Resveratrol compounds inhibit human holocarboxylase synthetase and cause a lean phenotype in *Drosophila melanogaster*

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Abstract

Holocarboxylase synthetase (HLCS) is the sole protein-biotin ligase in the human proteome. HLCS has key regulatory functions in intermediary metabolism, including fatty acid metabolism, and in gene repression through epigenetic mechanisms. The objective of this study was to identify foodborne inhibitors of HLCS that alter HLCS-dependent pathways in metabolism and gene regulation. When libraries of extracts from natural products and chemically pure compounds were screened for HLCS inhibitor activity, resveratrol compounds in grape materials caused an HLCS inhibition of >98\% in vitro. The potency of these compounds was piceatannol > resveratrol > piceid. Grape-borne compounds other than resveratrol metabolites also contributed toward HLCS inhibition, e.g., p-coumaric acid and cyanidin chloride. HLCS inhibitors had meaningful effects on body fat mass. When *Drosophila melanogaster brummer* mutants, which are genetically predisposed to storing excess amounts of lipids, were fed diets enriched with grape leaf extracts and piceid, body fat mass decreased by more than 30\% in males and females. However, Drosophila responded to inhibitor treatment with an increase in the expression of HLCS, which elicited an increase in the abundance of biotinylated carboxylases in vivo. We conclude that

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Author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; ELC, RA, DCT, and RZ conducted experiments; SO, PR, and FD supplied the PECKISH library and grape leaves, IZ wrote the manuscript.
mechanisms other than inhibition of HLCS cause body fat loss in flies. We propose that the primary candidate is the inhibition of the insulin receptor/Akt signaling pathway.

Keywords
Grapes; fat mass; Drosophila; holocarboxylase synthetase; inhibitor; resveratrol compounds

1. Introduction
Holocarboxylase synthetase (HLCS, E.C. 6.3.4.-) has essential functions in intermediary metabolism and gene regulation. The roles of HLCS in intermediary metabolism are mediated by five carboxylases, which attain biological activity through HLCS-dependent binding of the coenzyme biotin to distinct lysine residues in acetyl-CoA carboxylases (ACC) 1 and 2, pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), and 3-methylcrotonyl-CoA carboxylase (MCC) [1]. All five carboxylases catalyze the covalent binding of bicarbonate to organic acids. ACC1 and ACC2 convert acetyl-CoA to malonyl-CoA in key steps in cytoplasmic fatty acid synthesis and the regulation of mitochondrial fatty acid uptake, respectively. PC converts pyruvate to oxaloacetate in gluconeogenesis in the cytoplasm. PCC converts propionyl-CoA to methylmalonyl-CoA in the metabolism of odd-chain fatty acids in cytoplasm. MCC converts 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA in leucine metabolism in cytoplasm. Recently, 108 new biotinylated protein were discovered by mass spectrometry analysis [2]. The biological function of their biotinylation is unknown, despite some evidence that biotinylation plays a role in the functionality of heat shock proteins [2,3].

Theoretically, inhibition of HLCS-dependent biotinylation of ACC1 and ACC2 might lead to a decrease in body fat. For example, preventing the biotinylation of ACC2 in the outer mitochondrial membrane would lead to decreased production of malonyl-CoA in the mitochondrial microdomain. Malonyl-CoA is a strong inhibitor of carnitine palmitoyltransferase 1, thereby decreasing the uptake and β-oxidation of fatty acids in mitochondria [4]. Consistent with this observation, de-repression of carnitine palmitoyltransferase 1 by loss of ACC2 causes an increase in the β-oxidation of fatty acids. For example, energy expenditure and resistance to diabetes increase in ACC2 knockout mice compared with wild-type mice [5]. While these findings are controversial [6–8], a targeted inhibition of HLCS-dependent biotinylation of ACC2 in the outer mitochondrial microenvironment might prove to be a productive approach to improving metabolic health. Here, we tested the effects of a novel class of HLCS inhibitors, resveratrol metabolites, on carboxylase biotinylation and body fat mass in Drosophila melanogaster. Specifically, we sought to determine whether loss of carboxylase biotinylation is a more likely mechanism for decreasing body fat than the recently reported inhibition of insulin receptor-dependent insulin/Akt signaling pathway [9].
2. Materials and methods

