Research Article

Uncommon Trimethoxylated Flavonol Obtained from Rubus rosaefolius Leaves and Its Antiproliferative Activity

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This study shows the evaluation the antiproliferative effect of the extract, fractions, and uncommon compounds isolated from R. rosaefolius leaves. The compounds were identified by conventional spectroscopic methods such as NMR-H1 and C13 and identified as 5,7-dihydroxy-6,8,4󸀠-trimethoxyflavonol (1), 5-hydroxy-3,6,7,8,4󸀠-pentamethoxyflavone (2), and tormentic acid (3). Both hexane and dichloromethane fractions showed selectivity for multidrug-resistant ovary cancer cell line (NCI-ADR/RES) with total growth inhibition values of 11.1 and 12.6 μg/ml, respectively. Compound 1 also showed selective activity against the same cell line (18.8 μg/ml); however, it was especially effective against glioma cells (2.8 μg/ml), suggesting that this compound may be involved with the in vitro antiproliferative action.

1. Introduction

Many are the advances in drug discovery for cancer prevention and treatment, but still there is a need for new therapies, as the statistics for cancer incidence and death by cancer worldwide are noteworthy [1]. Nature is an important source of new molecules with biological activities; around 75% of the anticancer agents are derived from or inspired by natural products [2]. Rubus is the largest genus in the Rosaceae family, which is used especially as ornamental plants and for food, as it has tasty fruits. In the folk medicine, some species are used to treat different diseases, particularly diabetes [3, 4]. Phytochemical and pharmacological studies have shown that some species exert gastroprotective, antimicrobial, and cytotoxic effects, without genotoxic effects [5–11]. Rubus rosaefolius is popularly known as “red berry,” and its fruits are consumed as food. Previous phytochemical investigations revealed the presence of sterols and terpenoids, which exhibited important antibacterial and antinociceptive activities [12, 13]. In this study, we have isolated and structurally characterized an uncommon methoxylated flavonol from the methanolic extract of Rubus rosaefolius leaves. Flavonols are an important class of compounds with many biological activities, including anticancer [14]. It is postulated that presence of 3-OH is essential for inhibition of topoisomerase in several cancer cell lines and substantially influences several mechanisms of antioxidant activity [14]. Also, methylation of some hydroxyl groups enhances the cytotoxic activity of some flavonols [14–16]. Bearing in mind the potential of methoxylated flavonols as anticancer agents and the need of new antiproliferative therapies, we evaluated the antiproliferative effect of the crude extract, fractions, and the uncommon methoxylated flavonol (1) in eight human cancer cell lines.
2. Results and Discussion

Dichloromethane (DCM) and hexane (HE) fractions of *R. rosaefolius* leaves were subjected to a series of silica gel chromatography columns to obtain three compounds, an uncommon methoxylated flavonol (1) along with the known compounds 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (2) and tormentic acid (3) (Figure 1). All compounds were identified by 1D and 2D RMN analysis and comparison of physical and spectroscopic data with those of literature [17, 18]. Compound 1 was obtained as a yellow crystal and showed a [M]+ peak at m/z 360.3 in the electron impact ionization mass spectroscopy (EIMS), corresponding to a molecular formula C_{18}H_{16}O_{8}. The IR spectrum showed the presence of hydroxyl groups (3354 cm\(^{-1}\)), a conjugated carbonyl group (1624 cm\(^{-1}\)), and aromatic rings (1500–1600 cm\(^{-1}\)). The \(^1\)H NMR spectrum (Table 1) revealed three downfield singlets at \(\delta H 12.26, 10.38,\) and 9.56, all disappearing by addition of D\(_2\)O, suggesting the presence of three phenolic hydroxyls one of which chelated. Also displayed two ortho coupled doublets (2H each) at \(\delta H 8.24\) and \(\delta H 7.17\) suggesting p-substituted aromatic rings, and three methoxyl groups at \(\delta H 3.79\) (3H), 3.80 (3H), and 3.85 (3H). The \(^{13}\)C NMR and HMBC correlations in DMSO-\(d_6\) are reported in Table 1 and indicated that the protons at 8.24 (H-2′, 6′) correlated with the carbons at \(\delta 129.2\) (C-2′, 6′), 146.3 (C-2), and 160.5 (C-4′). Similarly, protons at 7.17 (d, \(J = 8.7\) Hz, H-3 5′) correlated with carbons at \(\delta 114.2\) (C-3 5′), 123.4 (C-1′), and 160.5 (C-4′), confirming a p-substituted B ring. Methoxyl protons observed at \(\delta 3.85, 3.80,\) and 3.79 showed correlations with aromatic carbons at \(\delta 127.7\) (C-8), 131.0 (C-6), and 160.5 (C-4′), respectively, demonstrating that the methoxyl groups were located at C-6 and C-8 of ring A and C-4′ of ring B (Figure 2). All the data and comparison with those reported previously and combined with other partial structures, enabled this compound to be identified as 5,7-dihydroxy-6,8,4′-trimethoxyflavonol (1). At the best of our knowledge, compound 1 was obtained by synthesis [19] and isolated only once as natural product [20].

