Dichloromethane Extract of the Leaves of *Arbutus pavarii* Pamp. Exhibits Cytotoxicity Against the Prostate Cancer Cell Line PC3: A Bioassay-guided Isolation and Identification of Arbutin and Betulinic Acid Methyl Ester

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ABSTRACT

Objectives: To assess the cytotoxicity of *Arbutus pavarii* Pamp. (fam. Ericaceae), a Libyan medicinal plant, against human cancer cell lines and to carry out bioassay-guided isolation and identification of compounds.

Materials and Methods: Shed-dried and ground leaves of *A. pavarii* were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), and assessed for cytotoxicity against several human cancer cell lines using the MTT assay. The cytotoxicity of the DCM extract against the normal human prostate cell line PNT2 was also assessed to determine the selectivity index (SI). The DCM extract was subjected to vacuum liquid chromatography (VLC) to produce eight fractions, which were then tested against the prostate cancer cell line PC3, as the DCM extract was considerably cytotoxic to this cell line. A reversed-phase preparative HPLC analysis of the active VLC fraction was carried out to purify the major compounds present in the active fraction, and the structures of the compounds were elucidated by spectroscopic means.

Results: The DCM extract displayed most prominent cytotoxicity against the PC3 cell line with an IC<sub>50</sub> value of 26 mg/mL. However, this extract was much less cytotoxic to the normal human prostate cell line PNT2 (IC<sub>50</sub> = 90 mg/mL) with a selectivity index of 3.5. VLC analysis produced eight fractions, and among them, fraction VLC-5 was most active against the PC3 cell line. Prep-HPLC-based purification of VLC-5 afforded the isolation of arbutin (1) and betulinic acid methyl ester (2), the structures of which were elucidated by spectroscopic means.

Conclusion: The DCM extract of the leaves of *A. pavarii* exhibited significant cytotoxicity to PC3 cells, but much less cytotoxicity against normal human prostate cell line. The isolated compounds from the active fraction, arbutin (1) and betulinic acid methyl ester (2), which
were previously shown to possess cytotoxic properties, could be responsible for the cytotoxicity of the DCM extract.

**Key words:** *Arbutus pavarii*, Ericaceae, betulinic acid methyl ester, arbutin, prostate cancer, PC3, anticancer, Libya

**INTRODUCTION**

*Arbutus pavarii* Pamp., commonly known as “Shmeri”, “Shmar” and “Libyan Strawberry”, is an endemic Libyan medicinal plant of the family Ericaceae. This evergreen shrub grows almost exclusively in the Al-Jabel Al-Akhdar mountainous region in Libya. It is a forage species of plant for honeybees to produce a specific type of honey, and has long been a component of Libyan traditional medicinal preparations for the treatment of both gastritis and kidney diseases. The berries of this plant are rich in minerals, nutrients, carbohydrates and ascorbic acid, and the aerial parts have their application in the tanning process. Previous phytochemical investigations performed on this species revealed the presence of simple phenolic compounds like arbutin (1) and gallic acid, and flavonoids and tannins, such as, apigenin, epicatechin, hesperidin, kaempferol, naringin, quercetin and rutin, as well as some triterpenes and sterols. Rutin and arbutin appear to be the most abundant compounds within the aerial parts of this plant, and arbutin (1) is considered as a chemotaxonomic marker for the genus *Arbutus* L. Only a few published reports on the bioactivities of this species described preliminary antioxidant, antimicrobial and cytotoxic activities of the crude extracts of this species. However, there is hardly any report on bioassay-guided isolation of any active compounds, and subsequent assessment of bioactivity of those compounds. We now report, for the very first time, on the assessment of cytotoxicity of the dichloromethane (DCM) extract against several human cancer cell lines, and a bioassay-guided isolation and spectroscopic identification of the major compounds, arbutin (1) and betulinic acid methyl ester (2), from the DCM extract of the leaves of *A. pavarii*.