2.1. HLCS activity

Two types of assays were employed to quantify HLCS activity. The basic principle is the same for both assays: Recombinant, full-length human HLCS (rHLCS) is prepared and purified as described [10]. Briefly, 30 nM rHLCS is incubated with biotin, cofactors, and the recombinant polypeptide p67, which comprises the 67 C-terminal amino acids in PCC, including the biotin-binding site K694 [11]. HLCS-dependent binding of biotin to p67 is assessed using streptavidin as probe. In the high-throughput variation of the assay, p67 is adsorbed to the plastic surface in 96-well plates for subsequent biotinylation by HLCS and quantification using IRDye®-800CW-streptavidin and an Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA). In the low-throughput variation of the assay, samples are incubated in a test tube, resolved by gel electrophoresis, and p67-bound biotin in transblots is probed with IRDye®-800CW-streptavidin and the Odyssey infrared imaging system. HLCS- or p67-free samples are used as negative controls in both assays. The gel-based assay has the advantage of convenient variation of assay parameters at the expense of comparably low throughput.

2.2. HLCS inhibitors

The PECKISH library of natural compounds (Christian-Albrechts University of Kiel, Kiel, Germany) was used for screening for HLCS inhibitors [12]. Briefly, the library contains aqueous extracts from >880 raw materials, including but not limited to fruits, spices, and leaf extracts disbursed in 96-well plates; plates and raw extracts were stored at −80°C. A subset of 72 extracts was used to screen for HLCS inhibitor activity. Grape leaves (Vitis vinifera, variety Gruner Veltliner) were obtained from Waldviertel’s Natureck (Plank am Kamp, Austria) and James Arthur Vineyards (varieties St. Croix and Edelweiss, Lincoln, NE). Extracts were prepared stirring 1 g of dried leaves into 10 mL of boiling distilled water and soaking the leaves for 10 min. Red (Langers, Inc) and white grape juices (Best Choice, Inc.) and red and white table grapes were obtained at local food stores. Pomace are the solid remains from pressing grapes for juice. Fresh pomace was obtained from James Arthur Vineyards (variety Edelweiss) and extracted as described for grape leaves. Chemically pure gallic acid, quercetin, ferulic acid, chlorogenic acid, ascorbic acid, phytic acid, caffeic acid, fumaric acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, catechin, cyanidin chloride, coenzyme Q10, hydroxyphenylethanol, ellagic acid, p-coumaric acid, citric acid, resveratrol, piceatannol and piceid were purchased from Sigma-Aldrich (St.Louis, MO, USA), Cayman Chemical (Ann Arbor, MI, USA) and Selleckchem (Houston, TX, USA) and used to prepare a second compound library with a focus on grape metabolites and for subsequent in-depth testing of individual compounds. For some compounds, the concentrations needed to cause a 50% inhibition of HLCS (IC_{50%}) were estimated by testing serial dilutions of inhibitors and calculating IC_{50%} through non-linear regression with the Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

2.3. Identification of phenolics

Separation and identification of major phenolics was accomplished with a Waters Millipore HPLC system (W.R. Grace and Co., Albany, OR) using a Waters 600s Controller with a
Vydac reverse phase C18 column (Millford, MA, USA) as described previously [13] with some modifications. Separation was achieved with a gradient mobile phase of (A) 50 mM (NH$_4$)$_2$PO$_4$ at pH 2.6, (B) acetonitrile and 50 mM (NH$_4$)$_2$PO$_4$ pH 2.6 at 80:20 (v:v) and (C) 200 mM H$_3$PO$_4$, pH 1.5 with 100% A for 4 minutes, 92% A and 8% B for 6 minutes, 14% B and 86% C for 12.5 minutes, 16.5% B and 83.5% C for 5 minutes, 25% B and 75% C for 22.5 minutes, 80% B and 20% C for 5 minutes, and 100% A for 5 minutes all at a flow rate of 1 ml per minute. Detection was performed using a Waters 2996 Photodiode array detector scanning at 280 nm, 320 nm and 380 nm. Extracts of the grape leaves were passed through a 0.45 µm filter and were kept at 4°C before injection at 10 µl with a Waters 717Plus Autosampler. Sample areas were compared to a standard curve prepared from select phenols ranging from 50 µM to 62.5 µM.

