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Original article

Erythroid induction activity of *Aquilegia fragrans* and *Aquilegia pubiflora* and identification of compounds using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Aquilegia fragrans (AF) and *Aquilegia pubiflora* (AP) are the two medicinally important species of genus *Aquilegia* used for the treatment of various diseases and infections. This paper describes the potential of fetal hemoglobin induction activity of the methanolic extracts of AF and AP in K562 cell line. AF and AP have shown 27.147 ± 1.376 and 32.786 ± 1.048 percent erythroid induction, respectively at 15.625 (μg/mL) concentration which suggested that both plants can be the source of potential fetal hemoglobin inducers and may be used for the treatment of β-thalassemia. Phytochemical analyses of both species were also evaluated by using high-resolution LC-ESI-QTOF-MS/MS techniques. A Total of thirty compounds were identified using positive and negative ionization modes. The identification was based on the matching of high-resolution masses, isotopic pattern, and MS/MS fragmentation. Several statistical analyses were performed to evaluate the distribution of compounds in both species. Identified compounds belong to various classes including flavonoids, steroids, lignans, terpenoids, benzofuran and coumarins. The established chemical fingerprints will be helpful in standardization and quality control of plant extracts.

1. Introduction

β-thalassemia, an inborn genetic disorder, is characterized by total absence or insufficient synthesis of β-globin chain (Thein, 2018). Due to reduced synthesis or total absence of β-globin chains, excessive α-globin chains, get accumulated in cells and form inclusion bodies that affect the membrane integrity and cause the premature death of erythroid cells (Sankaran and Orkin, 2013). This leads to ineffective erythropoiesis, which results in chronic anemia associated with β-thalassemia (Nienhuis and Nathan, 2012).

Current treatment options for β-thalassemia are mostly symptomatic, including blood transfusion and iron chelation therapy. Bone marrow transplantation and gene therapy could be promising, but these methods have limitations due to the high cost and availability to common man, especially in developing countries (Cavazzana-Calvo et al., 2010). Therefore, current research focuses on a cost-effective and safe therapeutic approach to treat β-thalassemia, which is fetal hemoglobin (HbF) induction. Fetal hemoglobin, a predominant form of hemoglobin during fetal stage of development, and about one year after birth, it is gradually replaced with adult hemoglobin as predominant form. This developmental suppression of fetal hemoglobin can be reversed by using inducers to ameliorate the clinical symptoms of thalassemia

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(Rees et al., 1999). Several potential HbF inducers, like hydroxyurea, butyrates, erythropoietin, and 5-azacytidine have been discovered, but their therapeutic usage is limited due to their cytotoxicity and carcinogenic properties (Osti et al., 1997). Therefore, there is a strong need to search for potential HbF inducers which are affordable and associated with minimal side effects.

Plants have been the source of lead molecules that are being used for the treatment of various diseases. *Aquilegia* (common name columbine) is one of the phytochemically important, phenotypically diverse and experimentally a tractable genus that belongs to family Ranunculaceae. *Aquilegia fragrans* is a perennial herb that mostly grows in northern regions of Pakistan, north-west Himalaya, eastern Afghanistan, north-western India and highly distributed at higher regions of Kashmir valley (Hussain et al., 2011). *A. fragrans* is commonly called as sweet-scented columbine and commonly called as Jangli kuth and Kalumb. The roots of *A. fragrans* are used to treat wounds and various inflammatory diseases like, gout, cystitis, eczema, psoriasis, blood sugar, kidney stones, and veterinary diseases such as bovine mastitis (Ganie et al., 2019). Dried flower paste is used for the relief of headache while seeds and the whole plant are used to cure jaundice and pneumonia, respectively (Khan et al., 2018). *Aquilegia pubiflora* is another medicinally important herb widespread in the Himalaya in India, Afghanistan, and northern Pakistan. It is commonly known as Hairy-flowered columbine while locally called as Domba and Thandi buti. This species is reported to have many medicinal values including anti-asthmatic, astringent, stimulant, cardio-tonic, febrifuge, jaundice, and dyspepsia. Dried roots are used to treat toothache, eye diseases, snakebite and in homeopathy, especially for the nervous system (Dhar and Samant, 1993; Hazrat et al., 2011).

Previous phytochemical studies on *Aquilegia* species, including *A. vulgaris* L., *A. panicii, A. oxysepala, A. canadensis, A. glandulosa, A. excimia* were reported based on various extraction, isolation and analytical methods. These studies have shown identification of flavonoids (Refaat et al., 2015), alkaloids, cyanogenic constituents, cycloartane-type glycosides (Yoshimitsu et al., 1999); butenolides (Guerriero and Pietra, 1984); terpenoids, tannins and unusual unsaturated fatty acids (Bylka and Matlawska, 1999). Until now, reported protocols for phytochemical analysis of *Aquilegia* species by mass spectrometric techniques were either low-resolution or restricted to a nonpolar class of compounds. Previously, no descriptive literature has been published on *A. fragrans* and *A. pubiflora* related to their phytochemistry.

This research aims to screen the potential *of A. fragrans* and *A. pubiflora* as fetal hemoglobin induction for β-thalassemia treatment and the identification of their secondary metabolites by chemical profiling using LC-ESI-QTOF tandem mass spectrometry.

