New cytotoxic chalcone derivatives from *Astragalus ponticus* Pall.

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**ABSTRACT**

*Astragalus ponticus* Pall. species was investigated for its antiproliferative effects on HeLa cells. Two new chalcones (B5 and B8) along with eight known compounds (B1, B2, B3, B6, B7, B10, B14 and B15) were isolated by following bioactivity guided isolation methods. In addition, from non-active fraction, three cycloartane glycosides (B11, B12 and B13) were isolated. Molecular structures of these isolated compounds were revealed by using spectroscopic methods like MS, 1D and 2D NMR and a single crystal X-ray diffraction analysis. New compounds B5 and B8 showed the highest antiproliferative activities against HeLa cells (IC\(_{50}\) values of 36.6 and 20.6 \(\mu\)M, respectively) while the rest showed high and low activities. Non-endemic species attract relatively low attention from the scientific community but this study demonstrates that valuable new compounds, which might be used as ingredients in medicinal preparations, can be obtained from these materials.

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**1. Introduction**

Since the beginning of Ayurvedic applications (3000 BC), natural product research has been evolving systematically in terms of extraction methods, isolation techniques,
identification methods and finally, usage area (Sarker et al. 2006). In light of inspection of archeological remnants, use of plants as medicine can be traced back to almost 60,000 years ago (Fabricant and Farnsworth 2001). Natural products are not only good sources of valuable medicinal materials but also lighthouses and inspirations for the scientific community to design and synthesis new drug candidate molecules (Molinski 2014).

Endemic species are considered by researchers as valuable and potential sources of new compounds with possible novel skeletons. However, the biggest handicap of working with endemic species is that the studies may lead to loss of the individual species from the natural habitat because of overexploitation. And this is one of the main drawbacks of natural products research: it is not always possible to find materials easily and in enough amounts (Harvey 2008). Almost a quarter of medicinal plants are threatened with extinction (Li and Vederas 2009). Considering the effect of human population on the number of plant species, over collecting the endemic species might give huge damages to natural sources.

As a subgroup of flavonoids, chalcones are one of the most interesting and attractive natural products (Zhang et al. 2013). Chalcones are structurally open-chain flavonoids: two aromatic rings are bonded through a three-carbon \( \alpha, \beta \) unsaturated system (Zhang et al. 2013; Kupcewicz et al. 2014). Since this class of compounds possesses various biological activities, such as antimalarial, anticardiac, anticancer, etc., chalcones have also gained special attention from synthetic chemists (Farooq and Ngaini 2019). Anwar et al. (2018) suggest that anticancer activities of chalcones result from the \( \text{C-}\alpha \) and \( \text{C-}\beta \) unsaturated system, together with the substituents on ring A and B: While the substituents like hydroxy and methoxy on ring A hinder the cancer cell growth, these substituents on ring B stop the cell division by interacting with the cell nucleus. In addition to this, chalcones show their effects through mechanisms like anti-initiation, apoptosis induction, antiproliferation, antimetastasis and antiangiogenesis (Zhang et al. 2013).

*Astragalus ponticus* Pall. is a member of *Astragalus* genus, which is one of the biggest genera within *Fabaceae* family with 2500–3200 species worldwide (Soltani et al. 2021). Some of the members of Astragalus genus, e.g. *A. membranaceus* and *A. complanatus*, are recorded in Chinese Pharmacopoeia (Sinclair 1998; Hu et al. 2009). We report here the results of our study on non-endemic *A. ponticus* Pall. species. In short, bioactivity guided chromatographic separation studies gave two new chalcones, 4-methoxy-2,3,4'-trihydroxychalcone (\( \text{B5} \)) and 4',5-dihydroxy-2,2'dimethoxychalcone (\( \text{B8} \)), and eight known compounds, 3-deoxysappanchalcone (\( \text{B1} \)) (Achenbach et al. 1988), pendulone (\( \text{B2} \)) (Rahman et al. 2011), 2-(2,5-dihydroxybenzyl)-6-methoxyaurone (\( \text{B3} \)) (Xiao et al. 2014), isoliquiritigenin (\( \text{B6} \)) (Veitch et al. 2003), cyclogaleoginoside B (\( \text{B7} \)) (Wang and Chen 2017), isomucronulatol (\( \text{B10} \)) (Guo et al. 2016), ergosterol peroxide (\( \text{B14} \)) (Trigos and Ortega-Regules 2002) and millepurpan (\( \text{B15} \)) (Gatouillat et al. 2014), in addition to three known compounds, astrasieveriansian II (\( \text{B11} \)) (Tabanca et al. 2005), astrasieveriansian VI (\( \text{B12} \)) (Tabanca et al. 2005) and astragaloside I (\( \text{B13} \)) (Zheng et al. 2015), from non-active fractions. These compounds were tested for their *in vitro* antiproliferative activities against human cervical cancer (HeLa) cells. Furthermore, structure-activity relationships for the compounds were discussed.
2. Results and discussion

