INCREASED CYTOTOXICITY OF 3,5 DIHYDROXY -7- METHOXYFLAVONE IN MIA PACA-2 AND PANCREATIC CANCER CELLS WHEN USED IN CONJUNCTION WITH PROLIFERATIVE COMPOUND 3,5 DIHYDROXY-7-METHOXYFLAVANONE BOTH DERIVED FROM CHROMOLAENA LEIVENSIS (HIERON)

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Abstract
Over 5000 flavonoids have been identified so far and many of these are known to have antineoplastic properties. The relationships between the targeting activities by these compounds on cancer cells and the specific features that determine their molecular structures are not completely elucidated. Here we report the differential cytotoxic effects of two unsubstituted ring B flavonoids that differ solely in the presence of a C2, C3 double bond in ring C, on human cancer cells of the lung (A549), pancreas (MIA PaCa-2, Panc28), colon (HCT 116, CaCo-2), Liver (HepG2), and breast (SKBr3). These compounds were extracted from Chromolaena levensis (Hieron) a plant belonging to the genus Chromolaena reputed to have antitumor activities. 3, 5 dihydroxy-7-methoxyflavone induce apoptosis in cancer cells of the lung A549, pancreas MIA PaCa-2 and Panc28, and colon HCT116, but not on Caco-2; whereas 3,5 dihydroxy-7-methoxyflavanone display proliferative effects in A549, Panc 28, MIA PaCa, and HCT116 cells at low concentrations, and slight cytotoxicity only on CaCo-2, a cancer cell line with a higher differentiation status than other cells tested. At the concentrations studied (5-80µM) neither compound demonstrated activity against cancer cells of the liver (HepG2) or breast (SKBr3) as indicated by MTT cell viability assays. When used in combination with 3,5 dihydroxy-7-methoxyflavone in pancreatic cancer cells, the targeting preference of 3, 5 dihydroxy-7-methoxyflavanone is altered, and a significant increase in inhibition of cell viability is observed 48 hours after dosing. The presence or absence of the C2, C3 double bond in ring C, accounts for electrochemical and structural changes that dictate differential specificity towards cancer cells. 3,5 dihydroxy-7-methoxyflavone has a more planar structure, whereas the absence of the double bond in C2, C3 causes ring B to adopt a perpendicular orientation to the plane formed by rings A and C and the OH group at C3.

Key words: flavonoids, Chromolaena leivensis, 3,5 dihydroxy -7- methoxyflavone, 3,5 dihydroxy -7- methoxyflavanone, cancer, pancreatic cancer, lung cancer.

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Introduction
Flavonoids are polyphenolic compounds with a phenylbenzopyrone structure (C6-C3-C6) commonly found in plants (1). Over 5,000 flavonoids have been discovered (2) and are divided into subgroups depending upon the level of oxidation of ring C, a benzo-γ-pirone (3). These subgroups include isoflavones, flavonols, flavanones, flavanones, flavanols, anthocyanidins, and chalcones (1, 4). Flavonoids have been recognized to possess several bioactive properties such as antioxidant (5, 6), antiviral (7, 8), and anti-diabetic, as well as the ability to protect the gastrointestinal tract (9), and cardiovascular system (4). In cancer research, some flavonoids have been found to have chemoprotective properties (10-12), while others have been reported to display antineoplastic activities (10, 13, 14).

Previously, it has been reported that plants belonging to the genus Chromolaena inhibit cell survival in cancer cells. Indeed, extracts derived from leaves and inflorescences of C. odorata have shown cytotoxic activity against cancer cells of the lung (15). Furthermore, studies on the same species have reported that p-hydroxybenzoic acid, p-Coumaric acid, flavones, flavanones and chalcones protect skin cells from oxidative damage and facilitate the repair process (16) suggesting the absence of a cytotoxic effect of these bioactive compounds on normal cells. In this study, we report on the cytotoxic activity on cancer cells by two flavonoids extracted from Chromolaena leivensis (Hieron), a wild plant commonly recommended as an anticancer treatment in some areas of the Andean region of Colombia, and a component of the traditional ethnobotanical knowledge of this region (17). We had previously isolated these compounds from the leaf extract of C. leivensis (Hieron), and identified them as 3,5 dihydroxy -7-methoxyflavone or CHL 1, and 3,5 dihydroxy -7-methoxyflavanone or CHL 2 (Figure 1).