2. Experimental

2.1. Sample collections

A. fragrans was gathered from village Lawat of Athmaqam tehsil in Neelum district (34°41′18.5 ~ N 73°58′12.3 ~ E′) while *A. pubiflora* was obtained from Makra village, Patekha tehsil in Muzaffarabad district (34°27′14.1 ~ N 73°32′56.2 ~ E′) of Azad Kashmir, Pakistan. Plants were identified by the Taxonomist from the Herbarium, University of Karachi, Pakistan and voucher numbers were assigned after deposit of plant material. Voucher no. 95,314 was given for *A. fragrans* while voucher no. 95314B was given for *A. pubiflora.*

2.2. Reagents and chemicals

For the mobile phase, methanol (HPLC grade) was acquired from "Merck" KGaA, (Darmstadt, Germany). Formic acid was bought from "DaeJung Chemical and Metals", (Korea) and type I water was obtained from Barnstead™ GenPure (USA) water purification assembly. Reagents for erythroid induction activity are Fetal Bovine Serum (Gibco), RPMI-1640 (Gibco), Pen/Strip (Gibco), DMSO (Sigma Aldrich, France), Benzidine hydrochloride (Perkin Chemicals, China), Hydrogen Peroxide-30% (Sigma Aldrich, Germany), Glacial acetic acid (Lab Scan), Trypan Blue 4% (Sigma Aldrich, UK), Hydroxyurea (Sigma Aldrich USA).

2.3. Sample preparation

Shade dried whole plants were ground using liquid nitrogen into homogenized powder. Exactly 1 g of each sample was weighed and extracted with 10 mL methanol in a 15 mL falcon tube using a ultra-sonicator for 30 min which were then centrifugated for 15 min at room temperature at 12,000 RPM in order to remove solid particles. 1 mL of upper layer from each sample was filtered through a PTFE syringe-driven filter (0.22 μm) and transferred to 1.5 mL centrifuge tubes for LC-MS analysis. Filtered sample extracts were further diluted by taking 50 μL of sample and diluted with 950 μL (20 times) of methanol in HPLC vial.

2.4. Erythroid induction activity

K562 cells (CCL243, ATCC,), were cultured in RPMI-1460 complete medium containing 10% FBS, and 1% penicillin/streptomycin solution, in a 5% CO_2 at 37 °C under humid conditions. 10 mg/mL stock solutions of methanolic extracts of each plant were prepared in sterile deionized water as a solvent to prepare a stock solution. To determine the erythroid induction, the cells were seeded at density of $4x10^4$ cells/mL in culture and treated in concentrated depend manner range from 250 μg to 15.625 μg of each extract. The cells were then placed in 5% CO_2 incubator at 37 °C for five days.

For erythroid induction benzidine-hydrogen peroxide was used. Briefly, 0.2% benzidine solution was prepared in 0.5 M acetic acid, and 20 μL of hydrogen peroxide was added per 1 mL of ice-chilled 0.2% benzidine solution. On day five, the cells were washed with phosphate buffer saline (PBS) and resuspended in it. Equal volume of benzidine-hydrogen peroxide solution was added to cell suspension in each well and plate was placed in the dark for about 3 min at room temperature (Theodorou et al., 2016). Then the cells were observed under an inverted compound microscope to determine percent benzidine positive cells.

2.5. Analysis of cell viability

The cell viability was determined by using alamarBlue fluorescence assay. This assay utilizes the non-toxic resazurin dye, in healthy cells resazurin undergoes reduction by mitochondrial enzymes and produces a highly fluorescent pink color. This fluorescence intensity of resorufin has been reported as a direct indication of the number of healthy living cells in the medium (Rampersad, 2012).

To determine the viability, cells were seeded (1×10^4 cells/mL) in 96 wells plate and treated with extracts in a dose-dependent manner and incubated for periods of up to 48 h at 37 °C. After incubation, 10 μL alamarBlue dye (Sigma, USA) (25 mg/100 mL PBS) was added to each well and was incubated in dark for 4 h. Cell viability and growth kinetics were determined by measuring the fluorescence intensity at 560 nm (excitation wavelength) and 570 nm (emission wavelength) with SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, USA) using SoftMax Pro software (Molecular Devices USA). The cell viability was calculated using the following formula.

$$\%\text{CellViability} = (Ft - Fb)/(Fc - Fb) \times 100$$

where Fb, Fc, and Ft describes for fluorescence of blank (only medium), untreated cells, and treated cells, respectively. The data were analyzed and presented as mean ± SEM (Error for Mean) using Graph Pad Prism 5.

2.6. LC-ESI-QTOF-MS/MS analysis

The samples were analyzed using LC-ESI-QTOF-MS/MS mass spectrometer (Bruker MaXis-II, Bremen, Germany), coupled to Thermo Fisher Scientific™ Ultimate™ 3000 series UPLC system, (USA), attached with an autosampler, solvent degasser, column thermostat. Chromatography was optimized using five different columns including columns Zorbax (Eclipse XDB-C18), Zorbax (Eclipse XDB-phenyl), Zorbax (Eclipse XDB-CN), Poroshell (120 EC-C18), Nucleodur Gravity column (RP-C18). For efficient chromatographic separation, the Macherey-Nagel RP-C18 Nucleodur Gravity column (3.0 × 100.0 mm, 1.8 μm particle size) was used, at 45 °C. Mobile phase A was an aqueous solution (0.1% formic acid) while methanol (0.1% formic acid) was used as mobile phase B with 0.70 mL/min flow rate. The solvent gradient system was started with 20% B, raised to 70% from 0.0 to 3.0 mins, and kept

Table 1
Erythroid Induction activity of *A. pubiflora* and *A. fragrans*.