Compound B5’s $^1$H and $^{13}$C NMR spectra reveal that this compound has a chalcone skeleton (All the NMR spectra for each compound can be found in the supplementary material). Its DEPT spectrum shows seven quaternary, eight methines and one methyl carbon. α and β protons, at 7.76 and 8.11 ppm respectively, have a coupling constant of 15.4 Hz, meaning that these protons are trans to each other (Tatsuzaki et al. 2006). C-6 methine carbon resonates at 131.5 ppm: such a shift to lower field shows that there is an oxygen substitution (either as –OH or –OR) at the meta position to this carbon (Soláňová et al. 1976; Hwang et al. 2011). HMBC correlation between C-β and the proton at 7.57 ppm confirms that this proton is located at position 6. H-6 proton gives a COSY correlation ($J = 8.7$ Hz) with H-5 at 6.51 ppm; and H-5 gives a splitting with a coupling constant of 2.5 Hz with the proton at 6.45 ppm, meaning this proton is located at position 3. HMBC correlation between H-β and the carbon at 159.5 ppm reveals that this carbon is the one at position 2. Methyl protons at 3.80 ppm give a HMBC correlation with the carbon at 163.4 ppm, so it can be said that this methoxy group is located at position 4. With these remarks, all the connections at ring B were concluded. Having HMBC correlations with C-3', C-4' and C-6', and not having any correlation with C-5' for the proton at 7.37 ppm means this proton is located at position 2' and it shows a para correlation with the proton at 6.81 ppm (H5', $J = 2.9$ Hz). The proton at 7.0 ppm shows a COSY correlation with H-5' proton ($J = 8.9$) meaning this proton is located at position 6'. H-6' shows a strong HMBC correlation with the carbon at 156.1 ppm but a weak correlation with the carbon at 149.0 ppm, indicating that these carbons are located at positions 4' and 3', respectively. After all these assignments, compound B5 was identified as 4-methoxy-2,3',4',5'-trihydroxychalcone (Figure 1).

B8's core structure was found as a chalcone by examining its $^1$H and $^{13}$C NMR spectra. DEPT spectrum shows seven quaternary, eight methines and two methyl carbons. H-α proton at 7.73 ppm and H-β proton at 8.12 ppm are located trans to each other with a coupling constant of 15.6 Hz (Tatsuzaki et al. 2006). Three correlations can be seen in B8’s COSY spectrum: One for α, β protons, one for each of A, and B rings. HMBC correlation of the proton at 7.16 ppm with C-β at 139.3 ppm shows this proton is located at position 6 of ring B. In the same manner, H-β at 8.12 ppm has a HMBC correlation with C-6 at 114.0 ppm. H-4 proton at 6.86 shows a meta interaction ($J = 2.9$ Hz) with H-6 proton at 7.16 ppm and an ortho interaction ($J = 8.9$ Hz) with H-3 proton at 6.91 ppm. Methyl protons at 3.86 ppm show a HMBC correlation with the carbon at 152.6 ppm. H-β proton at 8.12 ppm also shows a correlation with this carbon, meaning this carbon is located at position 2 and methoxy group is connected to

![Figure 1. Molecular structures of B5 and B8.](image-url)
C-2. The location of the proton at 7.97 ppm is decided as 6' of ring A because (i) Characteristic lower field resonation of the C-6', a result of the connection of two -OR groups meta to this carbon (Solǎnová et al. 1976; Hwang et al. 2011), and (ii) HMBC correlation to the carbonyl carbon. H-6' shows an ortho interaction (\(J = 9.0 \text{Hz}\)) with 5' proton at 6.54 ppm and 5' proton shows a meta interaction (\(J = 2.5 \text{Hz}\)) with 3' proton at 6.45 ppm. In the light of these findings, B8 was identified as 4',5-dihydroxy-2,2'dimethoxychalcone (Figure 1).