Preliminary tests of the compounds using human erythroleukemia K562 and melanoma A375 cells showed cytotoxic activity against these cells, lending initial support to the notion that C. leivensis (Hieron) may have anticancer properties (18). In this study we further explore the antineoplastic effects of these two compounds on human cancer cells derived from lung (A549), colon (HCT 116 and CaCo 2), pancreas (MIA PaCa-2 and Panc 28), liver (HepG2), and breast (SKBr3). These two flavonoids are compounds with unsubstituted ring B and differ solely in the presence of the C2=C3 double bond in ring C, thus displaying differences in the electrochemistry and the structure of the molecules. Previous studies of isomeric flavonoids with unsubstituted ring B, have shown differential targeting by these compounds of highly tumorigenic cells with varying differentiation status (19). Furthermore, it was shown that both of these compounds depend on distinct apoptotic pathways to exert their cytotoxic activities on the specific target cells (20). We hypothesized that CHL 1 and CHL 2 may have differential cytotoxic activities on cancer cells, and that these differences may help elucidate the contributions of the presence of the C2=C3 double bond in flavonoids with unsubstituted ring B, to distinct antineoplastic targeting.

Methods
Procedure to obtain 3,5 dihydroxy -7-methoxyflavone and 3,5 dihydroxy -7-methoxyflavanone.

Both flavonoids were isolated, purified and identified as previously described (18). Briefly, flowering plants were collected in the outskirts of Bogota Colombia (40°33’00.7”N 74°15’00.5”W) and a control sample was sent to the National Herbarium of Colombia for identification and was determined to be Chromolaena leivensis (Hieron). King & H. Rob, number COL-535 219 Colombian National Herbarium. The extraction was carried out in Soxhlet with 95% ethanol. The extract was mixed with silica gel (1:2) and extracted solid-liquid with petroleum ether, toluene, dichloromethane, ethyl acetate and methanol, successively. A portion of the Toluene fraction was subjected to column chromatography with silica gel (Merck Kieselgel), eluting with petroleum ether, toluene and methanol in various proportions. The flavonoids were obtained by fractional crystallization and were identified by physical, chemical and spectroscopic assays.

Cell Lines and Culture Conditions.
Colon (HCT 116, CaCo 2), pancreas (MIA PaCa-2), liver (HepG2), breast (SKBr3), and lung (A549) cancer cell lines were obtained from the American Tissue Type Culture Collection (Mannassas, VA). All cell lines were cultured following the ATCC recommendations. Pancreatic cancer cell line, Panc 28, was a gift from Dr. Paul Chiao from the University of Texas M.D. Anderson Cancer Center, Houston, TX, and was cultured in the same manner as recommended for MIA PaCa-2 cells, in Dulbecco's modified Eagle's medium with high glucose (GIBCO/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin/streptomycin.
**Cell Viability Assay.**

Cells were plated in 48 well plates and allowed to grow to ~75% confluent before being treated with either vehicle, CHL 1, CHL 2, or combination of CHL 1 and CHL 2. The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma- Aldrich) and dosed at final concentrations of 0, 5, 10, 20, 40, and 80 µM. Pancreatic cancer cells were also treated with gemcitabine hydrochloride (Sigma- Aldrich) dissolved in DMSO and dosed at final concentrations of 0, 1, 2, 4, 8, and 16 µM (21-24). The maximum final concentration of DMSO in the treated well was ≤0.27% of the total volume in the well. After 24 or 48 hours of incubation, a solution of 5mg/mL of 3-(4, 5-methyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; from Sigma Aldrich) was added at 10% of the volume of each well, and then incubated for 3.5 hours. Formazan products were solubilized with acidified 2-propanol and optical density was measured at 570 nm with a Biotek Synergy HT plate reader (Winooski, VT). Data from assays displaying a decrease of cell viability ≥50%, were evaluated by nonlinear regression analysis (GraphPad Prism, La Jolla, CA), and represented as the effective concentration required to decrease 50% of cell viability (EC50).

