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ORIGINAL ARTICLE

Characterization of phenolics, amino acids, fatty acids and antioxidant activity in pulp and seeds of high altitude Himalayan crab apple fruits (*Malus baccata*)

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Abstract Phytochemicals in fruits and vegetables have achieved immense significance owing to the increasing evidence which signifying their activity for antioxidant and prevention of chronic diseases. The amount of phloretin (88.39 μg mg^{-1}) and phloridzin (83.03 μg mg^{-1}) were found to be higher among other phenolics determined using UPLC. DPPH, $ABTS^{+}$, metal chelating and ·OH radical assays were used to evaluate antioxidant activity. *Malus baccata* pulp portion showed higher antioxidant activity than seed portion. HPLC analysis for free amino acids showed that serine (9.06 μg mg^{-1}), alanine (8.03 μg mg^{-1}), tyrosine (10.33 μg mg^{-1}), and cysteine (76.86 μg mg^{-1}) were only detected in pulp portion while seed comprised of histidine (3.96 μg mg^{-1}) only. Seed portion was also determined for their fatty acid composition including palmitic acid (0.89%), ethyl palmitate (0.56%), methyl petroselinate (0.90%) and linolein (3.93%) using GC–MS analysis. HPAEC technique detected fructose and sucrose in a fair amount of 21 and 17.3 mg g^{-1} in pulp, while 9.4 and 4.24 mg g^{-1} in seed portion, respectively. The present study suggested that *M. baccata* fruit is a rich source of phenolic and other chemical components which can be used in food products and nutraceutical formulations.

Keywords *Malus baccata* · Phytochemicals · Antioxidants · Fatty acids · Free amino acids · Sugars · UPLC · HPLC · GC–MS

Introduction

Fruits are the major source of minerals, vitamins, and fiber, which provide essential nutrients for human health. Increased consumption of fruits reduces the chances of various chronic diseases such as cancer and cardiovascular diseases. Fruits also contain polyphenolic compounds that exhibited many biotic activities like anti-cancer, antimicrobial, anti-inflammatory and anti-oxidative (Basak and Guha 2017). Many important fruits are already investigated for the phenolic compounds because of their key role in preventing oxidation process in human. Phenolic compounds are also high in demand in the food industries and cosmetics due to its health-promoting benefits of these compounds but there is little information about such wild edible fruits varieties that are locally available and underutilized. Most of these health benefits compounds are associated with their higher content in vitamin C, phenolics (especially flavonoids), antioxidants and dietary fibers (Das et al. 2012).

One of the fruits of *Malus* species found in the Upper Himalayas of Himachal Pradesh, India is usually recognized as 'wild apples' or 'crab apples' and the term is derived due to their small and astringent fruits. The crab apples, botanically classified as *Malus baccata* (Lin.)

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belong to the genus *Malus* (Rosaceae) is another species of apple. The genus *Malus* is consist of about 30–35 species of deciduous shrubs or trees. It is a small sized, cold-field edible fruit and has traditional medicinal values. They are widely distributed throughout the world and in the parks of Europe and South America that are basically used for the ornamental purposes. Wild crab apple fruits are the rich source of phenolic compounds including anthocyanins (Sharma and Nath 2016). Crabapples are generally used in the preparation of jellies, jams, and beverages. Fruits of *M. baccata* L. were also described to contain long chain alcohols like *a*-sitosterol, campesterol and ursolic acid and their D-glucosides (Mulabagal et al. 2007). Crabapples are supposed to possess similar composition like nutrients and phenolic compounds, as reported in apples (Li et al. 2014). The phenolic compounds with a huge range of structure and functions generally possess an aromatic ring containing one or more hydroxyl groups. The valuable effects derived from phenolic compounds could be the main reason of antioxidant potentials of foods and also a natural source of antioxidants. Numerous extraction techniques have been used for the extraction of phenolics from plant materials, such as incubator shaker technique, supercritical fluid extraction, ultrasonic assisted extraction, soxhlet extraction and pressurized liquid extraction (Ince et al. 2014; Rahaiee et al. 2015). On the other hand, ultrasonic extraction technique has been extensively used for the extraction of phenolic compounds because of its low cost and simple instrumental use (Altemimi et al. 2017). The present study also deals with ultrasonic extraction to extract the crab apple pulp and seeds. Apart from phenolics, amino acids are also essential in the biosynthesis of proteins and precursors in the formation of many secondary metabolites that involved in the gene expression, cell signaling, and homeostasis and antioxidant activity (Wang et al. 2017). The composition of sugars and amino acids differ in pulp and seeds are expected to enhance the food formulation with food ingredients and juices. Free amino acids also assist plant to adopt extreme stress conditions like low and high temperature, salinity, ultraviolet radiations and drought resistant. There are many techniques that facilitate the determination of sugars in different food matrices (Pereira da Costa and Conte-Junior 2015). HPAEC-PAD is prescribed as the best analytical technique to determine carbohydrates.

The main objective of this study was to identify and quantify the phenolic compounds, free amino acids, fatty acids in seeds and pulp of underutilized wild crab apple from the upper Himalayas and to discover how these phytochemicals contribute to their antioxidant activities.

Plant material

Fruits of wild crab apples (*M. baccata*) were collected from the high altitude Himalayan region at Keylong (3080 m), Lahaul-Spiti, Himachal Pradesh, India. Freshly collected fruits of *M. baccata* were plugged, and washed with distilled water and dried with blotting paper, followed by freezing through liquid nitrogen and kept under − 80 °C for further use. Frozen samples were thawed at room temperature before extraction. Both the seeds and pulp were separated manually through hands using gloves followed by drying at 50 °C in hot air oven (Macro Scientific Work, Pvt, Ltd. India). Samples were further crushed with a grinder (Philips, India) into the fine powder.