Initially, we evaluated the antiproliferative activity of the crude extract against four cell lines such as U251, MCF7, NCI-H460, and 786-0. The concentrations which inhibited 50% cell growth were 28.9, 29.6, 36.1, and 225.2 \(\mu\)g/mL, respectively. Considering these results, fractions and compound 1 were tested for their antiproliferative activity against eight human cancer cell lines and a nontumor cell line in four concentrations (0.25, 2.5, 25, and 250 \(\mu\)g/mL), using

Table 1: \(^1\)H and \(^{13}\)C NMR data of compound 1 in DMSO-\(d_6\).

| Position | \(^1\)H NMR | \(^{13}\)C NMR | HMBC |
|----------|-------------|---------------|------|
| 2        | 146.3       |               |      |
| 3        | 135.8       |               |      |
| 4        | 176.4       |               |      |
| 5        | 147.3       |               |      |
| 6        | 131.0       |               |      |
| 7        | 150.6       |               |      |
| 8        | 1277        |               |      |
| 9        | 144.5       |               |      |
| 10       | 102.4       |               |      |
| 1'       | 123.4       |               |      |
| 2′, 6′   | 8.24 d (9.0)| 129.2         | C-2, C-2′ 6′, C-4′ |
| 3′, 5′   | 7.17 d (9.0)| 114.2         | C-1′, C-3′ 5′, C4′ |
| 4′       | 160.5       |               |      |
| OMe-4′   | 3.80 s      | 55.4          | C-4′ |
| OMe-6    | 3.79 s      | 60.2          | C-6  |
| OMe-8    | 3.85 s      | 61.1          | C-8  |
| OH-3     | 9.56 s      |               |      |
| OH-5     | 10.38 s     |               |      |
| OH-7     | 12.26 s     | C-6, C-9, C-10|

\(\delta\) in ppm; \(J\) in Hz; \(^1\)H NMR at 300 MHz and \(^{13}\)C NMR at 75 MHz.
Table 2: Antiproliferative activity of doxorubicin, hexane (HE), dichloromethane (DCM), and ethyl acetate (EA) fractions and compound 1 against human cancer cell lines.a.

| Cell lines                                | Total growth inhibition (TGI) (µg/mL)b |
|-------------------------------------------|---------------------------------------|
| Glioma (U251)                             | 4.1                                   |
| Breast (MCF-7)                            | 0.62                                  |
| Multidrug-resistant ovary carcinoma (NCI-ADR/RES) | 9.1                                    |
| Kidney (786-0)                            | 0.46                                  |
| Lung, non-small cells (NCI-H460)          | 4.3                                   |
| Ovary (OVCAR-3)                           | 2.4                                   |
| Colon (HT-29)                             | 1.3                                   |
| Leukemia (K-562)                          | >25                                   |
| Nontumoral keratinocyte (HaCat)           | 0.79                                  |