Drug Conc: (μg/mL)	AP Extract	AF Extract	Control	Hydroxy Urea
250	15.33 ± 1.4	17.31 ± 3.9	–	–
125	18.71 ± 0.9	18.25 ± 3.1	–	–
62.25	21.2 ± 1.3	25.77 ± 5.6	–	–
31.25	25.22 ± 2.0	27.13 ± 1.2	–	–
15.625	27.15 ± 5.1	31.30 ± 10	–	–
Control	–	–	10.84 ± 2.1	–
Hydroxy Urea	–	–	–	37.06 ± 0.1

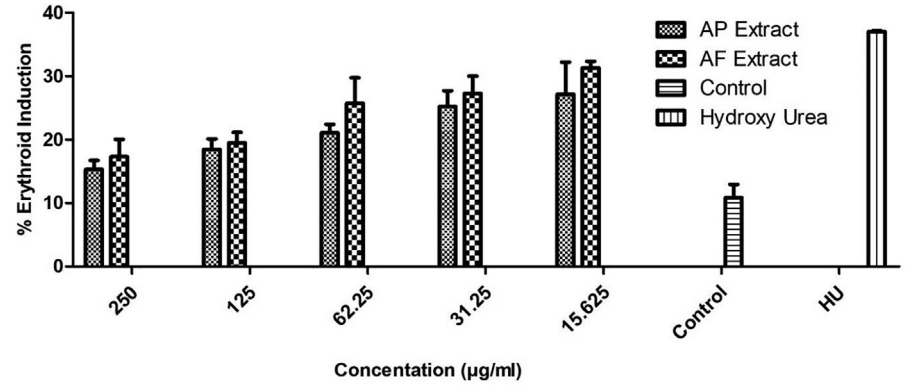

Fig. 1. Erythroid induction activity of *Aquilegia pubiflora* and *Aquilegia fragrans* compared with standard drug hydroxy urea and control. The results are represented as mean % benzidine positive cells at different concentrations.

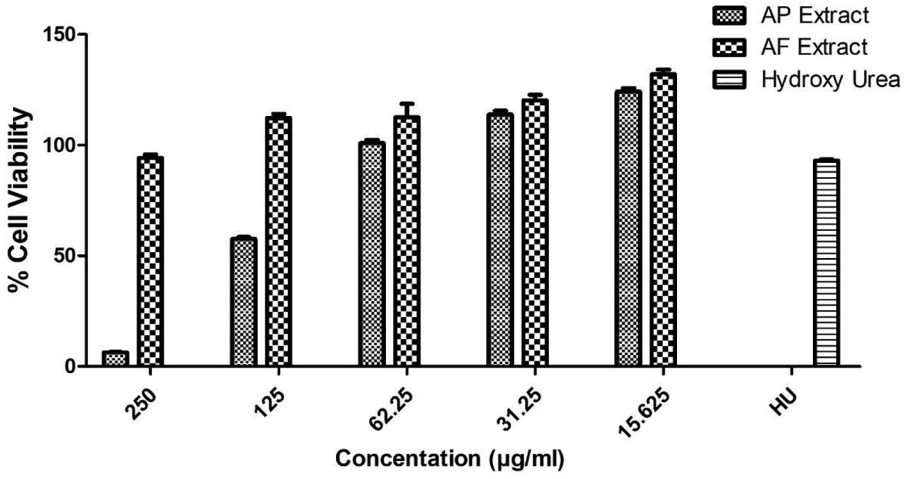

Fig. 2. Cell viability of K562 cells against AP, AF and hydroxy urea at different concentration levels, represented as percent viability of control.

constant for 3.00 min and then again raised to 90% from 6.0 to 8.0 mins then lowered back to 20% at 9.0 min with 1 min equilibrium.

Tandem mass spectrometric analysis was performed using electrospray ionization through positive and negative ionization modes separately. The ion source parameter were set as voltage at 4500 V, drying gas at 270 °C temperature and 12.0 L/min flow rate, nebulizer gas with 45.0 psi pressure, mass range at 100 to 2000 *m*/*z* while scan speed at 5 Hz for MS, and 12 Hz for MS/MS analysis. Acquisition software, 'Bruker Compass TargetAnalysis' was used for quick screening of metabolites.

2.7. Identification of compounds

Compounds identification was based on various features including exact mass matching, error > 5 ppm, MS/MS spectral data was compared with available ESI-MS libraries. An *in-house* library was prepared from Dictionary of Natural Products on DVD (DNP ver. 26.2) based on previously identified compounds of *Aquilegia* species. Then these identified compounds were screened against high-resolution mass spectra using Target Analysis version 1.3 (Bruker Daltonics, Bremen Germany). MS/MS-based identification was performed using different available ESI mass spectral libraries including MassBank of North America, MassBank of Europe, mzCloud and NIST-MS/MS library through comparison of exact masses, isotopic pattern and MS/MS fragmentation pattern.

3. Results and discussion

3.1. Erythroid induction assay

Both, *A. fragrans* and *A. pubiflora*, investigated in this study showed erythroid induction activity in a concentration-dependent manner. The results of Erythroid induction are given in Table 1 and Fig. 1. Both extracts have shown substantial erythroid induction activity at indicated concentration as compared to untreated control group. The extracts have shown more erythroid induction at lower concentrations which decreases with the increase in extract concentration. However, the *A. fragrans* extract was found more potent at all the concentrations as compared to *A. pubiflora.* The maximum activity was observed as 27.15 ± 5.1 and 31.30 ± 1.0 percent benzidine positive cells by *A. fragrans* and *A. pubiflora* extracts, respectively at 15.625 (μg/mL) concentration.

3.2. Evaluation of cell viability

In the cell viability experiment, AF and AP extracts have shown concentration-dependent response. Cell viabilities were represented as percent of control and data is expressed as mean ± SEM for experiments (n = 3). The gradual increase in cell viability was observed with a decrease in the concentration of treatment along. AP extract showed 6.3% cell viability (93.7% cytotoxicity) and 57.6% cell viability (42.6% cytotoxicity) at