Chemicals and standards

Caffeic acid, chlorogenic acid, ferulic acid, epicatechin, catechin, phloridzin, phloretin, cinnamic acid, gallic acid, *p*-coumaric acid, protocatechuic acid, rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonate) (ABTS), potassium persulphate, ferrous sulphate, 1, 10-phenanthroline, potassium ferricyanide, trichloroacetic acid were purchased from Sigma-Aldrich, India. Acetonitrile, ethanol, hydrochloric acid, and methanol were of HPLC grade procured from Merck India Pvt. Ltd. Ferric chloride, ferrous chloride, ferrozine, and hydrogen peroxide were purchased from Himedia.

Materials and methods

Ultrasonic extraction

The ultrasonic extraction technique was performed using an ultrasonic probe (Autotune series ultrasonic atomizer) with an ultrasonic power (50 W) and a frequency (20 kHz) controlled by a phase-locked loop system. The probe had a titanium horn having a diameter of 2.5 cm and throwing an ultrasonic wave directly from the horn. Fine powdered samples (20 g) of seeds and pulp were mixed with 60% ethanol (200 mL v v^{-1}) in a beaker in which the ultrasonic probe was immersed. Extraction was carried out at the temperature at 25 °C for 10 min. The suspensions were centrifuged for 10 min at 12,000*g* and the supernatant was collected followed by filtration using 0.45 µm syringe filter to remove solid material and other impurities (CE Minisart RC 15, Sartorius, Goettingen, Germany). The filtrate was collected and evaporated using a rotary evaporator (Rotavapor R-300, BUCHI). Finally, the samples were freeze-dried through lyophilizer (Labconco) for further analysis.

Determination of total phenolics and flavonoid content

Total phenolic content was determined using Folin–Ciocalteu method (Joshi et al. 2015). Ethanolic extracts of the pulp and the seeds were treated with Folin–Ciocalteu reagent (500 µL, 1 N) followed by saturated sodium bicarbonate (100 µL). The final volume was made up to 25 mL using distilled water. The reaction mixture was then incubated for half an hour at room temperature. Absorbance was recorded at 730 nm against blank using UV–Vis spectrophotometer (Eppendorf). The distilled water used as a blank. Gallic acid was used as the standard and expressed in mg gallic acid equivalents (GAE) per g extract.

In total flavonoid content, ethanolic extract of the pulp and the seeds (1 mg mL^{-1}), sodium nitrite (5%) followed by aluminum chloride (10%) were mixed. Before adding 1 M sodium hydroxide the reaction mixture was incubated for 5 min. the final volume was made up to 10 mL with distilled water. Absorbance was recorded at 510 nm against blank. Quercetin was used as a positive control and expressed in terms of standard equivalent (µg mg^{-1} of the extracted compound).

Determination of antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity of the extract was determined using the method of Joshi et al. (2015) with some modifications. A stock solution of DPPH (0.1 mM) was prepared in 60% ethanol and kept in dark for overnight at room temperature. To prepare working solution, the stock solution of DPPH was diluted with 60% ethanol to an absorbance of 0.9 ± 0.05 at 517 nm. The pulp extract and the seed extracts (100 µL each) with different concentration (20–200 µg mL^{-1}) were mixed separately with 1.9 mL of working DPPH solution and kept in dark for 30 min at room temperature. Absorbance was recorded at 517 nm using Kinetic Bio Spectrometer (Eppendorf).

ABTS radical scavenging activity

ABTS radical scavenging activity of the extract was determined using the method of Joshi et al. (2015). A stock solution of ABTS radicals was prepared using 5 mL of 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonate) (ABTS) solution (7 mM) mixed with 88 µL of potassium persulphate solution (140 mM). The mixture was placed for overnight in dark at room temperature to generate $ABTS^{\cdot+}$ cation (2.45 mM). To prepare working solution of $ABTS^{\cdot+}$, the stock solution of $ABTS^{\cdot+}$ was diluted with absolute ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. 20 µL of the pulp extract and the seed extract was allowed to react with 980 µL of $ABTS^{\cdot+}$ working solution and incubated in dark for 10 min at room temperature. Absorbance was recorded spectrophotometrically at 734 nm.

Ferrous ion chelating ability

The ferrous ion (Fe^{2+}) chelating ability of extract was determined using the method of Agrawal et al. (2016). The pulp extract and the seed extracts (1 mL) were mixed with ferrous chloride (2 mmol L^{-1}) (0.05 mL) and ferrozine (5 mmol L^{-1}) (0.2 mL). The mixture was vortexed and kept standing for 10 min. The absorbance of the mixture was recorded at 562 nm. Distilled water was used as blank.

Hydroxyl radical (·OH) scavenging activity

Hydroxyl radical-scavenging activity of the extract was determined using the method of Agrawal et al. (2016). The pulp extract and the seed extract (25 µL) were mixed with ferrous sulphate (3 mM) (25 µL) and 1, 10-phenanthroline (3 mM, dissolved in 0.1 M PB (pH 7.4) (25 µL). To initiate the reaction 0.01% (v v^{-1}) hydrogen peroxide (25 µL) was added to the mixture and incubated for 1 h at 37 °C. The absorbance of the mixture was recorded at 536 nm.