**Doxorubicin** | **HE** | **DCM** | **EA** | **Compound 1** |
|----------------|--------|---------|--------|----------------|
| 35.4           | 51.1   | >250    | 2.8    |
| 45.2           | 17.8   | >250    | 55.8   |
| 12.6           | 11.1   | >250    | 18.8   |
| 30.7           | 19.3   | >250    | 15.8   |
| 56.3           | 135.0  | >250    | 14.1   |
| 46.1           | 89.7   | >250    | 14.5   |
| 28.2           | 24.4   | >250    | >250   |
| 25.8           | >250   | 17.5    |

*a* Assessed by the sulforhodamine B (SRB) assay. *b* TGI values represent the concentration necessary (µg/mL) for total inhibition of cancer cell proliferation. Values were determined through nonlinear regression analysis using the ORIGIN 8.0 (OriginLab Corporation). Dose range tested: 0.25 to 250 µg/mL. Experiment was conducted in triplicate.

As shown in Table 2, DCM and HE fractions demonstrated antiproliferative activity for most of the human cancer cell lines evaluated. DCM fraction was more potent than HE fraction, showing selectivity for breast (MCF-7), multidrug-resistant ovary carcinoma (NCI-ADR/RES), kidney (786-0), and colon (HT-29), with TGI values of 2.8, 18.8, 15.8, 14.1, 14.5, and 17.5 µg/mL, respectively. On the other hand, ethyl acetate fraction (EA) did not demonstrate significant activity in any of the concentrations tested.

Regarding the anticancer activity of hexane fraction, it can be partially attributed to compound 3, which is reported to have a potent free radical scavenging ability and considerable antiproliferative activity against MCF-7 and HepG2 cell lines [21]. Considering that compounds 1 and 2 were isolated from DCM fraction and that there are already reports of the anticancer activity of tormentic acid and its derivatives [22, 23], only compound 1 was evaluated against the same cell lines. As can be observed, compound 1 demonstrated in vitro antiproliferative effects for glioma (U251), multidrug-resistant ovary carcinoma (NCI-ADR/RES), kidney (786-0), lung (NCI-H460), ovary (OVCAR-3), and leukemia cells (K-562), with TGI values of 2.8, 18.8, 15.8, 14.1, 14.5, and 17.5 µg/mL, respectively (Table 2). Despite being less potent than DCM fraction for breast cancer cell line (MCF-7), the isolated compound exhibited better in vitro antiproliferative activity, being especially potent for glioma, with a TGI value close to that of the positive control, doxorubicin. Glioblastoma multiform (GBM) is the most common primary tumor of the central nervous system (CNS), and its occurrence in older age groups has increased in recent decades, with treatment or cure presenting a challenge to the scientific community [24].

Although extract and fractions antiproliferative effect may be attributed to the presence of several compounds, these results suggest that this uncommon methoxylated flavonol may be more closely involved with the in vitro anticancer activity. Although some studies have demonstrated the anticancer activity of *Rubus* genus, no reports have been found for *R. rosaefolius*, this study being the first one to describe the antiproliferative activity of this specie.

### 3. Material and Methods

#### 3.1. General

IR spectra were recorded on a BOMEM-100 with Fourier-Transform Infrared (FT-IR, BOMEM, St. Jean Baptiste, QB, Canada) spectrometer using KBr. EIMS were recorded on Shimadzu Gas Chromatograph (QP-2010S series, Kyoto, Japan) coupled with a mass spectrometric detector equipped with a NIST08 software database. 1D and 2D NMR spectra were recorded on a Bruker AV-300 (Bruker, Karlsruhe, Germany) in DMSO-<sub>d6</sub>. Chemical shifts (δ) are expressed in ppm, and coupling constants are given in Hz.

#### 3.2. Chromatographic Conditions

Recoated aluminum plates silica gel 60F<sub>254</sub> (E. Merck, Darmstadt, Germany) for TLC were used and visualized under UV at 254 and 366 nm and...
by spraying with anisaldehyde sulfuric and FeCl₃ reagents. Column silica gel 70–230 mesh and flash silica gel 230–400 mesh (E. Merck, Darmstadt, Germany) were used for the column chromatography. Melting points were determined on a Micro-Quimica APF-300 apparatus (Micro-Quimica, Florianópolis, SC, Brazil) and are uncorrected. The purity of the isolated compound was examined by thin layer chromatography (TLC) using Merck silica gel precoated aluminum plates (thickness = 200 μm) and several solvent systems of different polarities. All the reagents and solvents used were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany).

