Chemical Constituents, in vitro Antioxidant and Antiproliferative Activities of *Perralderia coronopifolia* Coss. subsp. eu-coronopifolia M. var. typica M. extract

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Abstract: Phytochemical investigations of extracts from the aerial parts (leaves and flowers) of *Perralderia coronopifolia* Cosson resulted in the isolation of nine secondary metabolites corresponding to three flavonoids: rhamnazin (1), chrysoeriol D (3), and (2R, 3R) taxifolin (4). Two monoterpene glycosides: myrtenol-β-D-glucopyranoside-6-O-acetate (2) and myrtenol-β-D-glucopyranoside (7), a disaccharide: sucrose (9) and three di-O-caffeoylquinic acid derivatives: methyl 3, 5-di-O-cafeoyl quinate (5) and methyl 3,4-di-O-cafeoyl quinate (6) as a mixture and 1,5-di-O-cafeoylquinic acid (8). The structures were identified by spectroscopic methods such as 1H and 13C NMR, COSY, HSQC and HMBC experiments, HRESI-MS and comparison with literature data. Myrtenol-β-D-glucopyranoside-6-O-acetate (2) was isolated in pure and native state for the first time. The other compounds are new for the genus *Perralderia* Cosson. The ethyl acetate extract showed a high antioxidant effect, especially DPPH radical scavenging activity with IC₅₀ = 7.01 ± 0.28 µg/mL compared to ascorbic acid (IC₅₀ = 5.01 ± 0.1 µg/mL). This extract also showed antiproliferative activity against HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells.

Keywords: Asteraceae; *Perralderia coronopifolia*; flavonoids; caaffeoylquinic acids; terpenoids; antioxidant activity; antiproliferative activity. © 2015 ACG Publications. All rights reserved.

1. Introduction

The North African genus *Perralderia* (Asteraceae – Inuleae – Inulinae) includes three species. They grow in the elevated regions of Morocco, Algeria and Libya in the north-western part of the African continent, primarily in the mountainous regions. *Perralderia paui* Font Quer is found in the North of Morocco, *P. garamantum* Asch occurs in Libya and *P. coronopifolia* Coss. which includes two subspecies, subsp. *purpurascens* (Coss. ex Batt.) M. and subsp. *eu-coronopifolia* M. with two varieties, var. *typica* M. and var. *dessignyana* (Hochr.) M. grow in Algeria [1, 2]. As part of our ongoing program of research of new molecules with potential biological activity from plants [3-10], we report our results on *P. coronopifolia* Coss. subsp. *eu-coronopifolia* M. var. *typica* M. growing in the Algerian Sahara. Our first selection criterion for this plant was based on the fact that considering the difficult conditions to which
Saharan plants are subjected (hydrous insufficiency and oxidative stress), they are likely to accumulate specific secondary metabolites to survive, to adapt and to develop. Moreover, in the best of our knowledge, no reports on the isolation of any secondary metabolites from this plant are available to date. In the present work on the chemical constituents of the chloroform and ethyl acetate soluble parts of the aqueous-EtOH extract of the aerial parts (leaves and flowers), an acylated monoterpenic glucoside has been isolated in pure and native state for the first time, together with eight known secondary metabolites. We report here the chemical study of these compounds, the free radical DPPH scavenging potential, the antiproliferative activities against HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells of the ethyl acetate soluble part.