Identification of phenolics using ultra performance liquid chromatography (UPLC)

UPLC analysis of phenolics was performed on a Waters Acquity UPLC-H class system consisting of a binary solvent manager, an autosampler, a column heater and eλ photodiode array detector (PDA). The injection volume was 1 µL and wavelength of eλ PDA detector was set at 280 nm. Phenolics were separated on Water AccQ Tag C18 column (21.1 × 50 mm, 1.7 µm particle size) and the column heater was set at 35 °C. Gradient program was 0–40 min 2–20% B, 40–50 min 20% B, 50–55 min 20–80% B, 55–60 min 80% B with solvent A as 0.1% TFA in water, and acetonitrile as solvent B at a flow rate of 1.0 mL min^{-1}. Before injection, all the samples were passed through a 0.45-µm Millipore membrane filter. The chromatographic data for each sample were recorded and quantification of separated compounds was based on different standards curves.

Fatty acid profile

Five gram of the pulp and the seeds were refluxed at 60 °C in hexane for 16 h using Soxhlet apparatus. After extraction, the solvent was removed under reduced pressure by rotary evaporator at 40 °C. Then the dried samples were re-

dissolved in 1 ml GC grade hexane and injected into GC column.

GC–MS analysis

The concentrated hexane extract was analyzed on a Shimadzu QP2010 GC–MS system with 2010 GC. A GC column BP-20 SGE (30 m × 0.25 mm id, film thickness 0.25 µm) was used with helium as a carrier gas. The split ratio was 1:50 and the injector temperature was 220 °C. GC oven temperature was programmed to hold at 70 °C for 4 min and then to increase to 220 at 4 °C min^{-1}, finally holding at 220 °C for 5 min. Column flow rate was 1.10 mL min^{-1}. Ion source temperature was 200 °C and interface temperature was set at 220 °C. MS was scanned at 70 eV over 40–600 a.m.u. Sample injection volume was 2 µL. Identification of compounds was performed using mass spectral libraries Wiley 7 and NIST 02 (Mc Lafferty 2000).

Determination of free amino acid profile using high-performance liquid chromatography (HPLC)

Detection and quantification of amino acids were done on Waters 996 HPLC system equipped with photo diode array (PDA) detector, Waters 717 plus autosampler, Waters 600 controller, Waters TM pump, Waters inline degasser AF. The column used was Lichrospher RP-18 column (250 mm × 4.6 mm, 5 µm, Merck, Germany) fitted with suitable guard column. Mobile phase A consisted of 0.14 M sodium acetate with 500 µL triethylamine, pH was adjusted to 6.7 with glacial acetic acid and methanol (90:10). Mobile phase B comprised of acetonitrile: water (60:40) vv^{-1}. The binary gradient varied from 0 to 75% B in 15 min followed by 75–100% in 15 min. The flow rate was 1 mL min^{-1}. Amino acids were monitored at 355 nm.

High-performance anion exchange chromatography for sugar analysis (HPAEC)

The lyophilized powdered extracts (1 mg mL^{-1}) mixed with HPLC grade milli-Q water was carried out for sugar determination. HPAEC connected via pulsed amperometric detection (PAD) was used to analyze the sugars in the sample. The analysis was performed on a Metrohm IC system. Separation of all standard mixture was achieved with the help of Hamilton RCX-30 column (4.6 × 250 mm length, 7 µm particle size). An isocratic system was employed using sodium hydroxide (100 mM) solution in HPLC grade milli-Q water as a mobile phase including injection volume of 300 µL with a flow rate of 1 mL min^{-1}. The pressure was maintained at 10.6 MPa. The analysis settings for sugars are provided by the company on its website (http://www.metrohm.com/). Different standards of sugars including arabinose, sucrose, sorbitol, fructose, and glucose were made to run for the study.

Statistical analysis

Data are expressed as the mean ± standard deviation from replicates (n = 3) in each experiment. Statistical analysis was done in MS Excel (Microsoft Windows 2007) using Student's *t* test. All statements of significant difference were based on the probability of 95% confidence level ($p < 0.05$).

Results and discussion

Pulp and seeds of crab apple contained various kinds of phenolics including *p*-coumaric acid, gallic acid, cinnamic acid, caffeic acid, protocatechuic acid, rutin, phloretin, and phloridzin which contributed to antioxidants, possibly so far well detected and quantified. A suitable extraction procedure was used to recover maximum phenolics as much as possible. From previous reports, ultrasonication assisted extraction yields higher polyphenols as compared to other extraction techniques. Hence, in present study, crab apple's polyphenols were extracted using ultrasonication technique and antioxidant potential was determined spectrophotometrically.

Total phenolics and flavonoids content

Phenolic compounds are predominantly responsible for the antioxidant activity of various plant materials. There was no significant difference found in total phenolic content of the pulp extract (114.00 ± 0.70 mg GAE g^{-1}) and the seed extract (112.23 ± 1.1 mg GAE g^{-1}) of *M. baccata* ($p > 0.05$). On the other hand, total flavonoid was found to be slightly higher in pulp extract (171.66 ± 0.5 mg RE g^{-1}) than in seed extract (154.16 ± 0.8 mg RE g^{-1}) at significant level ($p < 0.05$). This result clearly indicated the pulp extract contained higher antioxidants compounds than seed extract. The amount of total phenolic content was found to be higher than flavonoid content in comparison to the data earlier reported by Chen et al. (2014) on *Malus domestica* fruit species during maturation on the tree. Previous reports showed total phenolics content of 1.78 ± 0.02 and 1.17 ± 0.03 in seeds and pulp, respectively in crab apple fruits (Sharma and Nath 2016). While, TPC and TFC content of the pulp and seed extract of the *M. **baccata*** falls in the range of previously studied ten crab apples (Li et al. 2014). In an another study of Limoncella' apple also showed closer results of total phenolic, total

flavonoids content and antioxidant activity (D'Abrosca et al. 2007). Total values of certain phytochemical groups, such as the TPC and TFC measured by spectrophotometric methods in the present study, are for close estimation and comparison purpose. However, accurate analysis of individual phenolics compounds in this study was done using UPLC analysis. An alternative and complementary approach to the TPC is the total phenolic index (TPI) has already been discussed by (Li et al. 2015).