3.3. In Vitro Anticancer Activity Assay. Human tumor cell lines, U251 (glioma), MCF-7 (breast), NCI-ADR/RES (multidrug-resistant ovary), OVCAR-3 (ovary), HT-29 (colon), K-562 (leukemia) were kindly provided by the United States National Cancer Institute (NCI). Nontumor cell line HaCat (human keratinocytes) was donated by Professor Dr. Ricardo Della Coletta, FOP/UNICAMP. Stock cultures were grown in medium containing 5mL of RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum (GIBCO BRL). Penicillin (100 U/mL) and streptomycin (100 μg/mL) were added to experimental cultures. Cells in 96-well plates (100 μL cells well-1) were exposed to sample concentrations (when samples are added) thus allowing the calculation of the concentrations that promote total growth inhibition (TGI). These concentrations are calculated from T0, in which the amount of protein at the end of sample incubation (T) is equal to the amount at the beginning (T0) [26]. Using the concentration-response curve for each cell line, TGI (total growth inhibition) was determined through nonlinear regression analysis using software ORIGIN 8.0 (OriginLab Corporation) [26]. Experiment was conducted in triplicate.

3.4. Plant Material. The R. rosaefolius plant material was collected in the town of Itaí, SC, Brazil (26° 54’ 28” S; 48° 39’ 43” W) in March 2013 and identified by Dr. Ademir Reis (Botanical Department of the Federal University of Santa Catarina). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itaí-SC) under number V.C. Filho 035.

3.5. Extraction and Isolation. Air-dried powdered leaves (580 g) of R. rosaefolius were exhaustively extracted with methanol (MeOH) at room temperature for seven days. The macerates were filtered and concentrated under reduced pressure in a rotatory evaporator, yielding 174.0 g of crude methanol extract. The extract was suspended in MeOH-H2O (90:10) mixture and subjected to liquid-liquid partition using solvents of increasing polarity such as HE, DCM, and EA. Part of the DCM fraction (4.5 g) was subjected to column chromatography over silica gel and eluted with DCM-MeOH (100:0:0-100:0) in increasing order of polarity, yielding 164 fractions which were combined based on TLC profiles. Fraction 31–41 (1.0 g) was rechromatographed using the same solvent system, yielding new 99 subfractions, which were pooled according to their similar chromatographic profiles. Subfractions 14–28 were then eluted with a mixture of DMSO:Acetone (75:25), yielding 35 subfractions in which subfractions 10–30 presented as a yellow crystal (35 mg) and were identified as 5,7-dihydroxy-6,8,4’-trimethoxyflavonol (I) by NMR, DEPT, HMBC, IR data in comparison with those reported previously [19, 27]. Also in relation to DCM fraction isolation, fractions 100–123 (985 mg) from first column were combined and rechromatographed using DCM-MeOH (100:0:0-100:0) as solvent system, yielding 125 subfractions, which were pooled according to their similar chromatographic profiles. Subfractions 45–47 presented as a pure colorless crystal (18 mg) and were identified as tormentic acid (3) based on IR, Mass, and NMR data in comparison with those reported previously [18]. Similarly, part of the HE fraction (9.04 g) was subjected to column chromatography over silica gel and eluted with DCM-MeOH (100:0:0-100:0) in increasing order of polarity to afford 123 fractions, which were combined based on their TLC profiles. Fraction 49–68 (2.12 g) was rechromatographed using the same solvent system, yielding new 105 subfractions. Of these, fraction 69–71 (14.0 mg) was rechromatographed using the same solvent system, yielding new 20 subfractions. Subfractions 1–6 presented as a yellow crystal (4.7 mg) and were identified as 5-hydroxy-3,6,7,8,4’-pentamethoxyflavone (2) based on IR, Mass, and NMR data in comparison with those reported previously [17].