Crystal data, refinement parameters, selected bond distances, bond angles and hydrogen bond interaction tables for B8 are given in supplementary material (Tables S1–S3). Compound MERCURY structure, hydrogen bonding and crystal packing structures are given in Figures 2, S27 and S28, respectively. X-ray suitable crystals of compound B8 were obtained by leaving the NMR sample at room temperature for one month in an airtight NMR tube. The analysis of crystallographic data revealed that B8 shows orthorhombic, \(P2_12_12_1\) space group. The shortest bond distance is carbonyl group (C(10)-O(3)) measured as 1.257 Å. Two hydroxy group oxygen-carbon distances (C(5)-O(2) and C(12)-O(4)) are calculated as 1.370 and 1.346, respectively. From the packing structure, some intra- and intermolecular hydrogen bonding interactions were observed. The only intramolecular hydrogen bonding interaction is observed between C(10)-O(3)----H4 atoms measured as 1.806 and one intermolecular hydrogen bonding interaction is observed between C(10)-O(3)----H2 atoms with 1.964 Å bond distances. (CCDC 1985022 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving. html or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

Four chalcones, two of which are new in the scientific literature, were tested for their antiproliferative activities against human cervical cancer cells (HeLa); accordingly, following results were obtained: IC\(_{50}\) values of 116.2, 36.6, 105.4, 20.6 \(\mu\text{M}\) for compounds B1, B5, B6 and B8, respectively (Results were given in the supplementary material).

Activities of the isolated chalcones followed an increasing manner in accordance with their oxygen substitution, depending on the position (Figures S56–S59). Each B5
and B8 has four oxygen substitutions on their rings A and B and has the highest activities (IC50 values 36.6 and 20.6 \(\mu M\) respectively), whereas B1 and B6, with three oxygen substitutions, showed lower activities (IC50 values 116.2 and 105.4 \(\mu M\)). Only difference between B1 and B6 is the substitution on 2’ location: B1 is with a methoxy and B6 is with a free hydroxy group. From their study on chalcones, Boumendjel et al. (2008) obtained an implication, which states the free hydroxy group at the 2’ location diminishes activities of chalcones as this group forms a hydrogen bond with carbonyl oxygen and flattens the main structure by inhibiting the free rotation of \(O = C – C1’\). However, in our case, instead of a fall in the activity by a 2’-hydroxy placement, we obtained a relatively higher result for B6 than B1. In a study by Rao et al. (2004) on Jurkat (human leukemia) and U937 (human lymphoma) similar SAR results were reported. In a supporting manner, although B8 has a free 2’-hydroxy group, it is the most active chalcone of isolated four chalcones in this study. Again, Boumendjel et al. (2008) suggested that ring A of chalcones is an important pharmacophore with a big impact on the pharmacodynamics characteristic of the whole molecule and methoxy substitution on ring B would have a lesser effect. Our results showed that activities of B1 and B6, each having only one hydroxy substitution on the ring B, are much lower than the activities of B5 and B8 with one hydroxy and one methoxy substitution on the ring B. This phenomenon can also be explained by the findings of Rao et al. (2009). In this study, it was suggested that an increase in the lipophilicity of ring B may cause an increase in the affinity to the cell membrane, thus may end up with higher activity. In our case, relatively more lipophilic methoxy groups on ring B, compared to free hydroxyl ones, caused a rise in the activities of B5 and B8. By comparing the IC50 values of B1, B6 and B8, we can deduce that a methoxy substitution at the 4’ location increases the activity. Chalcones show their anticancer effects through different cellular mechanisms, one of which is antimitotic effect (Zhang et al. 2013). Karthikeyan et al. (2015) suggest that methoxy groups on ring B, especially at positions 2, 4, and 6, are likely to be responsible for an increase in antimitotic activity of chalcones. Compound B8 has a methoxy group at position 2 of ring B, while compound B5 has a free hydroxy group at the same position. By comparing the structures and activities of these, correlatively to Karthikeyan et al. (2015)’s proposal, it can be deduced that these compounds show their activities through antimitotic mechanism.

Effect of isoflavanes B10 (isomucronulatol) and B15 (millepurpan) on HeLa cells can be seen in S61–S62. B10 and B15 showed weak activities with IC50 values of 121.1 and 161.5 \(\mu M\) for B10 and B15, respectively. It seems that an addition of a methoxy group at ring B caused a drop in activity. Peng et al. (2019) reported a result that is similar to our findings. The researchers isolated a series of isoflavanes from Spatholobus suberectus and tested them for their cytotoxic activities against human breast cancer cells. According to findings, establishment of a methoxy group at 2’ location results in a fall in the activities, whereas displacement of this methoxy by a free hydroxy group ends up with loss of activity at all.