2.4. Body fat percentage in Drosophila melanogaster brummer mutants

A homolog of mammalian adipose triglyceride lipase, the lipid storage droplet-associated triacylglycerol lipase Brummer, is encoded by the brummer (bmm) gene in Drosophila melanogaster [14]. Loss of Brummer impairs the mobilizations from fat bodies in flies, i.e., brummer mutants are characterized by a large body fat mass [14]. Brummer mutants 15828 and 15959 were obtained from the Vienna Stock collection (Vienna, Austria) and reared on instant fly food (Formula 4–24 Plain, Carolina, Inc.; Burlington, NC, USA). The flies can live a maximum of 90 days with an average lifespan of 45 days. Seven days after eclosure, male and female virgins were separated and fed diets containing 0.05% or 1% (by weight) grape leaf extracts (Gruner Veltliner) or piceid (0.012 or 0.12 µmol/L) for 21 days. Soraphen A is an inhibitor of ACC1 and ACC2 [15] and was used as a positive control (5 µmol/L, 21 days). Flies were frozen (−80°C) and homogenized in a 5% Tween 20 solution. Triacylglycerols in the heat-inactivated (5 min at 70°C) extracts were incubated with Infinity Triglycerides solution (Thermo Scientific) at 37°C for 35 min and quantified colorimetrically (540 nm) using triolein as standard [16]. Biotinylated carboxylases were isolated from fly homogenates as described previously [17] and analyzed by streptavidin blot analysis.

2.5. Statistics

Bartlett’s test was used to confirm that variances were homogenous. One-way ANOVA and Fisher’s Protected Least Significant Difference was used to determine whether differences among treatment groups were significantly different. The unpaired t-test was used for pairwise comparisons. Statview 4.5 was used for all calculations [18]. $P < 0.05$ was considered statistically different. Data are reported as means ± SD.

3. Results

3.1. HLCS inhibitors

When the PECKISH library was screened for HLCS inhibitor activity using the 96-well plate assay, 21 extracts inhibited HLCS to an activity of <2% compared with inhibitor-free controls (see Fig. 1 for a representative image), including grape leaf extracts. The pool of candidate inhibitors was narrowed down as follows. First, extracts that caused a shift in the assay pH were disregarded. Representative examples include extracts from oranges and maté leaves (Ilex paraguariensis). Second, compounds that do not play a quantitatively
meaningful role in diets were disregarded. Representative examples include *Cassia fistula* and *Syzygium cumini*. Grape leaf extract passed these screening steps and was further investigated, partially because of the links of grape compounds such as resveratrol and its metabolites to cancer prevention, adipocyte differentiation, inhibition of inflammatory processes, and low body fat mass [9,19–24].

The effects of grape leaf extract on HLCS activity were confirmed in independent experiments. First, raw material from the PECKISH library was re-extracted and analyzed using the gel-based assay. When aqueous extracts from 1 mg of grape leaves (in 10 µL) were incubated with HLCS (50 µL final volume), biotinylation of p67 was considerably lower compared with inhibitor-free sample (Fig. 2A); an HLCS-free sample was used as negative control and produced no signal. Effects of mate leaf extract, oranges extracts, and a synthetic HLCS inhibitor are shown for completeness. Second, new raw material was obtained from Waldviertel’s Natureck Inc., extracted, and analyzed for inhibitor activity. The results were essentially the same as for previous assays (not shown). Third, leaf extracts were prepared from local varieties of grapes (*St. Croix* and *Edelweiss*). These extracts also were potent inhibitors of HLCS although their activities were less than that of extracts from *Gruner Veltliner* (Fig. 2B). Fourth, grape juices and extracts from crushed table grapes were tested for inhibitor activity. Juices and white grapes inhibited HLCS to a meaningful extent (Fig. 2C,D); crushed red grapes also were effective inhibitors of HLCS but, at the highest concentrations tested, these effects might have been caused by shifts in the assay pH (not shown). Fifth, HLCS inhibitor activity was also detected in pomace (Fig. 2E).