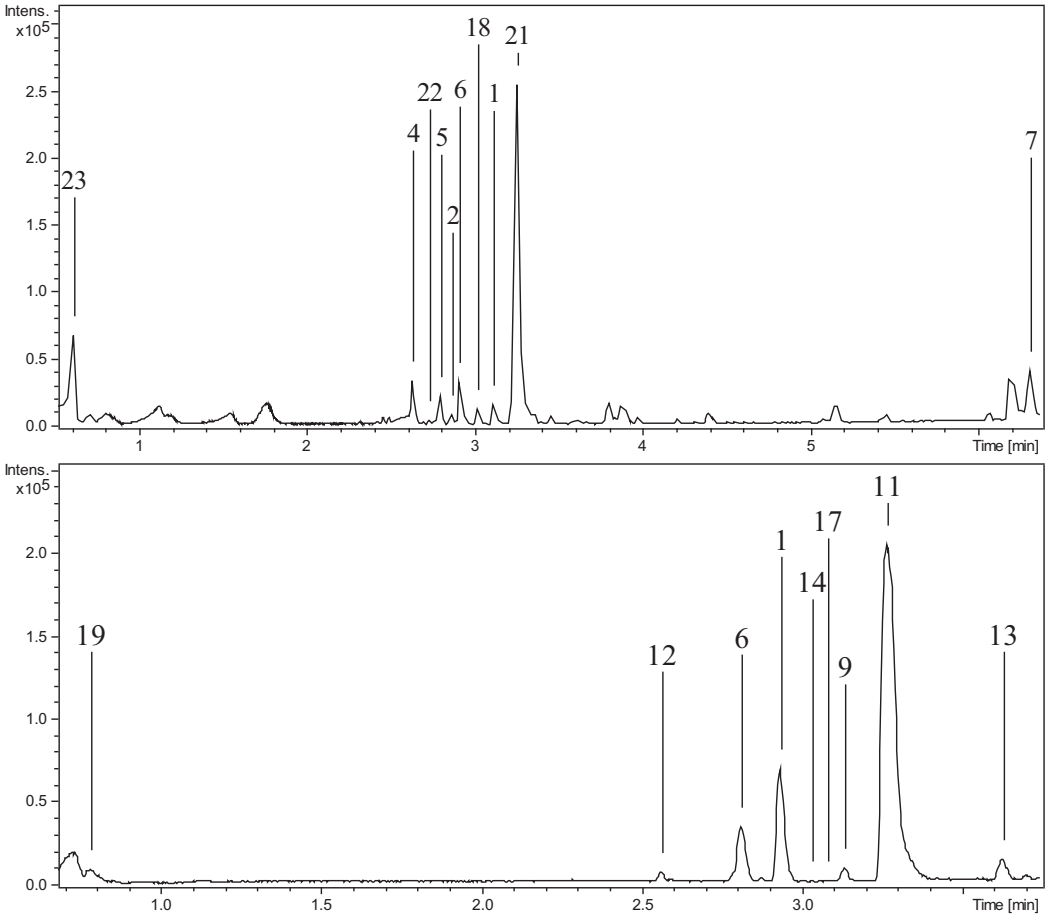

Fig. 3. Base Peak Chromatograms of: AF in positive mode, AF in negative mode, AP in positive mode and AP in negative mode, respectively. Peaks are labeled with the codes of compounds identified in it.

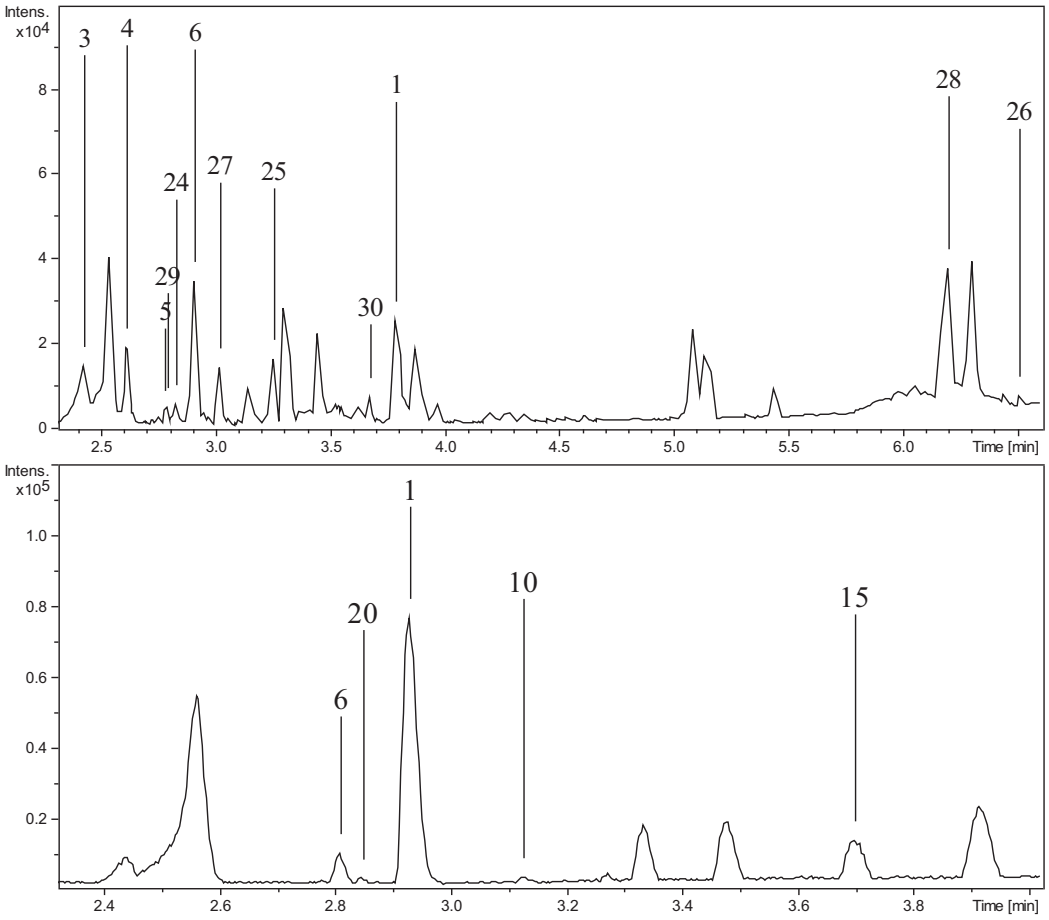

Fig. 3 (*continued*)