**MATERIALS AND METHODS**

**General experimental procedure**

1D and 2D NMR spectroscopic analyses were carried out on a Bruker 600 MHz AMX Ultrashield NMR spectrometer using the deuterium locking. Chemical shifts are in d ppm and coupling constants J in Hz. Vacuum liquid chromatography (VLC) was performed on silica gel 60H (Sigma-Aldrich, UK) column under vacuum. Solvents for extraction and chromatographic work were of analytical grade, obtained from Fisher Scientific, UK, and used without further purification.

**Plant materials**

The leaves of *Arbutus pavarii* Pamp. (Ericaceae) (Figure 1) were collected from the Al-Jabal Al-Akhdar region in Libya (latitude and longitude: 32° 35' 51'' North and 21° 28' 22'' East) in 2016, and a voucher specimen for this collection (D6854201) has been retained at the Herbarium of the Faculty of Science, Tripoli University, Libya. Shed-dried leaves were ground to a fine powder using a coffee grinder.
A portion (150 g) of the ground leaves was Soxhlet-extracted, sequentially, with \( n \)-hexane, dichloromethane (DCM) and methanol (MeOH), 900 mL each and 10 cycles each solvent. All extracts were filtered using Whatman filter paper, evaporated to dryness using a rotary evaporator (Cole-Parmer, UK) and stored at 4°C.

**Vacuum liquid chromatography (VLC)**

VLC was carried out following the method described by Sarker and Nahar.\(^9\) The DCM extract (2.4 g) was subjected to VLC fractionation on a silica 60H column under vacuum using a step gradient comprising \( n \)-hexane, ethyl acetate (EtOAc) and MeOH of different proportions and in the order of increasing polarity, e.g., 100\% \( n \)-hexane, 10, 30, 50, 80\% EtOAc in \( n \)-hexane, 100\% EtOAc and 50\% MeOH in EtOAc.

**Isolation of compounds by preparative high-performance liquid chromatography (prep-HPLC)**

Preparative HPLC separation was performed on an Agilent 1200 preparative HPLC comprising a binary gradient pump, photodiode array detector and a computer with control and data analysis software. In the preparative isolation of compounds, an ACE prep-column [150 × 21.2 mm, 5 \( \mu \) m, Hichrom Ltd, UK; MeOH-water linear gradient: 50-100\% acetonitrile in water (both containing 0.1\% 0.1\% trifluoro acetic acid, TFA) in 30 min, then 100\% acetonitrile for 10 min, flow rate: 10 mL/min, monitored simultaneously at 215, 254, 280 and 320 nm] was used with a volume of injection of 200 \( \mu \)L.

**Identification of compounds**

The identity of the isolated compounds (1) and (2) (Figure 2) was confirmed by spectroscopic means, particularly, by MS and 1D and 2D NMR data analyses.

**Figure 2: Structures of arbutin (1) and betulinic acid methyl ester (2)**

**Cytotoxicity assay (MTT assay)**

Human cancer cell lines A549 (human lung carcinoma), EJ138 (human bladder carcinoma), HepG2 (human liver hepatocellular carcinoma), MCF7 (human breast adenocarcinoma) and
PC3 (human prostate cancer) were used in this study. Additionally, the normal human prostate cell line PNT2 was used. These cell lines were obtained from the European Collection of Authenticated Cell Cultures. All the cancer cell lines were adherent epithelial cells derived from human carcinoma or adenocarcinoma. Cytotoxicity of the extracts of *A. pavarii* at different concentrations (0.0, 0.005, 0.01, 0.05, 0.1 and 0.5 mg/mL), after treatment for 24 h, was assessed against these cell lines using the MTT assay.10,11 The VLC fractions were assessed for cytotoxicity using the same assay but at different concentrations: 0.0, 0.0004, 0.002, 0.01, 0.05 and 0.25 mg/mL. The IC₅₀ values of these extracts against the tested cell lines (where appropriate), and that of the VLC fractions against the PC3 cell line were calculated applying the method described by Patel *et al.*12 Any IC₅₀ value of greater than 100 mg/mL was considered non-cytotoxic. The cytotoxicity of the *n*-hexane extract could not be assessed because of its insolubility in DMSO.