**Apoptosis Assay.**

Cells were seeded on cover slips and grown to ~75% confluent, and treated with vehicle (DMSO), or 40 µM of either CHL 1, CHL 2, combination, for 4 or 24 hours. TUNEL (TdT-mediated dUTP nick end labeling) was performed according to manufacturer’s instructions (Roche; Mannheim, Germany). Briefly, cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% sodium citrate and 0.1% Triton. Nuclei of the cells were stained with DAPI and fluorescence images obtained with an EVOS microscope (AMG; Bothell, WA).

**X-ray Diffraction Analysis (XRD).**

Data collection, cell refinement, and data reduction: MSC/AFC65 diffraclometer control software (Molecular Structure Corp., The Woodlands, TX). Program used to solve structure: SHELXS97; program used to refine structure: SHELXL97; molecular graphics: SHELXTL-PC (G.M Sheldrick, Institute of Inorganic Chemistry Göttingen, Germany).

**Analysis of Antioxidant activity.**

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was conducted by adding DPPH at a final concentration of 0.05mM to the flavonoids at different concentrations. The samples were allowed to incubate in the dark for 60 minutes and the absorbance was measured at 540nm in a Cary 50 (Varian, Cary, NC). The activity is expressed as the percent of inhibition of DPPH, and radical scavenging activity is expressed as AOC50 (the concentration of substrate which causes a 50% loss of DPPH activity, or antioxidant capacity).

**Statistical Analysis.**

The effect on cell viability was analysed for significant difference using two-way ANOVA followed by a Tukey’s HSD test, in which compound or combination of compounds and concentration were considered to be study factors. (SAS 9.2; SAS Institute, Cary, NC). Statistical significance was set at p 0.05.

**Results**

**Flavone CHL 1 exerts a significantly higher cytotoxic effect via apoptosis on lung cancer A549 cells as compared to flavanone CHL 2.**

To test the potential cytotoxic activity of the compounds against lung cancer, lung alveolar basal epithelial carcinoma A549 cells were treated with either CHL1 or CHL2 at concentrations ranging from 5-80 µM. MTT assays done 24 hours after treatment showed that CHL1 effectively decreased cell viability of A549 cells (Figure 2A). Conversely, treatment with CHL2 caused an increase in cell viability at 5, 10, and 20µM, displaying cytotoxicity only at concentrations higher than 40µM (Figure 2B). The half maximal effective concentrations of the compounds are 24.19 ± 1.34 µM for CHL 1, and for CHL 2 it is beyond the tested concentrations at 120.80µM (Figure 2C). To determine whether treatment with the flavone cause apoptosis in A549 cells, TUNEL assay was performed. The presence of fragmented DNA in A549 cells treated with 40µM of CHL1, suggest that the observed loss of cell viability is the result of the activation of apoptosis as shown in figure 2D, green channel. As expected no DNA fragmentation is observed on cells treated with CHL 2 at this concentration (Figure 2D).

**Flavone CHL 1 but not flavanone CHL 2 inhibits cell viability and causes apoptosis on pancreatic cancer Panc28 and MIA PaCo-2 cells.**

Previous studies of isomeric flavones which also displayed an unsubstituted ring B, have shown differential targeting of highly tumorigenic cancer cells with varying differentiation status (19). To test whether CHL 1 and CHL 2 display similar targeting,
better differentiated pancreatic cancer Panc 28 cells and poorly differentiated MIA PaCa-2 cells were treated with CHL 1 and CHL 2 at concentrations ranging from 5-80µM. Cell viability assays via MTT showed that CHL 1 has a cytotoxic effect on both pancreatic cancer cell lines with half maximal effective concentrations of 71.75 and 52.3 µM for Panc28 and MIA PaCa-2 cells respectively (Figure 3A and C). Conversely, CHL 2 failed to inhibit cell viability on either pancreatic cancer cell line (Figure 3B), with a proliferative effect at low concentrations, as evidenced by increased cell viability at 5 and 10µM in Panc28 cells. The cytotoxic effect observed on pancreatic cancer cells after treatment with CHL 1, may involve the induction of apoptotic signalling pathways as suggested by the presence of fragmented DNA (figure 3D, green channel) in Panc28 cells dosed with 40µM CHL 1, as shown by TUNEL assay. The results shown in figure 3D confirm the absence of effect by CHL 2 firstly observed via MTT. Similar results were obtained for TUNEL assay performed on MIA PaCa-2 cells dosed with 40µM of either CHL 1 or CHL2 (results not shown).