Resveratrol and its metabolites piceatannol and piceid account for the inhibition of HLCS by grape materials, but other compounds in grapes also contribute toward HLCS inhibition. When 19 compounds in grapes were assessed regarding their HLCS inhibitor activity by using the gel-based assay, 8 compounds inhibited HLCS by more than 90%, including the three resveratrol derivatives that were tested (Table 1). Piceatannol had a stronger effect on HLCS than resveratrol, which had a stronger effect than piceid, judged by dose-response studies comparing the three compounds (Fig. 3). IC₅₀ values were 0.28±2.12 µmol/L units for piceatannol, 3.70±0.0085 µmol/L units for resveratrol, and 21.30±0.014 µmol/L units for piceid. Note that grape compounds other than resveratrol and its metabolites also inhibited HLCS. For example, *p*-coumaric acid and cyanidin chloride had an effect similar to that of resveratrol (Table 1). The concentrations of resveratrol and other polyphenols varied between grape varieties (Table 2).

### 3.2. Body fat in Drosophila melanogaster brummer mutants

Grape leaf extracts and chemically pure piceid caused a significant loss of body fat in *brummer* mutants flies. When flies were fed diets containing an aqueous extract equaling 0.05 and 1% dried grape leaves for 21 days, the body fat mass was about 50% lower in males and females compared with controls (Fig. 4A–D). The same pattern was observed for *brummer* mutants 15828 (panels A and B) and 15959 (panels C and D). Likewise, when flies were fed diets containing 0.012 or 0.12 µmol/L piceid for 21 days, the body fat mass was about 30% lower in males and females compared with controls (Fig. 5A, B). Soraphen A,
inhibitor of ACC1 and ACC2, was used as positive control and caused a more than 60% decrease in body fat (Fig. 5C, D).

3.3. Biotinylation of carboxylases in Drosophila melongaster brummer mutants

Grape leaf extracts increased the amount of biotinylated carboxylases in brummer mutant flies. When flies were fed diets containing 1% dried grape leaves there was an increase in biotinylated ACC, MCC, PCC, and PC in males and an increase in biointylated PC in females. The absence of detectable ACC1, ACC2, MCC, and PCC was previously reported in female flies [17]. This was accompanied by an increase in HLCS protein (Fig. 6).

4. Discussion

This is the first report to identify naturally occurring inhibitors of HLCS. Our discoveries are relevant for human nutrition for two reasons. First, red and white grapes contain about 5 mg/L and 0.6 mg/L, respectively, of total resveratrol, piceid, and piceatannol [25–27]. The comparably low HLCS inhibitor activity of piceid compared with piceatannol and resveratrol is partially offset by its high levels in grape-borne materials [25]. Second, table grapes and grape juice are consumed in fairly large quantities in the United States and other countries. For example, the annual per person consumption of grapes (white and red) was 8.6 pounds in 2008 in the U.S., in addition to 5.7 pounds consumed in the form of grape juice [28]. Inhibitor activity was detected in all grape-based materials that were tested. Importantly, our studies provide unambiguous evidence that resveratrol and its metabolites inhibit HLCS not only in vitro but also cause body fat loss in Drosophila feeding studies.

While resveratrol metabolites inhibited HLCS efficiently in vitro, these effects were not seen in Drosophila feeding experiments in vivo. In fact, we found that there was an increase in biotinylated carboxylases in male and female flies fed diets containing 20% grape leaf extracts. This is due to an increase in HLCS expression. Interestingly, a similar pattern was observed when HEK-293 cells were treated with either piceatannol or grape leaf extract, or the synthetic HLCS inhibitor, β-ketophosphonate-5’-AMP (Online Supplementary Figs 1 and 2). Likewise, treatment of NIH/3T3 cells with the synthetic HLCS inhibitors β-hydroxyphosphonate-5’-AMP and biotinol-5’-AMP [29] resulted in an increase in biotinylated carboxylases (Online Supplementary Fig. 3). Consistent with our observations regarding natural HLCS inhibitors, HLCS expression increased ~3-fold when HEK293 cells were treated with biotinol-5’-AMP compared with solvent controls. We propose that HLCS is essential for cell survival, and cells and organisms respond to any perturbation of HLCS activity with an increase in HLCS expression. This proposal is based on the following rationale. The importance of HLCS is apparent in that no HLCS null individual has ever been reported, suggesting embryonic lethality, and mutations in the human HLCS gene cause a substantial decrease in HLCS activity and metabolic abnormalities [30,31]. Additionally, HLCS knockdown (~30% residual activity) in Drosophila melanogaster results in a reduced life span and heat tolerance [32] and aberrant gene regulation in human cell lines [33,34].