**RESULTS**

The Soxhlet extraction of the shed-dried ground leaves of *A. pavarii* gave three different extracts, *i.e.*, *n*-hexane, DCM and MeOH, with 4.4, 1.8 and 36.0% yields, respectively. Because of insolubility issues, *n*-hexane extract was not subjected to cytotoxicity assay (MTT assay), but the other two extracts were assessed for their cytotoxicity against five different human cancer cell lines, and the DCM extract was found to be the most cytotoxic extract (Table 1). Additionally, the DCM extract was tested for cytotoxicity against the normal human prostate cell line PNT2 (Table 1), and the selectivity index (SI), which is the ratio between the IC₅₀ values against the normal cells and the cancerous cells, was calculated.

**Table 1. Cytotoxicity of DCM extract of *A. pavarii* against five human cancer cell line and normal human prostate cell line PNT2**

<table>
<thead>
<tr>
<th>Cancer cell lines</th>
<th>Inhibitory concentration 50% (IC₅₀) in mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 (human lung carcinoma)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>EJ138 (human bladder carcinoma)</td>
<td>90</td>
</tr>
<tr>
<td>HepG2 (human liver hepatocellular carcinoma)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MCF7 (human breast adenocarcinoma)</td>
<td>100</td>
</tr>
<tr>
<td>PC3 (human prostate cancer)</td>
<td>26</td>
</tr>
<tr>
<td>PNT2 (normal human prostate cells)</td>
<td>90</td>
</tr>
</tbody>
</table>

As the DCM extract was significantly cytotoxic, and it showed the most prominent activity against the PC3 cell line, it was subjected to VLC fractionation resulting in eight different fractions (VLC-1 to VLC-8), which were then assessed for cytotoxicity against the PC3 cell line (Table 2).

The most active fraction VLC-5 was subjected to reversed-phase preparative HPLC on a C₁₈ preparative column using a linear gradient elution with a mobile phase comprising acetonitrile and water (both containing 0.1% TFA). Preparative HPLC analysis of a portion of the fraction VLC-5 resulted in the isolation of two major compounds 1 and 2 (Table 3), which were identified as arbutin (1) and betulinic acid methyl ester (2) by spectroscopic analyses, especially, MS, and 1D and 2D NMR, as well as by comparison with published data for these compounds.13-16

**Table 2. VLC fractions of the DCM extract of *A. pavarii* and their cytotoxicity against the PC3 cell line**

<table>
<thead>
<tr>
<th>VLC fractions</th>
<th>Mobile phase composition</th>
<th>Weight (mg)</th>
<th>IC₅₀ value in mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLC-1</td>
<td>100% <em>n</em>-Hexane</td>
<td>150</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VLC-2</td>
<td>10% EtOAc in <em>n</em>-hexane</td>
<td>65.2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VLC-3</td>
<td>30% EtOAc in <em>n</em>-hexane</td>
<td>41.7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VLC-4</td>
<td>50% EtOAc in <em>n</em>-hexane</td>
<td>165.7</td>
<td>40.0</td>
</tr>
<tr>
<td>VLC-5</td>
<td>50% EtOAc in <em>n</em>-hexane</td>
<td>477.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>
Table 3. Isolation and identification of arbutin (1) and betulinic acid methyl ester (2) from the fraction VLC-5 of the DCM extract of A. pavarii (retention time and weight)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention time ($t_R$) in min*</th>
<th>Weight in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbutin (1)</td>
<td>17.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Betulinic acid methyl ester (2)</td>
<td>32.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*A gradient elution with 50-100% acetonitrile in water (both containing 0.1% 0.1% TFA) in 30 min, then 100% acetonitrile for 10 min, flow rate: 10 mL/min.