2. Materials and Methods

2.1. General

UV spectra were recorded using a Shimadzu model UV-1700 spectrophotometer. NMR spectra were obtained by Bruker model Avance 400 and AMX-500 spectrometers with standard pulse sequences, operating at 400 and 500 MHz for $^1$H and 100 and 125 MHz for $^{13}$C, respectively. MeOH-d$_4$, DMSO-d$_6$ or CDCl$_3$ were used as solvents and TMS as internal standard. EIMS were taken on a Micromass model Autospec (70 eV) spectrometer. HRESI-MS was performed with a LCT Premier XE Micromass Waters spectrometer in positive ionization mode (Waters Corporation). Column chromatography (CC) was carried out with Si gel Fluka (cat. 60737) (40:3:1 ltrate was filtered using common filter paper and concentrated in vacuum at room temperature with EtOH and concentrated and dissolved in H$_2$O (80:20, v/v) for 48 h, three times. After filtration, the filtrate was concentrated and dissolved in H$_2$O (650 ml). The resulting solution was extracted successively with CHCl$_3$, EtOAc and n-butanol. The organic phases were dried with Na$_2$SO$_4$, filtered using common filter paper and concentrated in vacuum at room temperature to obtain the following extracts: CHCl$_3$ (2 g), EtOAc (7 g) and n-butanol (40 g).

The CHCl$_3$ extract was fractionated by CC (silica gel; n-hexane/EtOAc step gradients and then with increasing percentages of MeOH) to yield 24 fractions (Fr$_1$-Fr$_{24}$) obtained by combining the eluates on the basis of TLC analysis. Fraction Fr$_{15}$ (70 mg) (n-hexane/EtOAc, 65:35), was submitted to preparative plates of silica gel TLC (CHCl$_3$/EtOAc,1:1) and purified on a Sephadex LH-20 column to give hramnadin (1) (2 mg) [11]. Fraction Fr$_{17}$ (277 mg) (n-hexane/EtOAc, from 55:45 to 45:55) was chromatographed on a silica gel column (CHCl$_3$ /EtOAc) to give myrtelin-β-D-glucopyranoside-6'-O-acetate (2) (56 mg) [12]. The EtOAc extract (7 g) was chromatographed on a silica gel column (CH$_2$Cl$_2$/Me$_2$CO step gradients and then with increasing percentages of MeOH) to yield 25 fractions (Fr$_1$-Fr$_{25}$) according to their TLC behavior. Fraction Fr$_{7}$ (90.4 mg) (CH$_2$Cl$_2$/Me$_2$CO, 75:25) was submitted to preparative silica gel TLC (CHCl$_3$/MeOH/H$_2$O, 9.8:1:1:0.1) and purified over Sephadex LH-20 column eluted with MeOH to afford
chrysosplenol D (3) (3 mg) [13, 14] and (2R, 3R)-taxifolin or dystilin (4) (4 mg) [15]. Fraction Fr15 (82 mg) (CH2Cl2/Me2CO, 70:30) was fractionated on a Sephadex LH-20 column using MeOH as eluent to give 7 sub-fractions. Sub-fractions 3 and 4 which had similar composition were combined and submitted to preparative TLC (CHCl3/MeOH/H2O, 8:2:0.1) and further purified by Sephadex LH-20 column using MeOH for elution to yield a mixture of methyl 3, 5-di-O-caffeoyl quinate (5) [16, 17] and methyl 3, 4-di-O-caffeoyl quinate (6) (18 mg) [18]. In this paper, the numbering of the quinic acid ring follows the new IUPAC nomenclature recommendation. Fraction Fr15 (219 mg) (CH2Cl2/Me2CO, 66:34) was rechromatographed on a silica gel column (n-hexane/Et2O step gradients and then with increasing percentages of EtOAc) to give myrtenol β-D-glucopyranoside (7) (9 mg) [19, 20]. Fraction Fr20 (129 mg) (CH2Cl2/Me2CO, 16:84) was further applied to a Sephadex LH-20 column using MeOH for elution to obtain 8 sub-fractions. Sub-fraction 6 was purified over Sephadex LH-20 column to afford 1, 5-di-O-caffeoylquinic acid (8) (13 mg) [21]. Fraction Fr23 (332 mg) (100% Me2CO) gave after purification by crystallization in MeOH, sucrose (9) (7.5 mg) [22, 23] (Figure 1).