**CHL 1 and CHL 2 have a differential apoptotic effect on colon cancer cells.**

To determine whether cell differentiation status is a factor in the targeting and cytotoxic effect by CHL 1 and CHL 2 on colon cancer cells, better differentiated CaCo 2(25) and poorly differentiated HCT 116 cells were treated with either CHL 1 or CHL 2 at concentrations between 5 and 80µM. MTT assay data showed that CHL 1 decreased cell viability of HCT 116 cells with a half maximal effective concentration 64.48µM. However, it had no effect on CaCo 2 cells (Figure 4A and C). Conversely, the flavanone CHL 2 appear to inhibit cell viability of CaCo 2 with a half-maximal effective concentration of 83.22µM, with no effect on HCT 116 (Figure 4B and C). TUNEL assay data show that both compounds induce apoptosis in their targeted cells as evidenced by the presence of fragmented DNA (figure 5A and B, green channel), confirming the differential effect on cell viability of CHL 1 and CHL 2 observed via MTT assay (figure 4).

A greater and sustained decrease of cell viability is observed in pancreatic cancer cell lines 48 hours after treatment with the combination of CHL 1 and CHL 2 as compared to treatment with CHL 1 as a single agent.

To determine whether there is a beneficial effect by the treatment of the cells with the combination of the compounds, all cell lines were dosed and analysed via MTT assay at 24 and 48 hours after treatment. No changes were observed after 24 hours as compared to treatment with CHL1 alone on any of the cell lines tested. However, a stronger effect was observed on pancreatic cancer cell lines 48 hours after dosing. MIA PaCa-2 and Panc28 cells dosed with the combination of CHL1 and CHL2, display significantly lower cell viability as compared to treatment with either individual agent. (Figure 6A and 6B). Furthermore, the effect on cell viability observed at 48 hours after treatment with the combination of CHL1 and CHL2 on pancreatic cancer MIA PaCa-2 and Panc28 cells, was significantly greater than the effect observed at 48 hours after dosing the cells with gemcitabine hydrochloride (Figure 6C) at effective concentrations (22, 24). The effect on cell viability of combination treatment of CHL 1 and CHL 2 on lung cancer A549 and colon cancer HCT 116 and CaCo 2 cells, did not differ from the results obtained with the individual compound, and returned to control levels after 48 hours in the colon cell lines (data not shown).

**The ring B on flavanone CHL 2 has a perpendicular orientation to the plane formed by rings A and C and the OH group at C3.**

X-ray diffraction analysis of CHL 2 showed that the position of the phenyl ring has a perpendicular orientation relative to the flavonoid ring (figure 7A). Conversely, the CHL 1 molecule has a more planar orientation in agreement with previous molecular modelling studies of flavones with unsubstituted ring B (26). Due to the saturated heterocyclic ring and the consequent lack of conjugation between the A and B rings, CHL 2 is expected to have lower antioxidant activity than CHL 1(6, 27). However, since the 3-OH in the C ring is responsible for antioxidant activity we tested its radical scavenging activity by DPPH.