Arbutin (1): White powder; UV (MeOH) $\lambda_{max}$: 212 and 282 nm; ESMS $m/z$: 273 [M+H]$^+$; $^{1}$H NMR (600 MHz, in CD$_3$OD): δ 6.77 d ($J = 8.9$ Hz, H-2 and H-6), 6.97 d ($J = 8.9$ Hz, H-3 and H-5), 4.81 d ($J = 7.6$, H-1'); 3.50-3.91 (overlapped peaks, H-2', H-3’, H-4’, H-5’ and H2-6’); $^{13}$C NMR (150 MHz, in CD$_3$OD): δ 151.8 (C-1 and C-4), 118.8 (C-2 and C-6), 116.3 (C-3 and C-5), 103.2 (C-1’), 77.5 (C-3’), 77.0 (C-5’), 75.1 (C-2’), 71.5 (C-4’) and 62.7 (C-6’).$^{13-15}$

Betulinic acid methyl ester (2): White amorphous powder; UV (MeOH) $\lambda_{max}$: 208 nm; ESMS $m/z$: 493 [M+Na]$^+$; $^{1}$H NMR (600 MHz, in CD$_3$OD): δ 4.77 bs and 4.64 bs (H$_2$-29), 3.52 s (-OCOMe) 3.22 dd ($J = 4.6$ Hz and 11.4 Hz, H-3), 3.10 m (H-19), 1.51 t ($J = 3.6$, H$_2$-1), 1.80 m (H$_2$-2), 1.70 s (H$_3$-30), 0.98 s (H$_3$-7), 0.94 s (H$_3$-26), 0.87 s (H$_3$-25), 0.86 (H$_3$-24) and 0.84 (H$_3$-23); $^{13}$C NMR (150 MHz, in CD$_3$OD): δ 178.8 (C-28), 151.5 (C-20), 110.0 (C-29), 78.4 (C-3), 56.6 (C-17), 55.7 (C-5), 51.0 (C-9), 50.0 (-OCOMe), 49.0 (C-19), 47.4 (C-18), 42.8 (C-14), 41.0 (C-8), 39.5 (C-4), 39.2 (C-13), 38.6 (C-13), 37.5 (C-22), 37.2 (C-10), 34.8 (C-7), 32.2 (C-16), 31.0 (C-21), 30.0 (C-15), 28.1 (C-22), 28.1 (C-2), 25.9 (C-12), 21.0 (C-11), 19.5 (C-30), 19.2 (C-20), 18.9 (C-6), 16.9 (C-2'), 15.3 (C-24), 16.2 (C-25) and 15.0 (C-27).$^{16,17}$

DISCUSSION

The highest extraction yield was obtained by MeOH (36.0 %), which could be due to the presence of large amounts of polar phenolic and polyphenolic compounds (e.g., tannins) and their glycosides in the leaves. On the other hand, DCM produced the least extraction yield (1.8%), which is not surprising, as most non-polar compounds are generally extracted by n-hexane and the polar compounds by MeOH, leaving DCM to extract only some compounds of medium polarity, and a few left over or trailing nonpolar compounds from the n-hexane extract and a small proportion of polar compounds that are fully extracted in the MeOH extract.

In the MTT assay, the DCM extract showed significant cytotoxicity against the prostate cancer cell line PC3 (IC$_{50}$ = 26 mg/mL), and a moderate level of activity against the bladder cancer cell line EJ138 (IC$_{50}$ = 90 mg/mL) (Table 1). However, this extract did not show any cytotoxicity against three other cancer cell lines: HepG2, A459 and MCF7. Moreover, the cytotoxicity of the DCM extract against normal human prostate cell line PNT2 (IC$_{50}$ = 90 mg/mL) (Table 1) was much less than its activity against the prostate cancer cell line PC3. The selectivity index (SI) of the DCM extract was determined as 90/26 = 3.5, indicating its selective cytotoxicity toward cancer cells as opposed to normal cells. The MTT result also demonstrated that the cytotoxicity of the DCM extract was selective to certain cancer cells and was not cytotoxic to each cell line tested.