Antioxidant activities

The radical scavenging capacities of the pulp extract and the seed extract against different free radical were investigated. When an electron is accepted by the free radical species it loses this adsorption, results in visual discoloration from purple to yellow. Figure 1 showed the DPPH radical scavenging activity of the pulp extract and the seed extract with standard ascorbic acid. DPPH assay is an excellent method to estimate the antioxidant activity of potential antioxidative compounds. The higher number of –OH groups and those –OH group present in ortho position in the aromatic ring usually quenches more DPPH molecules on the molar basis. The antioxidant activity can also be measured due to the hydrophilic and lipophilic composition in sample indicated in ABTS assay. But previous reports also showed that ABTS is more accurate, rapid and robust for estimation of antioxidant potential (Rawat et al. 2011). There was significant difference found in the scavenging activity of both extracts and standard ascorbic acid. With increasing concentration in the range of 20–200 μg mL^{-1}, the DPPH activity of the pulp extract and the seed extract was found in the range of 28.92–77.08 and 8.45–30.47%, respectively, as compared to ascorbic acid 15.68–95.21% with the concentration range of 2–14 μg mL^{-1} (Fig. 1). Thus the extract of pulp at 200 μg mL^{-1} concentration can be used as a natural antioxidant instead of artificial antioxidants. IC_{50} values of DPPH radical scavenging activity of the pulp extract and the seed extract were estimated to be 0.09 and 0.51 mg mL^{-1}, respectively. Figure 1 represented the percent inhibition of ABTS radical cation by the pulp extract and the seed extract and standard ascorbic acid. Percent inhibition of the pulp extract (12–89.28%) was found significantly greater than the seed extract (8.28–72.42%) at the concentration range of 20–25 μg mL^{-1} ($p < 0.05$). ABTS radical scavenging activity based on IC_{50} was found to be 0.19 mg mL^{-1} for pulp and 0.23 mg mL^{-1} for seed extract. The results for the IC_{50} value of pulp showed consistency with previous studies revealing the higher antiradical scavenging activity of these compounds (Lebot et al. 2016). In the present study, the antioxidant activity of *Malus* species was much higher than the reported value of apple peel, indicating *Malus* fruits are the rich source of phenolics with high antioxidant activities for preparing extracts. Smaller fruits have higher phenolics content and exhibited stronger antioxidant activity among other fruits (Serce et al. 2010). This could be the reason for higher content of Himalayan crab apple compared to apple. When total phenolic content and antioxidant capacity of crab apple fruits compared, it has one of the highest values among the other fruits; among common small fruits. In addition, crab apple fruits are perhaps most comparable to blackberries, cranberry, and blueberries, which are known to have the highest value of antioxidant and total phenolic content among all fruits.

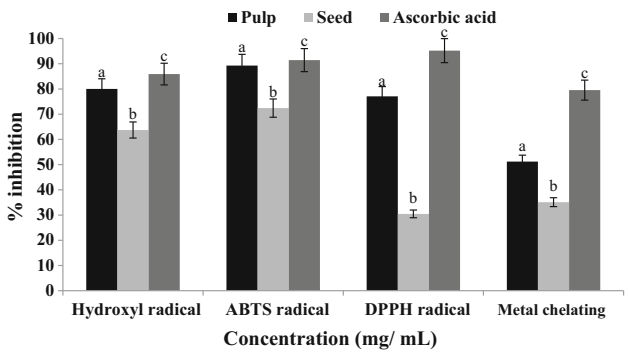

Fig. 1 Free radical scavenging activity of pulp and seed extract from *M. baccata*

The hydroxyl radical is a highly reactive free radical and capable of damaging almost every molecule found in living cells. The pulp extract and the seed extract were used to compare the preventing capacity against hydroxyl radicals. The results showed in Fig. 1 revealed that the pulp extract (79.47%) possessed significantly higher hydroxyl radical scavenging activity than seed extract (63.46%) ($p < 0.05$) at a concentration of 1 mg mL^{-1}. Thus, both phenolic and non-phenolic constituents of *M. baccata* modulate its antioxidant activity. Transition metals such as iron and copper ions are the strong agent to generate free radicals, such as hydroxyl radical and superoxide radicals which can catalyze the generation of reactive oxygen species. Fe^{2+} metal quantitatively forms the complexes with ferrozine. Complex formation is disrupted resulting in a decrease in the red color of the complex in the presence of chelating agents. Color reduction measurement makes possible the metal chelating activity estimation. The result depicted in Fig. 1 showed the pulp extract and the seed extract interacted with iron at the different level. At the concentration of 1 mg mL^{-1} the Fe^{2+} chelating ability of pulp extract (51.20%) was found to be significantly higher than seed extract (35.11%) ($p < 0.05$). Previous reports explained the phenolic compounds exhibited redox properties (i.e. act as reducing agents, hydrogen donators and singlet oxygen quenchers) and are also responsible for iron chelating

activity (Loizzo et al. 2012). These findings demonstrate that phenolic might be the key contributor to antioxidant activities of crab apple the pulp extract and the seed extract.

