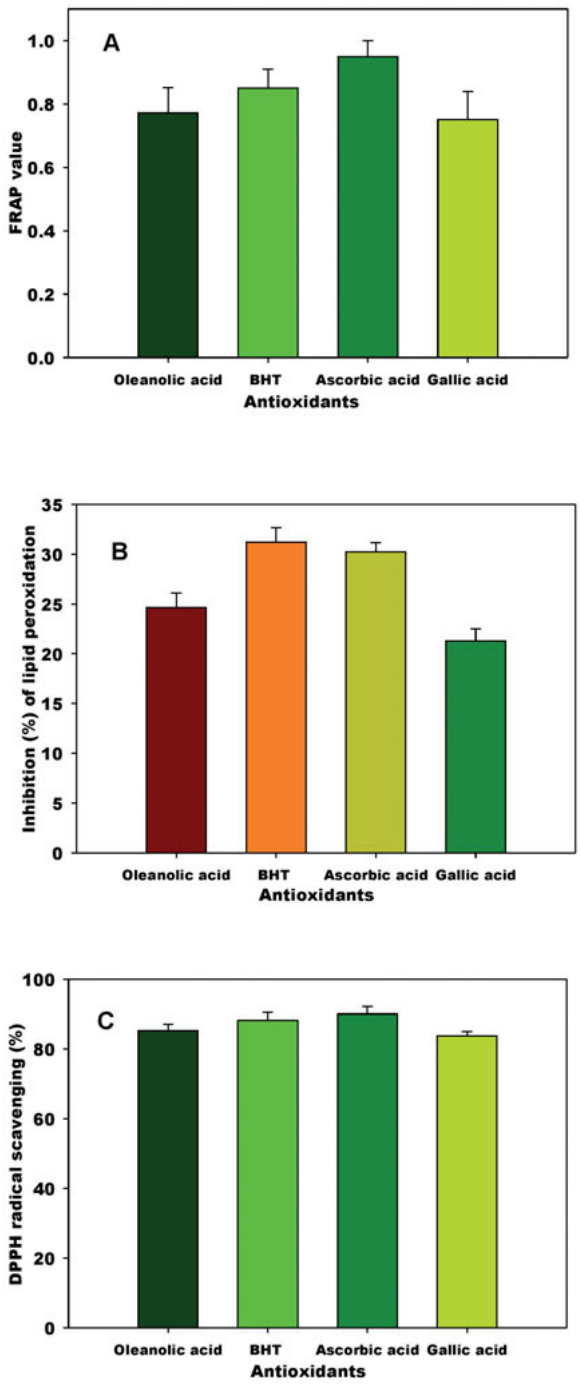

Figure 3. Comparison of antioxidant activities of oleanolic acid (purified from grape peel) with those of butylated hydroxytolutene or BHT, ascorbic acid and gallic acid. All tested samples contained 0.5 mg of pure compound / mL of dimethyl sulfoxide. Bars represent standard error of the mean (n=3) and those with different small letters are significantly ($P < 0.05$) different. Ferric reducing antioxidant power or FRAP value (A); lipid peroxidation inhibition (B) and anti-DPPH radical activity (C).

observed that oleanolic acid perhaps acts through indirect biological effect and protects human normal hepatocytes cells against cytotoxicity induced by tert-butyl hydroperoxide (tBHP) through increasing the generation of antioxidant and the expression of oxidative stress sensitive transcription factor-Nrf2, and mitogen-activated protein kinases, mainly c-Jun N-terminal kinases or JNK and protein kinases sub-family ERK. It can be observed in that oleanolic acid carries a phenolic hydroxyl group, which is generally attributed as the site for bioactivity or antioxidant ability of a bio-molecules against various ROS. This study demonstrates that grape peel is a potential source of oleanolic acid which is an important bioactive compound with possible health benefits.

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