3.5.1. 5,7-Dihydroxy-6,8,4’-trimethoxyflavonol (1). Yellowish solid; mp 295–297°C; IR (KBr) λ max 3354, 2970, 1664, 1269, 1220 cm⁻¹; 1H NMR (300 MHz, DMSO-d6) δ 12.26 (1H, s, C-7-OH); 10.38 (1H, s, C-5-OH); 9.56 (1H, s, C-3-OH); 8.24 (2H, d, J = 9 Hz, H-2’6’); 7.17 (2H, d, J = 9 Hz, H-3’5’); 3.85 (3H, s, C-8-OMe); 3.80 (3H, s, C-4’-OMe); 3.79 (3H, s, C-6’-OMe); 13C NMR (75 MHz, DMSO-d6) δ 176.4 (C=O, C-4), 160.5 (C-4’), 150.6 (C-7), 147.6 (C-5), 144.5 (C-9), 135.8 (C-3), 131.0 (C-6), 129.2 (C-2’6’), 127.7 (C-8), 123.4 (C-1’), 114.2 (C-3’5’), 102.4 (C-10), 61.1 (C-8-OMe), 60.2 (C-6-OMe), 55.4 (C-4’-OMe); EIMS m/z 360.3 [M]+ (calcd for C18H14O6: 360.3148).

3.5.2. 5-Hydroxy-3,6,7,8,4’-pentamethoxyflavone (2). Yellowish solid; mp 119–123°C; IR (KBr) λ max 3241, 2985, 1752, 1546, 1239 cm⁻¹; 1H NMR (300 MHz, CDCl3) δ 8.17 (2H, d, J = 9 Hz, H-2’6’); 7.06 (2H, d, J = 9 Hz, H-3’5’); 4.10 (3H, s, C-7-OMe); 3.95 (3H, s, C-6-OMe, C-8-OMe) 3.90 (3H, s, C-8-OMe); 3.87 (3H, s, C-3-OMe); 13C NMR (75 MHz, CDCl3) δ 179.3 (C=O, C-4), 161.9 (C-4’), 156.1 (C-2), 152.9 (C-7), 149.2
3.5.3. Tormentic Acid (3). White powder; mp 269–275°C; 1H NMR (30 MHz, CD3OD): δ 5.29 (1H, s, H-12), 4.62 (1H, br s, 19-OH), 3.66 (1H, dd, J = 11.1, 4.5 Hz, H-2), 2.93 (1H, d, J = 9.3 Hz, H-3), 2.50 (1H, s, H-18), 1.35 (3H, s, H-27), 1.19 (3H, s, H-29), 1.02 (3H, s, H-23), 1.00 (3H, d, J = 6.4 Hz, H-30), 0.94 (3H, s, H-26), 0.81 (3H, s, H-24), 0.79 (3H, s, H-25); 13C NMR (75 MHz, CD3OD) 182.4 (C28), 140.2 (C-13), 129.4 (C-12), 84.7 (C-3), 73.7 (C-19), 69.7 (C-2), 56.8 (C-5), 55.2 (C-18), 48.6 (C-9), 48.3 (C-1), 43.2 (C-20), 42.7 (C-14), 41.2 (C-8), 40.7 (C-4), 39.4 (C-22), 39.2 (C-10), 34.2 (C-15), 29.7 (C-29), 29.4 (C-23), 27.4 (C-21), 27.2 (C-30), 26.7 (C-16), 25.0 (C-27), 24.9 (C-11), 19.8 (C-6), 17.6 (C-24), 17.6 (C-26), 17.2 (C-25), 16.8 (C-29); EIMS m/z: 488.2 [M]+ (calc for C30H48O5: 488.6991).

4. Conclusions

In summary, our results showed the presence of an uncommon methoxyflavonol identified as 5,7-dihydroxy-6,8,4′-trimethoxyflavonol along with two known compounds, 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone and tormentic acid. They also showed that the methanolic extract, hexane, and dichloromethane fractions presented an interesting in vitro antiproliferative profile. Trimethoxyflavonol isolated from Rubus rosae folius leaves also showed promising antiproliferative profile, especially against glioma cell line, making this compound of great interest in the search for new chemotherapeutic agent.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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