250 μg/ml and 125 μg/ml concentrations respectively, but no cytotoxic effects were observed at concentrations ≤ 62.25 μg/ml against K562 cell line. Similarly, 94.5% cell viability (5.5% cytotoxicity) was observed at 250 μg/ml AF extract, but it had no cytotoxic effects at concentrations ≤ 125 μg/ml. Many bioactive plants are toxic on higher concentrations and their dose determines their therapeutic significance (Deshpande, 2002). Both AP and AF extracts showed pro-proliferative effects on K562 cells at lower concentrations (Fig. 2). Our findings suggest that the potent erythroid differentiation of K562 cells by methanolic extracts of *A. fragrans* and *A. pubiflora,* signify these plants as potential sources of HbF inducers for the treatment and efficient management of β-thalassemia.

3.3. Chemical fingerprinting of Aquilegia samples through identification of compounds

Plant samples were analyzed using high-resolution LC-ESI-QTOF tandem mass spectrometry. LC-MS method was optimized using five columns with different chemistry and dimensions. The best-resolved chromatogram (Fig. 3) was observed using RP-C18, column (3.0 × 100.0 mm, 1.8 μm) and thus selected for LC-MS analysis. Further optimizations of run-time, mobile phase gradient and flowrate were performed on the selected column. Based on exact masses and MS/MS matching, total thirty chemical compounds were identified including nineteen compounds through positive ionization mode and thirteen through negative ionization mode. For identification of compounds, every *m*/*z* values were checked for mass error (less than 5 ppm) and mSigma values (less than 50 units) which shows the matching of observed isotopic pattern with a calculated isotopic pattern of that molecular formula. The list of identified compounds through both positive and negative ionization modes are given in Table 2. Fig. 3 shows the Base Peak Chromatograms of both species through negative and positive modes of ionization. These chromatograms have peaks labeled with the codes of identified compounds which were found in each of these peaks. Except three compounds, all the compounds were identified in the retention time between 2 min and 4 min.

The reported biological activities of identified compounds were also tabulated such as cardioprotective, prevent endothelial dysfunction, reduce overexpression, inhibition of α-glucosidase enzyme, antidiabetic, antidepressant, anti-inflammatory, antimicrobial, antiviral, antifungal and protozoal, antineoplastic, antiplatelet and anticancer activity (Table 2).

3.4. Chemometric analysis of identified compounds

To visualize the distribution of identified compounds between both species, chemometric analysis were performed. Perseus software (version 1.6.2.1) was utilized to generate heatmap clusters. Heatmap from compounds identified using positive ionization mode revealed that compound **6** was found with high intensities in both plants and could be responsible for erythroid induction activity of these plants. Compounds **1** and **5** were found in higher intensity in AF sample while lower intensity in AP. Similarly, com-

Table 2
List of identified compounds in positive and negative ionization mode.

S#	Name	Formula	RT (min)	Observed Precursor mass (*m*/*z*)	Ion type	Calculated Precursor mass (*m*/*z*)	Error (ppm)	Fragments	Biological activities
1	Rhoifolin[a]	$C_{27}H_{30}O_{14}$	3.0	579.1702 577.1574	[M + H] $[M-H]^-$	579.1708 577.1563	1.1 1.9	271.060 269.044	Antioxidant, anti-inflammatory, anti-microbial, hepatoprotective and anticancer agent, inhibitor of α-glucosidase and α-amylase (Refaat et al., 2015).
2	Apiin[b]	$C_{26}H_{28}O_{14}$	2.8	565.1545	[M + H]	565.1552	1.2	325.071, 379.081, 4099.091, 511.124	Antiviral agent, anti-inflammatory, α-exo sialidase inhibitor (Liu et al., 2008a)
3	Apigeniin-6-*C*-glucoside-7-*O*-glucoside[c]	$C_{27}H_{30}O_{15}$	2.4	595.1673	[M + H]	595.1657	2.7	337.069, 397.091, 283.058, 313.070, 415.101, 577.153	Antifungal and anti-cancer (Smiljkovic et al., 2017)
4	3-*O*-Neohesperidoside kaempferol[a]	$C_{27}H_{30}O_{15}$	2.6	595.1650	[M + H]	595.1657	1.3	397.092, 415.103, 445.113, 475.124, 499.124, 541.135, 559.1045, 577.153	Anticancer (Wannes et al., 2017)
5	Luteolinoside[a]	$C_{21}H_{20}O_{11}$	2.8	449.1076	[M + H]	449.1078	0.5	313.071, 397.092, 415.103, 475.124, 529.135, 559.145, 541.135, 577.165, 595.165	Antioxidant
6	Vitexin[a]	$C_{21}H_{20}O_{10}$	2.9	433.1126	[M + H]	433.1120	1.4	283.059, 313.070, 337.070, 397.091, 379.081, 367.0809, 284.063	Antiplatelet, an α-glucosidase inhibitor, an antineoplastic (Can et al., 2013; Afifi and Abu-Dahab, 2012)
				431.0978	$[M-H]^-$	431.0971	1.6	311.055, 312.059, 341.065, 323.050, 342.069, 353.065, 413.082	
7	Sclareol[b]	$C_{20}H_{36}O_2$	6.3	273.2576	[M + H-$2H_2O$]	273.2565	4.0	163.148, 191.179, 149.132, 203.179, 177.162	Antimicrobial and anti-fungal (Noori et al., 2013)
8	Lawsonaringin[c]	$C_{20}H_{19}O_5Na$	6.2	363.1111	[M + Na]	363.1110	0.3	204.979, 217.054, 235.063, 261.043, 279.053	Anticancer, immunocytochemistry, apoptosis (Anwar et al., 2018)
9	Sissotrin[b]	$C_{22}H_{22}O_{10}$	3.6	445.1134	[M-H]	445.114	1.3	325.068	Estrogenic (Shajib et al., 2012)
10	Kaempferol-7-*O*-glucoside[a]	$C_{21}H_{20}O_{11}$	3.1	447.0936	[M-H]	447.0926	2.2	285.039	Antioxidant (Elbarbry and Shoker, 2007)
11	3,6,2′,4′-Tetrahydroxyflavone[b]	$C_{15}H_{10}O_6$	3.4	285.0405	[M-H]	285.0400	1.8	255.030	Not found
12	Saponarin[b]	$C_{27}H_{30}O_{15}$	2.6	593.1535	[M-H]	593.1512	3.9	473.108, 474.110, 503.120, 594.154	Antioxidant (Simeonova et al., 2014)
13	3′-Methoyxy-4′,5,7-trihydroxy flavonol[b]	$C_{16}H_{12}O_7$	3.7	315.0515	[M-H]	315.0510	1.6	300.027	prevents endothelial dysfunction, and reduce overexpression induced by angiotensin II (Sheu et al., 2004; Kanadaswami et al., 2005).
14	Peonidin-3-*O*-*β*-glucoside[b]	$C_{22}H_{22}O_{11}$	3.0	461.1086	[M-H]	461.1086	0.0	298.050, 341.050, 371.084	Antioxidant, and α-glucosidase inhibitor (Nile and Antioxidant, 2014)
15	Secoisolariciresinol[c]	$C_{20}H_{26}O_6$	3.7	361.1656	[M-H]	361.1656	0.0	346.104	Antidepressant, anti-cancer, phytoestrogen (Wang et al., 2013)