Determination of phenolics using UPLC

Phenolic compounds were quantified through peak assessment in seed and pulp extracts of *M. baccata* using modified UPLC analytical method. Flavonoid and phenolic acids were the most widespread phenolic compounds separated and detected by HPLC analysis (Donado-Pestana et al. 2015). Results were prepared by comparing the retention time and area under the curve of different phenolics compounds including *p*-coumaric acid, gallic acid, cinnamic acid, caffeic acid, procatechuic acid, rutin, phloretin and phloridzin. Species-specific for apples consists of 16 polyphenols compounds are evaluated including phloridzin, quercetin and its glycosides, procyanidins, catechin, phloretin, epicatechin, and several cyanidin glycosides and cinnamic, chlorogenic and caffeic acids (Andre et al. 2012). Phenolic were detected and quantified by plotting calibration curve for eight standards at different concentrations are shown in Table 1. Two phenolic compounds including phloretin and phloridzin were detected in the higher amount reported in other species of *Malus* including *Malus domestica* (Alberti et al. 2017). Phloretin was quantified as 88.39 and 37.90 μg mg^{-1} in seed and pulp, respectively. Similarly, phloridzin was quantified as 83.09 and 49.15 μg mg^{-1} in seed and pulp, respectively (Fig. 2). Higher content of phloridzin seed also detected in *Malus domestica* (Rana and Bhushan 2016) which clearly supported the crab apple is equally healthier food items. Our results were close to these reported data. Other phenolic compounds in pulp were detected as *p*-coumaric acid (4.34 μg mg^{-1}), gallic acid (16.90 μg mg^{-1}), caffeic acid (3.34 μg mg^{-1}) and rutin (3.37 μg mg^{-1}). Similarly, in seed extract, protocatechuic acid (2.15 μg mg^{-1}) and gallic acid (5.79 μg mg^{-1}) were detected. The results were also highlighted the higher content of phloretin and phloridzin in seed as compared to the pulp. However, a small amount of other phenolics including *p*-coumaric acid, gallic acid, rutin and caffeic acid in pulp were showed its own independent importance. The antioxidative importance of seeds and pulp extracts of crab apple as a substitute of other commercially utilized *Malus* fruits. These results are particularly significant for developing value-added products from the seeds and pulp of *Malus* fruits.

Quantification of fatty acids through GC–MS

Seeds were referred to as the prime source of fatty acids. However, major saturated fatty acid like palmitic acid (0.89%) and esters such as ethyl palmitate (0.56%), methyl petroselinate (0.90%) and linolein (3.93%) were identified and quantified using GC–MS in the seed extract of *M. baccata* (Fig. S1). Palmitic acid was also found in borage seed oil, boysenberry seed oil, blueberry seed oil, seabuckthorn seed, basil seed oil (Abedi and Sahari 2014). Fatty acid compositions of different parts (peel, pulp, and seeds) of ten native Amazonian fruits were characterized chemically and showed higher content in seed part of fruit (Berto et al. 2015). Soxhlet method is a better method for extraction of fatty acids and adopted in the present study. These fatty acids are merely carboxylic acids with long hydrocarbon chains, an important source of energy for many organisms and precursors for other secondary metabolites. The human body is capable of producing all the fatty acids except for linoleic acid and alpha-linolenic acids. These fatty acids have to be consumed from the diet

Table 1 Phenolic composition and amino acid composition in pulp and seed extract of crab apple using UPLC and HPLC, respectively

Extracts	*p*-Coumaric acid	Gallic acid	Cinnamic acid	Caffeic acid	Protocatechuic acid	Rutin	Phloretin	Phloridzin
Phenolic compounds (μg mg^{-1})								
Pulp extract	4.34 ± 0.05[#]	16.90 ± 0.02	1.82 ± 0.16	3.34 ± 0.02	1.02 ± 0.03	3.07 ± 0.01	37.90 ± 0.05	49.15 ± 0.06
Seed extract	nd	5.79 ± 0.03	nd	1.25 ± 0.01	2.15 ± 0.02	nd	88.39 ± 0.08	83.09 ± 0.03

Extracts	Serine	Histidine	Alanine	Glycine	Tyrosine	Cysteine
Amino acid profile (μg mg^{-1})						
Pulp extract	9.06 ± 0.11[#]	1.25 ± 0.35	8.03 ± 0.05	5.60 ± 0.52	10.33 ± 1.08	76.86 ± 0.32
Seed extract	nd	3.96 ± 0.15	nd	3.16 ± 0.28	4.35 ± 0.21	15.43 ± 0.33