2.4. Cell culture

HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells were grown in Dulbecco’s modified eagle’s medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and 2% Penicillin-Streptomycin (Sigma, Germany) at 37°C in a 5% CO2 humidified atmosphere.

2.5. Antiproliferative Activity

2.5.1. Preparation of Cell Suspension

HeLa cells in the culture flask were detached from bottom of flask by 10 mL Trypsin-EDTA solution. After detachment, 10 mL of medium was added into the flask and mixed thoroughly. This suspension was transferred to Falcon tubes and centrifugated at 600 rpm for 5 min. After removing the supernatant, 5 mL of medium was added to Falcon tube and mixed carefully. Cell concentration of this cell suspension was measured by CEDEX HiRes Cell Counter which uses Trypan Blue.

2.5.2. Preparation of E-Plate 96

50 µL of medium was added into each well of E-Plate 96. The plate was incubated in the hood then in the incubator for 15 min in each time. After this period, the E-Plate 96 was inserted to the RTCA-SP station and a background measurement was performed. Then 100 µL of the cell suspension (2.5x10^3 cells/100 µL) was added into wells, except in the last three wells where only 100 µL of medium was added to check if there would be an increase from culture medium. The plate was left in the hood for 30 min, and then inserted to xCELLigence station in the incubator. A measurement was performed for 80 min. Extract was dissolved in DMSO to a final concentration of 20 mg/mL. 25 µL of this solution was mixed with 475 µL of medium. The extract solutions (50, 20 and 10 µL equivalent to 250, 100 and 50 µg/mL concentrations, respectively) were added into the wells and the final volumes were completed to 200 µL with medium. No extract solution was added into control and medium wells. Then the plate was inserted to xCELLigence station in the incubator and a measurement for 48 h was started.

2.6. Cell Proliferation Assay

Cells were plated in 96-well culture plates (COSTAR, Corning, USA) at a density of 30,000 cells per well. Vehicle (DMSO), 5-FU, cisplatin and several samples in various concentrations (5-100 µg/mL) were added to each well. Cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer’s procedure. Briefly, cells were pulsed with BrdU labeling reagent for 4 h followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of anti- BrdU-POD for 1.30 h at room
temperature. The amount of cell proliferation was assessed by determining the A450 nm of the culture media after addition of the substrate solution by using a microplate reader (Ryro, China). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. All assays were repeated at least twice using against HeLa and C6 cells. Percentage of inhibition of cell proliferation was calculated as follows:

\[(1 - \text{A}_{\text{treatment}} / \text{A}_{\text{vehicle control}}) \times 100\]

Stock solution of the samples, 5-FU were dissolved in sterile DMSO and diluted Dulbecco's modified eagle’s medium (DMEM, 1:20). DMSO final concentration is below 1% in all tests.

2.7. Lactate Dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit by Roche Diagnostics GmbH, Mannheim, Germany according to protocol in the user's manual. At 100 concentrations that was the highest dose at antiproliferative activity test were determined cytotoxicity (%) against HeLa cell line. 5-FU were used as positive control. Samples and 5-FU were incubated with 100 µL of C6 cell suspension having 5×10⁵ cells/mL in 96-well plates at 37°C for overnight in 5% CO₂ atmosphere. All the control and tested substances were tested in triplicates and twice and mean ± SEM of the absorbance value were taken to calculate the percentage of cytotoxicity.