Table 2 (*continued*)

S#	Name	Formula	RT (min)	Observed Precursor mass (*m*/*z*)	Ion type	Calculated Precursor mass (*m*/*z*)	Error (ppm)	Fragments	Biological activities
16	Orientin[a]	$C_{21}H_{20}O_{11}$	2.8	447.0925	[M-H]	447.0927	0.4	227.050, 328.055, 357.062, 358.064	Antioxidant, antiaging, antiviral, antibacterial, antiinflammation, vasodilatation and cardioprotective, radioprotective, neuroprotective, antidepressant, antinociceptive (Lam et al., 2016).
17	Astragalin[b]	$C_{21}H_{20}O_{11}$	3.1	447.0932	[M-H]	447.0932	0.0	384.032, 285.039	anti-inflammatory, antioxidant, neuroprotective, cardioprotective, antiobesity, antiosteoporotic, anticancer, antiulcer, and antidiabetic (Bahadır Acikara et al., 2016).
18	Biochanin A[b]	$C_{25}H_{23}O_{13}$	3.1	532.1211	$[M-H]^+$	532.1201	2.0	297.032, 327.123, 351.123, 381.009, 411.031, 429.123	Tyrosine kinase inhibitor, phytoestrogen, antineoplastic and anticancer (Bhardwaj et al., 2014; Ying et al., 2001; Holbech et al., 2013)
19	N-Fructosyl pyroglutamate[b]	$C_{11}H_{17}NO_8$	0.7	290.0872	[M-H]	290.0876	1.4	201.059, 212.056, 170.045, 200.056	Not found
20	4,5-Dihydroxy-6-(hydroxymethyl)-3-[3,4,5-trihydroxy-6-(hyroxymethyl)oxan-2-yl] oxyoxan-2-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4one[a]	$C_{27}H_{30}O_{15}$	2.9	593.1527	[M-H]	593.1512	2.5	293.046, 413.087	Not found
21	Swertisin[b]	$C_{22}H_{22}O_{10}$	3.3	447.1286	$[M-H]^+$	447.128	1.3	285.112, 297.021, 309.198, 327.123, 337.022, 351.109, 375.081, 381.100, 393.198, 411.099	Anticancer, anti-inflammatory antioxidant, radioprotective, and hypoglycaemic (34)
22	7-Amino-4-methylcoumarin[b]	$C_{10}H_9NO_2$	2.7	176.0706	[M + H]	176.0706	0.0	173.060	Antifungal and antibacterial (Liu et al., 2008b)
23	Elmycin B[b]	$C_{19}H_{18}O_6$	0.6	365.1003	[M + Na]	365.0995	2.2	366.108, 347.094	Angucyclinone antibiotic (Fotso et al., 2008)
24	2-Cyclohexen-1-one, 4-[(1*E*)-3-(*β*-D-glucopyranosyl-oxy)-1-buten-1-yl]-4-hydroxy-3,5,5-trimethyl-, (4*S*)[c]	$C_{19}H_{30}O_8$	2.8	409.1824	[M + H]	409.1830	1.5	203.052, 410.187, 411.198	Not found
25	*β*-D-Xylopyranoside, 1,3,4,6,7,7,7,8,9,10,11,13,13,15,15-pentadecahydro-13-hydroxy-11-(1hydroxy-1-methylethyl)-1,1,7,8,13-pentamethyl-10,12-epoxy-2H,5H -cyclopropa[1′,8′a] naphth[2′,1′:4,5]indeno[2,1] oxepin-2-yl][c]	$C_{35}H_{54}O_9$	3.2	641.3660	[M + Na]	641.3599	9.5	491.297	Not found
26	Mycophenolic acid[c]	$C_{17}H_{20}O_6$	6.5	321.1369	[M + H]	321.1333	11	303.122	Immunosuppressant, antibiotic, antineoplastic and antimicrobial (Elbarbry and Shoker, 2007; Dzidic et al., 2006)
27	(3*Z*)-3-Hexen-1-yl 6-*O*-alpha-L-arabinopyranosyl-*β*-D-glucopyranoside[c]	$C_{17}H_{30}O_{10}$	3.0	417.1734	[M + H]	417.1731	0.7	418.176	Not found
28	2-Acetoxy-4-pentadecyl-benzoic acid[c]	$C_{24}H_{38}O_4$	6.2	413.2519	[M + Na]	413.2565	11	310.140	Not found
29	2-[2-Hydroxy-5-[(1*E*)3-methoxyphenyl]-3-(4-hydroxy-3-methoxyphenyl) propylhexopyranoside[c]	$C_{26}H_{34}O_{11}$	2.8	545.1993	[M + H]	545.1993	0.0	383.146, 546.293, 547.203	Not found
30	(3*S*,3*R*,4*S*,4*R*,7*R*,8*R*,9*R*)-3,4,8-Trimethyl-2,5-dioxo-2,3,3,4,4,5,7,8,9,9-decahydroazuleno [6,5–b] furan-4-yl (2*Z*)-2-methyl-2-butenoate[c]	$C_{20}H_{26}O_5$	3.7	369.1671	[M + Na]	369.1672	0.3	287.124, 370.171	Not found