Values are expressed as mean of three replicates ± standard deviation

nd not detected

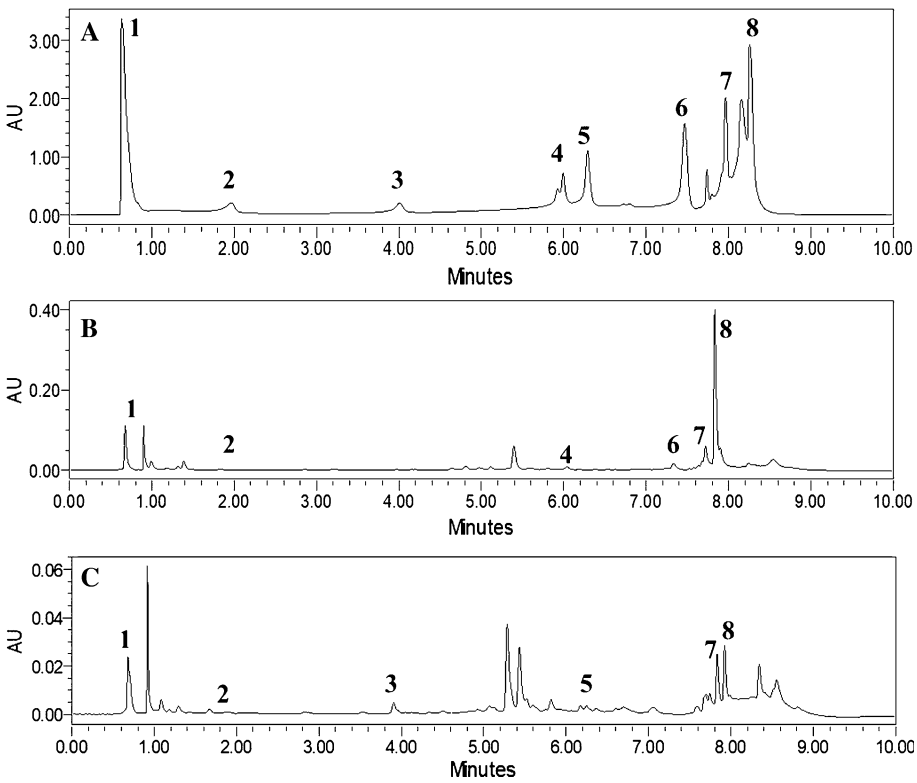

Fig. 2 UPLC profile of phenolic compound in *M. baccata*, **a** standard mixture; 1. Gallic acid, 2. Pro-catechuic acid, 3. Cinnamic acid, 4. Caffeic acid, 5. *p*-Coumaric acid, 6. Rutin, 7. Phloretin, 8. Phloridzin, **b** seed extract, **c** pulp extract

from different sources such as fruits, vegetables etc. Therefore, these locally growing Himalayan crab apples are a good source of fatty acids at low cost providing adequate nutritive values.

Free amino acid content in the pulp extract and the seed extract of crab apples

Free amino acids in plant exert an essential role in the human diet. They signify a source of nitrogen and nutritionally essential amino acids such as lysine (Lys), methionine (Met), and threonine (Thr). Recently, the pre-column derivatization on HPLC analysis has been widely used for amino acids detection due to the time-saving and sensitive method. To our knowledge, there are no published reports regarding the amino acid analysis of the pulp extract and the seed extract of *M. baccata* Fig. 3. Showed the chromatographic representation of the amino acid content of pulp (C) and seed (B) extract with their standard amino acid (A) expressed as mg 100 g^{-1}. Both qualitative and quantitative differences were found between the pulp extract and the seed extract. Serine (9.06 ± 0.11 μg mg^{-1}), alanine (8.03 ± 0.05 μg mg^{-1}), tyrosine (10.33 ± 2.08 μg mg^{-1}), and cysteine (76.86 ± 0.32 μg mg^{-1}) were only detected in pulp extract (Table 1). The seed contains only histidine (3.96 ± 0.15 μg mg^{-1}) which is an essential amino acid for the human body. Results are not consistent with previously reports which represent asparagine, aspartic acid, glutamic acid, and phosphoserine were main free amino acids present samples from Annurca apples (Maro et al. 2011). Recent reports suggested that the free amino acids also contributed significantly to antioxidant activity in Korean fermented red pepper paste (Lee et al. 2016). Food products rich in histidine (His) is beneficial for reduction of body weight loss in fat-rich diets fed mice through increasing energy expenses. Previous findings suggest that qualitative composition of amino acids in all parts were the same, but the quantitative contents in seeds, pulp differ from each other (Abudayeh et al. 2016).

Sugar content in the pulp extract and the seed extract of crab apples

Structure and functionality of the plant are mainly affected by sugar content as sugar does not only provide energy source but also the structure matter in plant growth. Figure 4 represented the chromatographic profile of sugar composition present in seed and pulp of *M. baccata* analyzed by HPAEC-PAD. The results represent the variation in sugar composition in both parts of the fruit. Pulp contained a higher amount of disaccharides while seed contains the higher number of disaccharides including glucose than other detected sugars. All the peaks were eluted between 3.52 and 11.9 min of retention time and detected as inositol, arabinose, glucose, fructose, and sucrose by