When considering that resveratrol metabolites do not appear to inhibit HLCS activity in vivo, the decrease in body fat mass in Drosophila observed in this study likely is due to the
mechanism proposed by Kwon et al. [9]. They propose that piceatannol inhibits differentiation of 3T3-L1 cells by delaying mitotic clonal expansion and in parallel preventing phosphorylation of the insulin receptor/Akt signaling pathway, leading to its inhibition. The substantial decrease in body fat mass in Drosophila fed a diet supplemented with grape leaf extract and piceid illustrates the potential benefits of these compounds for human health, considering the current epidemic of obesity and obesity-related diseases [35–37].

A few key observations in this study are worthwhile pointing out. First, no apparent difference was noted when comparing materials from red and white grapes. Second, the variety of grapes might be important regarding HLCS inhibitor activity, e.g., leaf extracts from Gruner Veltliner were more effective than extracts from Edelweiss. Third, bioactive compounds other than resveratrol and its metabolites, also contribute toward HLCS inhibitor activity in grape materials, e.g., p-coumaric acid and cyanidin chloride. Therefore, synergisms need to be considered in future studies, and experiments with chemically pure compounds should always be supplemented with studies using crude materials. Fourth, pomace contains meaningful amounts of HLCS inhibitors. It might be worthwhile considering purifying these compounds from pomace, which is typically considered as waste product in the production of wine and grape juice.

The following uncertainties remain. First, it is unknown whether HLCS inhibitors also affect gene repression through HLCS-dependent epigenetic mechanisms. We abstained from conducting such studies because effects of HLCS in gene repression are caused by HLCS/protein interactions that might not depend on the catalytic activity of HLCS [38–40]. Second, it is unknown which of the four domains in the HLCS domain interact with resveratrol and its metabolites [41]. Considering that the comparatively bulky glucose residue in piceid impaired inhibitor activity compared with resveratrol, one could assume that the resveratrol binding site in HLCS is rather specific [25]. We also do not know whether effects of resveratrol compounds are stereospecific; trans isomers or piceid and resveratrol are more abundant than cis isomers in grape materials [25]. Third, he expression of HLCS is regulated by three promoters [42,43], but the responsiveness of regulatory elements in these promoters to HLCS inhibitors is unknown. These uncertainties are currently being addressed in our laboratories.

Taken together, resveratrol metabolites caused a substantial 50% fat loss in flies. Our studies suggest that loss of biotinylation events do not contribute to loss of body fat, and that the observed effects can be attributed to inhibition of the insulin receptor/Akt signaling pathway as proposed previously [9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgements

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Highlights

Resveratrol metabolites inhibit holocarboxylase synthetase in vitro.

Inhibitors of holocarboxylase synthetase (HLCS) cause an increase in HLCS expression.