a-common in both species, b-only in AF, c-only in AP.

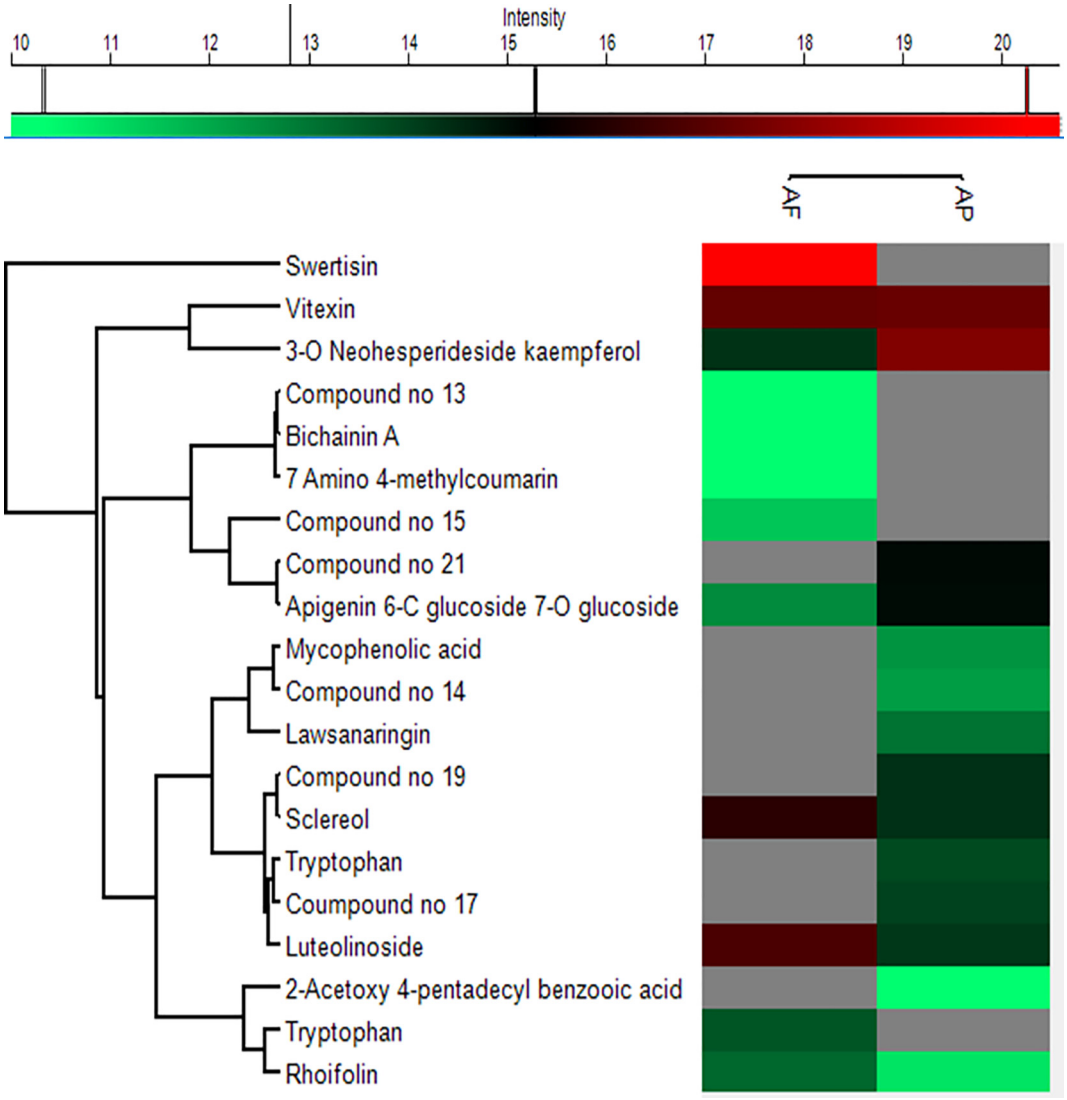

Fig. 4. Heatmap and hierarchical clustering based on peak areas of identified compounds in *A. pubiflora* and *A. fragrans* through positive ion mode.

pound **4** exhibited lower intensity in AF than AP. Compounds **9**, **12** and **13** were found with good intensities in AF while completely absent in AP. Compounds **8**, **14**, **16** and **19** were absent in AF sample but found in AP with low intensity. Compound **10** showed the highest intensity compared to all the compounds but present in AF only. Heatmap from the positive mode of ionization is given in Fig. 4.