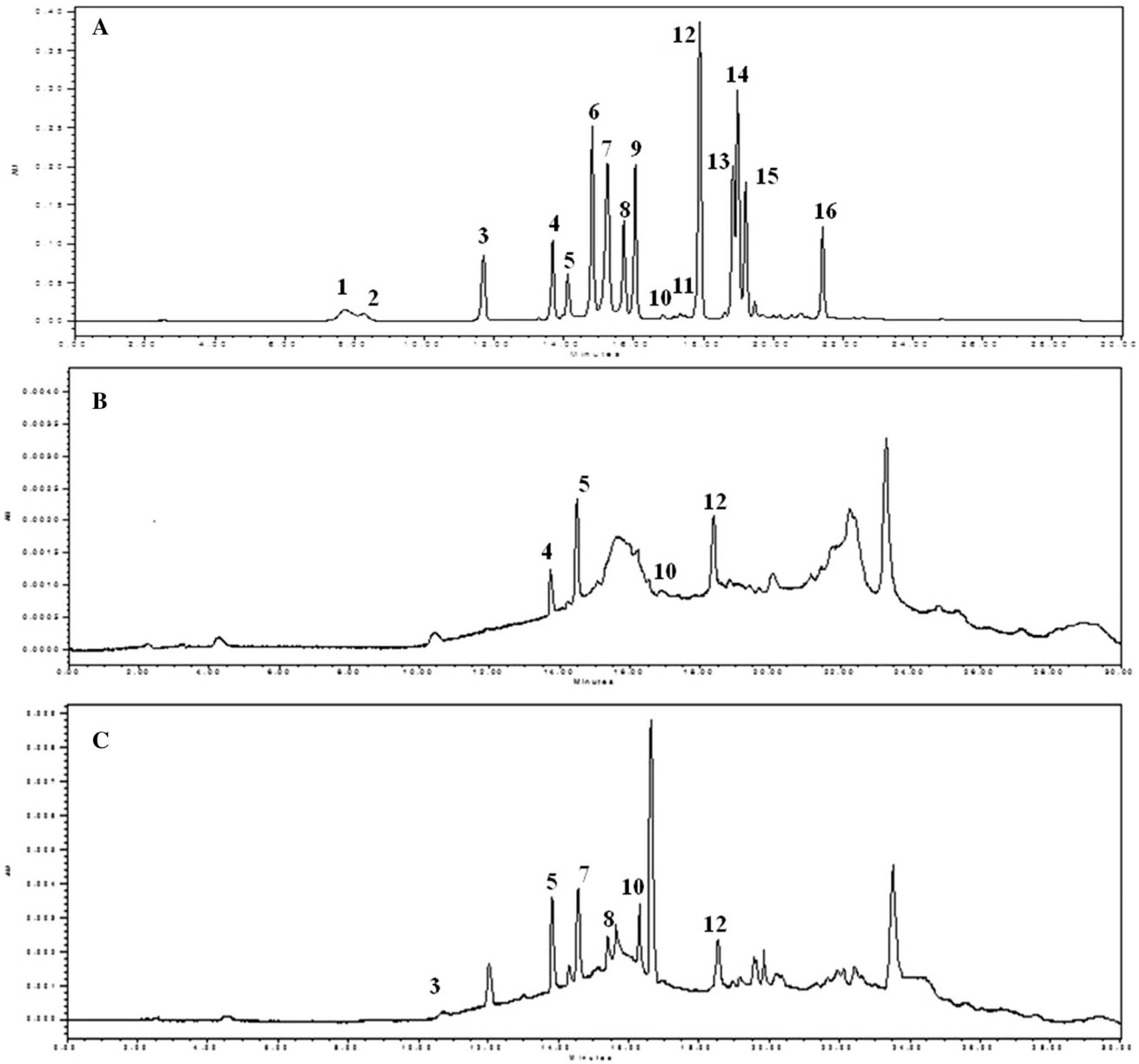

Fig. 3 HPLC profile of amino acids content in a Standard mixture; 1. Asp, 2. Glu, 3. Ser, 4. Gly, 5- His, 6. Arg, 7. Thr, 8. Ala, 9. Pro, 10. Tyr, 11. Met, 12. Cys, 13. Ile, 14. Leu, 15. Phe, 16. Lys, **b** seed extract, **c** pulp extract

comparing with reference standards. The predominant sugar was found fructose and sucrose in a fair amount of 21 and 17.3 mg g^{-1} respectively in the pulp part, while 9.4 and 4.24 mg g^{-1}, respectively in seed part. Similar results were reported by Liu et al. (2006) in intact apple fruits. Previous reports showed the dominating carbohydrates including sucrose, D-glucose, and D-fructose and the polyol, D-sorbitol in apple and apple juice (Zielinski et al. 2014). Findings of the current study on *M. baccata* from Himalayan region are generally in agreement with the finding of another study on different apple cultivars. However, some compounds were recorded to be higher, while some were lower. The differences are possible due to the *M. baccata* from high altitude region utilized in this study which affects its functional properties.

Conclusion

Among various characteristics of functional foods, underutilized *Malus* species could be consumed as new food ingredient containing nutritional outcomes, such as free amino acids as the signal molecule or neurotransmitter, vitamins, and minerals as the immune booster. While