**Discussion**

Previous studies on flavonoids have reported that the reduction of the C2, C3-unsaturated bond in the C ring with a 3-OH and a 4-carbonyl has no effect on the antioxidant activity (6). This is not the case in molecules with unsubstituted ring B, as it is shown in the present study where the reduction of the C2=C3 bond, showed a total loss of activity. Thus, effectiveness for radical scavenging and perhaps for the inhibition of viability of some cancer cells, requires the C2=C3 bond, regardless of the presence of the 3-OH group and the 4-carbonyl in the C-ring in these flavonoids. The presence of the C2=C3 bond...
also dictate structural changes in the molecule which subsequently may contribute to the observed cytotoxic activity. The flavone CHL 1 is most cytotoxic to A549 lung cancer cells with and ECSO of 24.19 followed by pancreatic cancer MIA PaCa-2 (ECSO 52.30), and colon cancer HCT 116 (ECSO 64.48). Moderate to complete absence of activity at the concentrations tested, was observed on pancreatic Panc28 (ECSO 71.75), colon CaCo-2, liver HepG2, and breast SKBr3 cancer cells. We have also shown that the inhibition of cell viability by CHL 1, may include the activation of apoptotic pathways as indicated by the presence of fragmented DNA via TUNEL assay. The flavanone CHL 2 has no significant activity on any of the cells tested, except for minimal activity on colon cancer CaCo-2 cells (ECSO 83.22). It was recently reported that flavone 3,5-dihydroxy-6,7,8-trimethoxyflavone (Flavone B) has a preference for poorly differentiated cancer cells (19) cells with significantly lower ECSO values for MIA PaCa-2 (ECSO 33.18) and HCT116 (ECSO 37.58), and no observed cytotoxic activity on better differentiated Panc28 and CaCo-2 cells (28). Flavone B differs from CHL 1, solely on the presence of two additional methoxy groups in positions C6 and C8. However, our results show absence of activity on both, better differentiated CaCo 2, as well as poorly differentiated HepG2 and SKBr3 cancer cells. These results suggest that the preferential targeting of poorly differentiated cells by unsubstituted ring B flavones with a 3-OH may require the presence of the additional two methoxy groups on positions C6 and C8. In the case of flavanone CHL 2, our results indicate that it has slight cytotoxic activity only on CaCo-2 of the cell tested. These observations are in agreement with previously reported studies that showed that flavanones have a lower anti-proliferative effect on cancer cells than flavones (29). Our data obtained 24 hours after treatment with flavone CHL 1 and flavanone CHL 2, lend support to this observation. The differential effects of the flavone versus the flavanone are further evidenced by the results obtained 48 hours after treatment with either compound or the combination of CHL 1 and CHL 2. While there is no significant change in viability of cancer cells of the lung (A549) or colon (HCT-116, CaCo-2), 48 hours after treatment with CHL 1, CHL 2, or the combination of both compounds as compared to the results obtained after 24 hours, this is not the case in pancreatic cancer cells. MIA PaCa and Panc28 pancreatic cancer cells treated with CHL 2 showed an increase in cell viability, whereas the flavone CHL 1 failed to have a significant effect as compared to the control (Figures 6A, 6B). Thus, there is no unexpected sustained effect of the flavone after 48 hours of treatment in cells of the pancreas. Surprisingly, treatment of MIA PaCa-2 and Panc 28 cells with the combination of CHL 1 and CHL 2 produced a significant decrease in cell viability 48 hours after treatment, as compared to the effect of either single compound, suggesting an interesting mechanism of action for the combination of these compounds. These results may indicate that the intensity of the inhibitory effect on cell growth by CHL 1 is not a factor in its behavior when dosed in combination with CHL 2 since the flavone is most effective in lung cancer cells, while requiring higher doses on pancreatic and colon cancer cells to achieve significant levels of cytotoxicity. Furthermore, it may also be evidence of specific targeting of pancreatic cells with varying phenotypic characteristics, requiring a longer time to inhibit cell viability.

We studied two unsubstituted ring B flavonoids with a 3-OH, that differ solely in the presence of a C2, C3 double bond in ring C. This difference accounts for electrochemical and structural changes that dictate differential specificity towards cancer cells. CHL 1 has a more planar structure, whereas the absence of the double bond in C2, C3 causes ring B to adopt a perpendicular orientation to the plane formed by rings A and C and the OH group at C3. Flavone CHL 1 demonstrated to induce apoptosis in all cells tested except CaCo-2, HepG2, and SKBr3, while flavanone CHL 2 is slightly cytotoxic only to CaCo-2, a cancer cell line with a higher differentiation status than other cells tested. CHL 2 targeting preference is altered when combined with CHL 1 and dosed to cancer cells of the pancreas MIA PaCa and Panc 28. The information presented here warrants further study of the mechanism of action of these compounds, and the relationships between chemical structures, type and position of substituent groups and their cytotoxic effects on cancer cells.

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**References**


**Figure 1.** Molecular structures of flavonoids CHL 1 and CHL 2 A. 3,5- dihydroxy-7-methoxy flavone or CHL 1 B. 3,5 dihydroxy -7- methoxyflavanone or CHL 2.