Cytotoxicity % = [(Triplicate absorbance – low control) / (High control – low control)] x 100

2.8. Statistical Analysis

The results of this investigation are the means ± SEM of six measurements for each cell type. Differences between treatment groups were compared by one-way analysis of variance (ANOVA) and p values < 0.01 and 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Structure elucidation

Compound 2 was previously found in Platychaete aucheri Boiss. (Compositae) but purified as its tetraacetate after acetylation reaction [12]. In our study, we obtained it in pure and native state. Its positive mode HRESI-MS spectrum presented [M+Na] ion at m/z 379.1732 (100%) according to the molecular formula C₁₈H₂₈O₇ (calculated for C₁₈H₂₈O₇Na: 379.1733), indicating a compound containing 5 unsaturations. The ¹³C- and DEPT NMR spectra of this compound confirmed the presence of the 18 carbon atoms. The ¹³C NMR spectrum showed six characteristic signals of a hexose group notably: a CH₂ at δC 63.6 (δH= 4.34, m, 2H) assigned to C-6’, a CH at δC 100.8 (δH 4.28, d, J=7.6 Hz, 1H) assigned to the anomeric carbon C-1’. The values of the chemical shift of this carbon and the coupling constant indicated an O-β-glycosylated compound. In the COSY spectrum, the anomeric proton H-1’ led to the assignment of H-2’ as a multiplet at δH 3.39 (δC 73.5). On the same spectrum, H-2’ showed a correlation with the proton corresponding to a triplet at δH 3.55 ppm (J=8.4 Hz) which was attributed to H-3’ (δC 76.3). The multiplicity of this signal and the value of the coupling constant indicated axial-axial interactions between H-3’/H-2’ and H-3’/H-4’. This observation led to a glucosylated compound. Moreover, in the HMBC spectrum, H₂-6’ showed correlation with the carbonyl of the carboxylic acid at δC 171.5 ppm, which showed a correlation with the protons of the methyl at δH 2.10 indicating the presence of an acetate group on the C-6 position of the glucosyl moiety. Considering the molecular formula C₁₈H₂₈O₇ and the presence the sugar moiety, the formula of the aglycone moiety of compound 2 was C₁₀H₁₅. Thus, compound 2 was a monoterpenoid glucoside 6-acetate. Besides the signals of the sugar moiety, the ¹³C- and DEPT NMR spectra of compound 2 showed two CH₃ (δC 20.9 and 26.2), three CH₂ (δC 71.8, 31.3 and 31.4), three CH (δC 40.8, 43.3 and 121.4 and two quaternary carbon atoms (δC 144.0 and 38.0). The values of the chemical shifts of the carbon atoms indicated that the sugar was attached to the CH₂ at δC 71.8 and also indicated the presence
of a double bond and two cycles in the monoterpene moiety. Complete $^1$H and $^{13}$C connectivity was established by extensive use and interpretation of 2D NMR experiments spectra (COSY, HSQC, HMBC and ROESY). The proton and carbon signals due to this monoterpene moiety were in good agreement with those of the aglycone of sacranoside A isolated from the underground part of *Rhodiola sacra* (Prain ex Hamet) S. H. Fu (Crassulaceae) [19]. Thus, compound 2 was myrtenol-$\beta$-D-glucopyranoside-6'-O-acetate.

The structures of known compounds were established by spectral analysis, mainly the HRESI-MS, the $^1$H-, $^{13}$C-, and 2D-NMR (COSY, ROESY, HSQC and HMBC) as well as by comparing their spectroscopic data with those reported in the literature.