B7, B11, B12 and B13 showed their antiproliferative activities with the IC50 values of 34.8, 105.7, 52.6 and 140.2 \(\mu M\), respectively (Results are given in the supplementary material). These compounds differ from each other only at the sugar moieties. According to the findings, an increase in the number of sugar groups leads to a
decrease in the activity: Monodesmoid B7 had the highest activity, while bidesmoid B11, B12 and B13 had lower activities. The reason for this result might be that the more polar –OH groups of sugar moieties may hinder the introduction of the molecule into the cytoplasm through non-polar cell membrane (Podolak et al. 2010). Reddy et al. (2017)’s results support this fact. A series of cycloartane type-triterpenoids were tested against several human cancer lines, including HeLa, and the result revealed that the highest activities against HeLa and other cell lines were shown by the compounds that do not bear any sugar moiety (Reddy et al. 2017). This might imply that the free 3- and 6-hydroxy groups have pharmacophore characteristics, which are necessary for the activity. Also, acetylation of 3-O-sugar moiety at 3' position induced a counter-effect on the activity. A similar result was found by Reddy et al. (2017). Finally, compounds B3 and B14 had no antiproliferative effect on HeLa cells (see the supplementary material).

3. Experimental

3.1. Spectroscopic data

3.1.1. 4-Methoxy-2,3',4'-trihydroxychalcone (B5)
Orange solid (Yield: 0.87 mg/100 g plant material). Its molecular formula was found to be C_{16}H_{14}O_{5} by examining its HRMS (MALDI/ToF) ([M-H]^{−}, m/z 285.0749, calculated 285.0768) (Figures S1 and S2) and NMR spectra. FTIR (cm^{−1}) 3334 (O-H), 1612 (C=O), 1557 (C=C), 1173 (C-O) (Figure S4). 1H-NMR (600 MHz, CD_{3}OD) δH 8.11 (d, 15.4, H-b), 7.78 (d, 15.4, H-α), 7.57 (d, 8.7, H-6), 7.37 (d, 2.9, H-2'), 7.00 (dd, 8.9, 2.9, H-6'), 6.81 (d, 8.9, H-5'), 6.51 (dd, 8.7, 2.5, H-5), 6.45 (d, 2.5, H-3), 3.80 (s, C4-OCH_{3}). 13C-NMR (150 MHz, CD_{3}OD) δC 194.1 (C=O), 163.4 (C-4), 159.5 (C-2), 156.1 (C-4'), 149.0 (C-3'), 141.8 (C-β), 131.5 (C-6), 123.8 (C-6'), 119.9 (C-1'), 118.1 (C-5'), 117.0 (C-α), 115.0 (C-1), 113.7 (C-2'), 106.1 (C-5), 100.6 (C-3), 54.4 (C4-OCH_{3}).

3.1.2. 4',5-Dihydroxy-2,2'dimethoxychalcone (B8)
Yellow solid (Yield: 0.24 mg/100 g plant material). Its molecular formula was found to be C_{17}H_{16}O_{5} by examining its HRMS (MALDI/ToF) ([M-H]^{−}, m/z 299.0920, calculated 299.0925) (Figures S13 and S14) and NMR spectra. FTIR (cm^{−1}) 3362 (O-H), 2943-2837 (C-H), 1630 (C=O), 1572 (C=C), 1216 (C-O) (Figure S16). 1H-NMR (600 MHz, CD_{3}OD) δH 8.12 (d, 15.6, H-β), 7.97 (d, 9.0, H-6'), 7.73 (d, 15.6, H-α), 7.16 (d, 2.9, H-6), 6.91 (d, 8.9, H-3), 6.86 (dd, 8.9, 2.9, H-4), 6.54 (dd, 9.0, 2.5, H-5'), 6.45 (dd, 2.5, H-3'), 3.86 (s, C2-OCH_{3}), 3.85 (s, C4'-OCH_{3}). 13C-NMR (150 MHz, CD_{3}OD) δC 192.4 (C=O), 166.3 (C-4'), 166.1 (C-2'), 152.6 (C-2), 150.9 (C-5), 139.3 (C-β), 131.4 (C-6'), 123.9 (C-1), 120.1 (C-α), 118.7 (C-4), 114.0 (C-6), 113.9 (C-1'), 112.4 (C-3), 107.0 (C-5'), 100.5 (C-3'), 55.2 (C2-OCH_{3}), 54.7 (C4'-OCH_{3}).

4. Conclusion

In our study on Astragalus ponticus Pall. species, two new and eleven known compounds, four of which are from signature class of cycloartane glycosides for Astragalus species. Structures of these compounds were elucidated by using spectroscopic
methods. These compounds had different core structures like aurone, chalcone, saponin, etc. Also, these compounds are screened for their antiproliferative effects on HeLa cell lines and new compounds with chalcone structures showed the highest activities, which can be further improved by synthetic alterations. Although the scientific community tries to focus on endemic species to find biologically active new compounds, this report emphasizes non-endemic ones can also be valuable sources of new natural products with pharmacological activities.

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