Through negative ionization mode, compounds **2**, **6**, **9**, **10**, **12** and **14** were present in both species. Compound **1** was found with higher intensity in AF, while compound **9** was at a lower intensity in AP. Compound **14** exhibited higher intensity in AP and lower intensity in AF. Compound **6** was present with higher intensity in AP while lower in AF. Compound **12** exhibited a very high intensity in AP as compared to AF sample. Compounds **1**, **3**, **4**, **5**, **7**, **11**, **13** were not found in AP while compounds **1** and **3** were present with higher intensities in AF. Heatmap from the negative mode of ionization is illustrated in Fig. 5.

Comparative studies of both plant species showed that in positive ion mode out of 19 identified compounds, 16 compounds were confined to one species while 5 compounds are similar in both species. Similarly, in negative ion mode, total thirteen compounds were identified among these 8 compounds were different and 6 compounds were similar in both species (Table 2). The previous phytochemical studies have reported cycloartane glycosides from *Aquilegia fragrans* while no phytochemical studies were performed on *Aquilegia pubiflora.* However, these compounds were not seen in the present study. The compounds identified here are mainly belonged to flavonoids class of natural products while some steroids, lignans, terpenoids, benzofuran and coumarins were also found. None of these identified compounds were previously reported to have erythroid induction activity.

4. Conclusion

Methanolic extract of *Aquilegia fragrans* and *Aquilegia pubiflora* have shown erythroid induction activity at lower concentrations, suggesting that the plants can be the potential source of fetal hemoglobin inducing compounds to treat β-thalassemia and other β-hemoglobinopathies. Moreover, a rapid and simple method was also developed for the chemical profiling and identification of compounds in selected *Aquilegia* species, using high-resolution LC-ESI-QTOF tandem mass spectrometry. Total nineteen compounds were identified through positive ion mode and thirteen through negative ion mode of mass spectrometer. Additionally, chemometric analysis was also performed to understand the distribution of identified compounds in both species. Identified compounds may be respon-

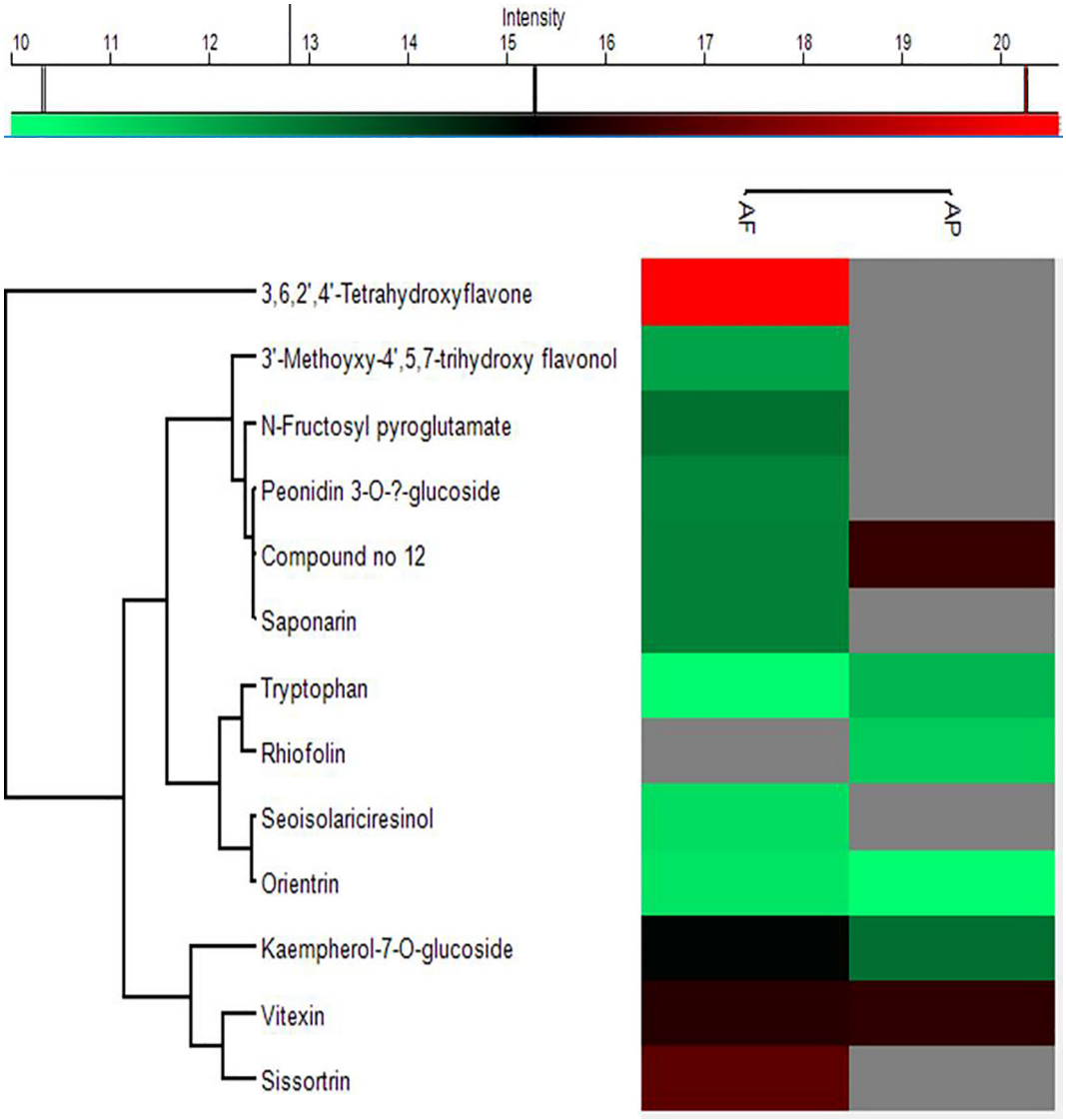

Fig. 5. Heatmap and hierarchical clustering based on peak areas of identified compounds in *A. pubiflora* and *A. fragrans* through negative ion mode.

sible for the aforementioned activity but require screening in purified form for further confirmation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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