As the DCM extract was most active against the PC3 cell line, this cell line was chosen for further bioassay-guided isolation processes, which started with the VLC fractionation of the DCM extract resulting in eight different fractions of different weights (Table 2). Fraction eluted with 50% EtOAc in n-hexane (VLC-5) was the highest yielding fraction (477 mg), followed by VLC-4 (165.7 mg), VLC-1 (150.0 mg) and so on (Table 2). The overall recovery of materials from the VLC process was 51.8%, which is quite usual in such operations.$^9$ All
eight fractions were subjected to the MTT assay using the PC3 cell line, and VLC-5 produced the most prominent cytotoxic effect against this cell line (IC_{50} = 30 mg/mL) (Table 2). Therefore, this fraction was chosen for preparative HPLC analysis aiming at isolating major compounds from this fraction. Fractions VLC-1, VLC-2, VLC-3, VLC-7 and VLC-8 were inactive against this cell line, while fractions VLC-4 and VLC-6, in addition to VLC-5, showed cytotoxicity with IC_{50} values of 40 and 98 mg/mL. As VLC-5 was the most active fraction, it was subjected to preparative HPLC analysis resulting in the purification of two compounds which were identified as arbutin (1)\textsuperscript{13-15} and betulinic acid methyl ester (2) by spectroscopic means.

Compound 1 was eluted first (t_{R} = 17.2 min) in the preparative HPLC run and obtained as a white powder. While the UV absorption maxima of this compound were 212 and 282 nm, the ESIMS analysis revealed the pseudomolecular ion [M+H]\textsuperscript{+} at m/z suggesting the molecular formula C_{12}H_{16}O_{7}. Analyses of the \textsuperscript{1}H NMR and the \textsuperscript{13}C NMR spectra of compound 1 revealed signals assignable to the protons/carbons of a para-di-substituted aromatic ring at δ_{H} 6.97 (d, J = 8.9 Hz, 2H) and δ_{C} 116.3, and δ_{H} 6.77 (d, J = 8.9 Hz, 2H) and δ_{C} 118.8 and all required signals for a b-D-glucopyranoside unit (data shown in the Results section). While a \textsuperscript{1}H-\textsuperscript{1}H COSY spectrum showed all \textsuperscript{1}H-\textsuperscript{1}H scalar couplings, a \textsuperscript{3}H-\textsuperscript{13}C HMBC displayed all major \textsuperscript{1}H-\textsuperscript{13}C long-range correlations and helped identification of this compound as arbutin (1) (Figure 2). The most noteworthy HMBC correlation was the 3\textsuperscript{J} correlation from the glucose anomeric proton (d_{H} 4.81) to the C-1 (d_{C} 103.2) of the aromatic ring. All data were comparable to the published data for arbutin (1).\textsuperscript{13-15}

Compound 2 was eluted beyond the gradient run duration and during the cleaning phase (t_{R} = 32.1 min) in the preparative HPLC run, and obtained as a white amorphous powder, having a very low UV absorbance maximum at 208 nm. The ESIMS exhibited the sodiated molecular ion peak at m/z 493 [M+Na]\textsuperscript{+} corresponding to the molecular formula C_{31}H_{50}O_{3}. The \textsuperscript{1}H and \textsuperscript{13}C NMR spectra showed characteristic signals for a betulinic acid skeleton with an additional methyl ester functionality\textsuperscript{16,17}, including the signals for six methyl groups (d_{H} 1.70, 0.98, 0.94, 0.87, 0.86 and 0.84; d_{C} 28.6, 19.5, 16.9, 16.3, 16.2 and 15.0), a hydroxyl group (d_{H} 3.22; d_{C} 78.4), an olefinic methylene functionality (d_{H} 4.77 bs and 4.64; d_{C} 151.0) and a methyl ester group (d_{H} 3.52; d_{C} 178.8 and 50.0). Thus, the compound was identified as betulinic acid methyl ester (2), and all data were comparable to the literature data for this compound.\textsuperscript{16,17}