*Myrtenol-$\beta$-D-glucopyranoside-6'-O-acetate (2)*: yellow oil; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) = 5.55 (1H, br s, H-3), 4.13 (1H, br d, $J = 12.4$ Hz, H-10a), 4.04 (1H, br d, $J = 12.4$ Hz, H-10b), 2.39 (1H, m, H-6a), 2.28 (2H, br d each one, $J = 20.8$ Hz, H-4a and H-4b), 2.18 (1H, m, H-1), 2.10 (1H, m, H-5), 1.29 (3H, s, H-9), 1.16 (1H, d, $J = 8.7$ Hz, H-7b), 0.82 (3H, s, H-8), 4.34 (2H, m, H-6$'$), 4.28 (1H, d, $J = 7.6$ Hz, H-1$'$), 3.55 (1H, t, $J = 8.4$ Hz, H-3$'$), 3.40-3.48 (2H, m, H-4$'$, H-5$'$), 3.39 (1H, m, H-2$'$), 2.10 (3H, s, OAc); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) = 144.0 (C, C-2), 121.4 (CH C-3), 71.8 (CH$_2$ C-10), 43.3 (CH, C-1), 40.8 (CH C-5), 38.0 (C, C-6), 31.4 (CH$_2$ C-4), 31.3 (CH$_2$ C-7), 26.2 (CH$_3$ C-9), 20.9 (CH$_3$ C-8), 100.8 (CH C-1$'$), 76.2 (CH C-3$'$), 73.7 (CH C-5$'$), 73.5 (CH C-2$'$), 70.1 (CH C-4$'$), 63.6 (CH$_2$ C-6$'$), 171.5 (C, COO), 21.0 (CH$_3$ OAc); HR-ESIMS (+): m/z 379.1732 (calcd. 379.1733 for C$_{16}$H$_{29}$O$_4$Na).

\[\text{Figure 1. Structures of the isolated compounds 1-9}\]
3.2. Biological activities

3.2.1. Antioxidant activity

The free radical scavenging activity of ethyl acetate extract was evaluated through its ability to quench 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical [24]. Many studies showed that chemical structure has an important impact on radical scavenging activity. The high antioxidant activity depends on their molecular structure, the number and the position of the hydroxyl groups [25]. Effective antioxidant activities were observed in comparison to ascorbic acid. Comparing IC_{50} values, we observed that ethyl acetate extract of P. coronopifolia showed a high scavenging effect against DPPH radicals with IC_{50}=7.01±0.28µg/mL compared to ascorbic acid (IC_{50}= 5±0.1µg/mL (Figure 2). This activity is possibly due to the presence of the flavonoids: taxifolin [26], rhamnazin [27] and caffeoylquinic acid derivatives found in this plant [28].

![Figure 2. DPPH scavenging activity of ethyl acetate extract and vitamin C. Values are mean ± SD of three samples analyzed, individually in triplicate](image)

3.2.2. Anticancer activity

Anticancer activity is highly studied for the medicinal plants and other natural sources [29]. However, phytochemical and cytotoxic, antitumor/anticancer investigations on P. coronopifolia have not been studied so far. The anticancer activity of ethyl acetate soluble part of the aqueous-EtOH extract of the aerial parts (leaves and flowers) of P. coronopifolia against HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells was tested using xCELLigence RTCA instrument. As shown in Figures 3 and 4, the extract exhibited different profiles on different concentrations. It has the most activity with the concentration of 250 µg/mL and it was about to reach to medium after 48 h post-treatment, while other concentrations [100 and 50 µg/mL] showed very low activity, nearly same with control. The profiles also showed differences at different time points. Low concentrations showed some activity against HeLa cells at 15th h (Figure 3) and against C6 cells at 3th h (Figure 4) after post-treatment, but after few hours they lost their capacity of inhibition. This may be caused because of the low ratio of bioactive molecules in the extract and within the time, cells started to proliferate after a short time of inhibition.
Chemical composition of *Perralderia coronopifolia*

3.2.3. Cell proliferation assay

The antiproliferative activities of samples and standard compound were determined against C6 and HeLa cell lines using BrdU cell proliferation ELISA assay [30, 31]. 5-FU was used as positive control. The antiproliferative activity of samples and positive control were investigated on eight concentrations (5, 10, 20, 30, 40, 50, 75 and 100 µg/mL). IC$_{50}$ and IC$_{75}$ values of the extracts and 5-FU were identified using ED$_{50}$ plus v1.0 programs (Table 1).

3.2.3.1. Antiproliferative activity of samples against HeLa cell.

The antiproliferative activity of the ethyl acetate extract was determined against HeLa cell. This extract has shown to increase the activity depending to the dose (Figure 5). The obtained results indicated...
that the lower concentrations (5 and 10 µg/mL) showed the proliferative activity and the higher concentrations (50, 75 and 100 µg/mL) showed high antiproliferative activity against HeLa cells compared to 5-FU.