**Figure 2.** Comparison of the effects of CHL 1 and CHL 2 on lung carcinoma A549 cells. The effects of CHL 1 (A) and CHL 2 (B) on lung cancer A549 cells were determined by MTT assays and are represented as a percent of the control absorbance at a wavelength of 570nm. All data were collected at 24 h after treatment. Data shown are from representative experiments (mean± SE, n = 3). * p<0.05, significant difference between control and other concentrations for each compound. C. Half maximal effective concentration (EC50)± SE for CHL 1 and CHL 2 treatment on A549 cells. The values were estimated by nonlinear regression analysis. D. Apoptotic effect of CHL 1 but not CHL 2 at a concentration of 40 µM, on A549 cells, as determined by TUNEL assay (green channel) 4 hours after treatment. DAPI (blue channel) is used to locate the nuclei of the cells. Control cells were treated with vehicle only (DMSO at a final concentration of ≤0.3%).
Figure 3. Comparison of the effects of CHL 1 and CHL 2 on pancreatic cancer MIA PaCa-2 and Panc28 cells. The effects of CHL 1 (A) and CHL 2 (B) on MIA PaCa-2 and Panc 28 pancreatic cancer cells were determined by MTT assays and are represented as a percent of the control absorbance at a wavelength of 570nm. All data were collected at 24 h after treatment. Data shown are from representative experiments (mean± SE, n = 3). * p<0.05, significant difference between control and other concentrations for each compound. C. Half maximal effective concentration (EC\textsubscript{50}) ± SE for CHL 1 and CHL 2 treatment on MIA PaCa and Panc28 cells. The values were estimated by nonlinear regression analysis. D. Apoptotic effect of CHL 1 but not CHL 2 at a concentration of 40 µM, on Panc28 cells, as determined by TUNEL assay (green channel) 4 hours after treatment. DAPI (blue channel) is used to locate the nuclei of the cells. Control cells were treated with vehicle only (DMSO at a final concentration of ≤0.3%).
Figure 4. Differential effects of flavone CHL 1 and flavanone CHL 2 on colon cancer CaCo 2 and HCT 116 cells. The differential effects of CHL 1 (A) and CHL 2 (B) on CaCo-2 and HCT 116 colon cancer cells were determined by MTT assays and are represented as a percent of the control absorbance at a wavelength of 570nm. All data were collected at 24 h after treatment. Data shown are from representative experiments (mean±SE, n = 3). * p<0.05, significant difference between control and other concentrations for each compound. C. Half maximal effective concentration (EC50) ± SE for CHL 1 and CHL 2 treatment on CaCo-2 and HCT 116 cells. The values were estimated by nonlinear regression analysis.

Figure 5. Apoptotic effect of CHL and CHL 2 on colon cancer CaCo2 and HCT-116 cells. Apoptotic effect of CHL1 and CHL 2 on CaCo 2 cells (A) and HCT 116 (B) after treatment with either flavonoid at a concentration of 40 μM, as determined by TUNEL assay (green channel) 4 hours after treatment. DAPI (blue channel) is used to locate the nuclei of the cells. As a control cells were treated with vehicle only (DMSO at a final concentration of ≤0.3%) and TUNEL assay was carried out parallel to the treated samples.
Figure 6. Differential effect of CHL 1, CHL 2, or the combination of both flavonoids 48 hours after treatment. Pancreatic cell lines (A) MIA Paca and (B) Panc 28 were treated with CHL 1, CHL 2, or an equal concentration of both flavonoids for 48 hours. (C) MIA Paca and Panc 28 cell lines were treated with gemcitabine hydrochloride. Cell viability was determined by MTT assays and are represented as a percent of the control absorbance at a wavelength of 570 nm. All data were collected at 24 h after treatment. Data shown are from representative experiments (mean±SE, n = 3). * p<0.05, significant difference between control and other concentrations for each compound. † and ‡ p<0.05, significant difference between the combination of CHL 1 + CHL 2 and CHL2; and CHL1+CHL2 and CHL1 and CHL2, respectively.

Figure 7. Molecular structure of CHL 2 and antioxidant activity of the flavonoids. A. Structure of the flavanone CHL 2 displaying the orientation of the rings was determined by X-ray diffraction analysis. The antioxidant activity of the flavanone CHL 2 (B) and the flavone CHL 1(C and D) were determined via DPPH assay at the various concentrations indicated. Data shown are from representative experiments (n=3).