Resveratrol metabolites cause a lean phenotype in Drosophila.
Fig. 1.
Representative example of HLCS activity in samples treated with extracts from the PECKISH library of natural compounds. HLCS activity was assayed using a 96-well plate format, values in individual wells denote HLCS activity (% of controls). Identifiers: B3 and B4 = vehicle controls; C3 = grape leaf extract; A4 = Cassia fistula; C7 = Syzygium cumini; row H is the calibration curve containing defined amounts of HLCS.
Fig. 2.
(A) Gel-based assay of HLCS activity in the absence and presence of grape leaf extract. A sample without HLCS was used as negative control. Extracts from maté leaves and oranges were not considered for subsequent studies, because of their inhibitor activity was caused by shifts in the assay pH as discussed in the text. (B) Comparison of leaf extracts from Gruner Veltliner, St. Croix, and Edelweiss. HLCS activity was measured in the presence of and aqueous extract of 500 µg grape leaves in a sample volume of 50 µL; controls were prepared using vehicle and by omitting HLCS. Lanes were electronically re-arranged to facilitate comparisons. (C) Effects of grape juices on HLCS activity. (D) Effects of crushed white
grapes on HLCS activity, quantified by gel densitometry. (E) Effects of pomace extract (variety Edelweiss) on HLCS activity.
Fig. 3.
Comparison of the effects of resveratrol, piceatannol, and piceid on the inhibition of HLCS.
Fig. 4.
Effect of grape leaf extract on body fat mass in male and female *Drosophila melanogaster brummer* mutants 15828 (panels A and B) and 15959 (panels C and D). Flies were fed a diet supplemented with 0.05 or 1% grape leaf solids (as extracts) for 21 days; controls were fed an extract-free diet. a,b Bars not sharing the same letter are significantly different ($P < 0.05$; n=4 tubes, each containing 40 flies).
Fig. 5.
Effect of piceid (panels A and B) and soraphen A (panels C and D) on body fat mass in male and female *Drosophila melanogaster brummer* mutant 15828. Flies were fed a diet supplemented with 0.012 µmol/L piceid, 0.12 µmol/L piceid, or 5 µmol/L soraphen A for 21 days; controls were fed piceid-free and soraphen A-free diets. Bars not sharing the same letter are significantly different (*P* < 0.05; *n*=4 tubes, each containing 40 flies).
Abundance of biotinylated holocarboxylases and HLCS in in male and female *Drosophila melanogaster* brummer mutant 15828. Flies were fed a diet supplemented with 0.05 or 1% grape leaf solids (GLS, as extracts) for 21 days; controls were fed an extract-free diet. Biotinylated carboxylases, HLCS, and β-actin (control) were probed using streptavidin, anti-HLCS, and anti-β-actin, respectively. ACC, acetyl-CoA carboxylases; MCC, 3-
methylcrotonyl-CoA carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase.
Table 1

Inhibition of HLCS by bioactive compounds in grapes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitor activity $^\ddagger$</th>
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<tbody>
<tr>
<td>Piceatannol</td>
<td>+++</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>+++</td>
</tr>
<tr>
<td>Piceid</td>
<td>+++</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>+++</td>
</tr>
<tr>
<td>Quercetin</td>
<td>+++</td>
</tr>
<tr>
<td>Cyanidin chlorid</td>
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</tr>
<tr>
<td>Gallic acid</td>
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</tr>
<tr>
<td>3-Hydroxybenzoic acid</td>
<td>+++</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>+++</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>+++</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>+++</td>
</tr>
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<td>Fumaric acid</td>
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<tr>
<td>Phytic acid</td>
<td>++</td>
</tr>
<tr>
<td>Hydroxyphenylethanol</td>
<td>+</td>
</tr>
<tr>
<td>Coenzym Q10</td>
<td>+</td>
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</tbody>
</table>

$^\ddagger$ Sorted by effect size.

$^\ddagger$ Symbols denote inhibitor activity (+++++, >90% inhibition of HLCS; ++++, >80% inhibition; +++ , >60% inhibition; ++ , >40% inhibition; +, <20% inhibition), judged by HLCS-dependent biotinylation of p67 in the gel-based HLCS assay and gel densitometry. Compounds were tested at concentrations of 0.05 to 5.7 mM.
### Table 2
Concentrations of polyphenols in grape leaves in two varieties.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Grüner Veltliner</th>
<th>Edelweiss</th>
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</thead>
<tbody>
<tr>
<td>Piceatannol</td>
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</tr>
<tr>
<td>Resveratrol</td>
<td>n.d.</td>
<td>0.64±0.68</td>
</tr>
<tr>
<td>Piceid</td>
<td>3.07±0.11</td>
<td>1.74±0.44</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>8.42±0.39</td>
<td>0.92±0.30</td>
</tr>
</tbody>
</table>

¹ Values are means ± standard deviations.

² n.d., not detectable.