This is the first report on the assessment of cytotoxicity of any DCM extract of the leaves of A. pavarii against various human cancer cell lines, as well as the bioassay-guided isolation of main compounds (1 and 2) from the bioactive fraction. While the distribution of arbutin is quite widespread within the genus Arbutus,\textsuperscript{18} this is the first report on the isolation of triterpene acid ester from A. pavarii. However, related pentacyclic triterpenes were previously isolated from the fruits of another species of this genus, A. unedo, for example, \alpha- and \beta-amyrin, betulinic acid, lupeol olean-12-en-3\text{b},23-diol.\textsuperscript{19} Similarly, the cytotoxic properties of the MeOH extract of the aerial parts of A. pavarii were previously demonstrated against both HepG2 hepatic carcinoma and T47D breast cancer cell lines, and the IC\textsubscript{50} values were determined as 19.7 and 19.0 mg/mL, respectively.\textsuperscript{3} The cytotoxicity of the MeOH extract of the aerial parts of this plant was also shown against lung (A549) and breast (MCF7) cancer cell lines with IC\textsubscript{50} values of below 30 mg/mL.\textsuperscript{4} Notably, this is the first report on the assessment of cytotoxicity of any DCM extract of A. pavarii against the bladder (EJ138) and prostate cancer (PC3) cell lines.

Arbutin (1) and its derivatives are known to possess cytotoxic as well as potential anticancer properties. Several arbutin derivatives with an acylated glucosyl unit were isolated from the leaves of Heliciopsis lobata and shown to possess cytotoxic property against the gastric cancer MGC803 cells and to inhibit MGC803 cell invasion.\textsuperscript{20} In a recent study looking into
the possible mechanism of action of arbutin (1), it was observed that this compound could display anticancer activity against rat C6 glioma cells by prompting apoptosis as well as by inhibiting inflammatory marker and phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling molecules. Moreover, arbutin (1) could generate excessive reactive oxygen species and disrupt the mitochondrial membrane resulting in induction of apoptosis in cells. Arbutin (1) and a few other phenolic compounds were assessed for their cytotoxic property against two human breast cancer cell lines, adriamycin-resistant MCF-7/Adr and wild-type MCF-7/wt, and arbutin (1) was found active only against the MCF-7/Adr cell line with an IC_{50} value of 5.58 mM. Hydroquinone, the aglycone of arbutin (1), showed antiproliferative activity against C6 and HeLa (human cervix carcinoma) cells, while arbutin (1) did not show antiproliferative activity in all tested doses. It can be noted here that C6 cells are spindle-like cells that can stimulate human glioblastoma when they are injected in the brain of neonatal rats, and this cell line is considered as the gold standard in glioma research.

Betulinic acid and its derivatives, e.g., betulinic acid methyl ester (2), are known to possess various bioactivities, including potential anticancer properties. In a study conducted by Quang et al., betulinic acid methyl ester (2) as well as betulinic acid were tested for cytotoxicity against HeLa, HepG2, SK-LU-1 (human lung carcinoma), AGS (human stomach gastric adenocarcinoma) and SK-MEL-2 (human melanoma) cell lines, and it was found that both compounds could exert non-selective and a moderate level of cytotoxicity against all five cell lines. Betulinic acid methyl ester (2) was particularly active against the SK-LU-1 cell line with an IC_{50} value of 60.84 mg/mL, and the IC_{50} values against other cell lines were in between 66.17 and 80.17 mg/mL.

CONCLUSIONS
Bioassay-guided approach afforded isolation of arbutin (1) and betulinic acid methyl ester (2) as the main compounds present in the cytotoxic fraction of the DCM extract of the leaves of *A. pavarii*. The DCM extract showed remarkable cytotoxicity against the prostate cancer cell line PC3, but much less toxicity against normal human prostate cell line PNT2, meaning cytotoxicity being more selective to cancer cells over normal cells. This is the first report on the isolation of betulinic acid methyl ester (2) from the leaves of *A. pavarii*. As both 1 and 2 were demonstrated previously to possess cytotoxic/antiproliferative properties against various human cell lines, and as they are present in the cytotoxic fraction of the DCM extract, it is reasonable to assume that the significant cytotoxicity of the DCM extract observed in the current study could be, at least partly, due to the presence of these two cytotoxic compounds. Further study involving these compounds and their structural analogues for their cytotoxic potential against human prostate cancer cell lines as well as mechanistic studies, e.g., apoptosis, necrosis and so on, could provide an insight into their structure-activity-relationships and possible mechanisms of action.

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REFERENCES
25.