The potency of inhibition at 100 µg/mL was: 5-FU > EtOAc extract.

Figure 5. Antiproliferative activity of the EtOAc extract and 5-FU against HeLa cell line. Each sample was tested twice in triplicates against HeLa cell lines. Data showed average of 2 individual experiments (p<0.01).

3.2.3.2. Antiproliferative activity of sample against C6 cell.

The antiproliferative activity of the ethyl acetate extract of *P. coronopifolia* was determined against C6 cell. The extract has shown to increase the activity as dose dependent manner. However, it was observed to have higher antiproliferative activity than 5-FU at 40-75 µg/mL (Figure 6).

The results of the present work are in agreement with previous evidences, which suggested that plant extracts are likely sources of substances potentially useful for the development of new drugs [32].

Figure 6. Antiproliferative activity of the EtOAc extract and 5-FU against C6 cell line. Each substance was tested twice in triplicates against cell lines. Data showed average of 2 individual experiments (p<0.01)

As shown in Figures 5 and 6, there is no proliferative activity even in lower concentrations against C6 cell lines as seen in lower concentrations against HeLa cell lines. The highest activities were observed against C6 cell lines than 5-FU in high concentrations, but not the same observations were obtained against HeLa cell lines.
Chemical composition of *Perralderia coronopifolia*  

The antiproliferative activity of ethyl acetate extract is expressed in terms of IC$_{50}$ and IC$_{75}$ and given in Table 1. The comparison of the IC$_{50}$ and IC$_{75}$ values for the two cell lines used in this study indicated that the extract showed a higher activity against C6 cell lines than HeLa cell lines.

**Table 1.** IC$_{50}$ and IC$_{75}$ values of EtOAc extract of *P. coronopifolia* and 5-FU, presented by antiproliferative assay against HeLa and C6 Cell Lines.  

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>HeLa cell</th>
<th>C6 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$</td>
<td>IC$_{75}$</td>
</tr>
<tr>
<td>EtOAc extract</td>
<td>45.49</td>
<td>63.71</td>
</tr>
<tr>
<td>5-FU</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*<5 µg/mL.

3.2.4. **Lactate Dehydrogenase (LDH) leakage assay**

Cytotoxicity % = 0% No cytotoxic effect was observed

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant or blood when the plasma membrane of cells is damaged. For this reason, the LDH assay is a widely used quantitative test for the toxicity of various molecules and drugs cells [33]. As shown in Table 2, the present study demonstrated that the EtOAc extract of *P. coronopifolia* has no cytotoxic effect.

**Table 2.** The percentage of cytotoxicity (Cytotoxicity %) of EtOAc extract of *P. coronopifolia* and 5-FU against C6 Cell Line using Lactate Dehydrogenase (LDH) leakage assay.  

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc extract</td>
<td>0</td>
</tr>
<tr>
<td>5-FU</td>
<td>19</td>
</tr>
</tbody>
</table>

In conclusion, this study showed that, the ethyl acetate soluble part of *P. coronopifolia* aqueous-EtOH extract had an antioxidant activity. This activity is possibly due to the flavonoids chrysosplenol D, taxifolin and caffeoylquinic acid derivatives which are the major components of this extract. A preliminary evaluation of the antiproliferative activity of this extract against HeLa and C6 cells revealed that it is quite promising for the cancer cells studied. They were tested by xCELLigence RTCA instrument and BrdU cell proliferation ELISA assay, respectively. 5-Fluorouracil was used as positive control. No cytotoxic effect was observed in this extract.

The relatively high content of bioactive compounds, positive antioxidant and antiproliferative properties of *P. coronopifolia* may justify the use of this plant as a new source of valuable antioxidants. *In vivo* studies are needed to confirm this pharmacological efficacy.

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