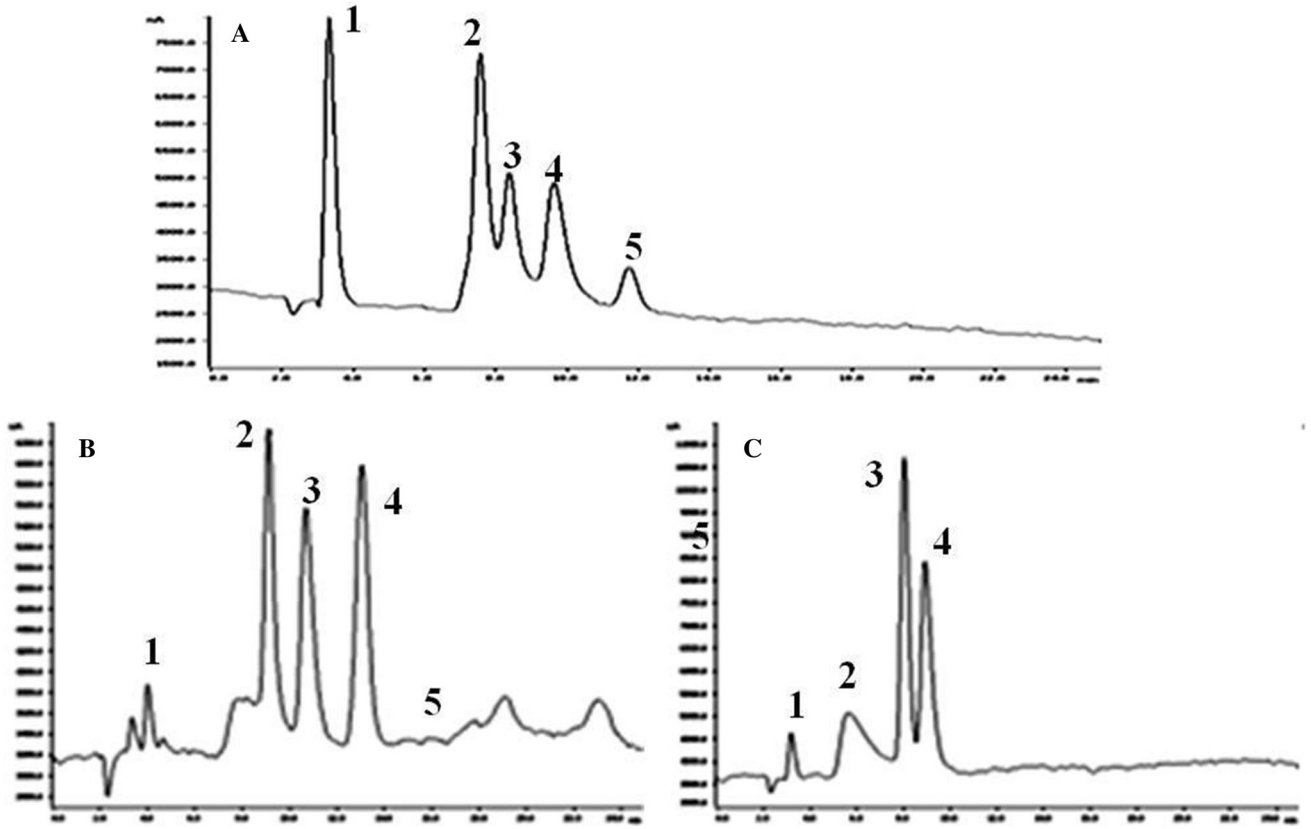

Fig. 4 High performance anion exchange chromatography (HPAEC) profile of sugars in **a** standard mixture; **b** pulp extract, **c** seed extract

flavonoids were resulted in protection against various reactive oxygen species causing chronic and degenerative diseases. Analytical results showed a wide range of bioactive molecules in seed and pulp extracts of underutilized Himalayan crab apple (*M. baccata*) responsible for health promotion. Extracts not only showed the fair amount of flavonoids but also fatty acid molecules like palmitic acid, ethyl palmitate, methyl petroselinate and linolein which were well known for its medicinal outcomes. Numerous essential amino acids were observed like serine, alanine, tyrosine and cysteine enhanced its nutritive value. While, sugar molecules were also analyzed in the extracts including inositol, arabinose, glucose, fructose, and sucrose. Overall, *M. baccata* is a good source of antioxidants, essential fatty and amino acids and sugars. Detailed phytochemical profiling showed that it is a good source for the production of health-promoting functional food and nutraceuticals products.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Abedi E, Sahari MA (2014) Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. Food Sci Nutr 2:443–463

Abudayeh ZHM, Lamazian HR, Sereda P, Chekman I, Khalifa IIA, Azzam KMA, Hassouneh LKM (2016) Comparative study of amino acid composition in the seeds, pulp and rind from *Citrullus colocynthis* fruits. Int J Pharmacogn Phytochem Res 8:433–437

Agrawal H, Joshi R, Gupta M (2016) Isolation, purification and characterization of antioxidative peptide of pearl millet (*Pennisetum glaucum*) protein hydrolysate. Food Chem 204:365–372

Alberti A, Zielinski AAF, Couto M, Judacewski P, Mafra LI, Nogueira A (2017) Distribution of phenolic compounds and antioxidant capacity in apples tissues during ripening. J Food Sci Technol 54(6):1511–1518

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA (2017) Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6(4):42

Andre CM, Jeffrey M, Greenwood EG, Walker MR, Sullivan M, Evers D, Perry NB, Laing WA (2012) Anti-inflammatory procyanidins and triterpenes in 109 apple varieties. J Agri Food Chem 60:10546–10554

Basak S, Guha P (2017) Use of predictive model to describe sporicidal and cell viability efficacy of betel leaf (*Piper betle* L.)

essential oil on *Aspergillus flavus* and *Penicillium expansum* and its antifungal activity in raw apple juice. LWT Food Sci Technol 80:510–516

Berto A, da Silva AF, Visentainer JV, Matsushita M, Fernandes E, de Souz NE (2015) Proximate compositions, mineral contents and fatty acid compositions of native Amazonian fruits. Food Res Int 77:441–449

Chen F, Li F, Lu L, Zhang X, Xu X, Li D (2014) Phenolic profile and changes in the antioxidant activity of crab apple (*Malus domestica* cv Royalty) fruit during maturation on the tree. Int J Food Sci Technol 49(7):1680–1688

D'Abrosca B, Pacifico S, Cefarelli G, Mastellone C, Fiorentino A (2007) 'Limoncella' apple, an Italian apple cultivar: Phenolic and flavonoid contents and antioxidant activity. Food Chem 104(4):1333–1337

Das L, Bhaumik E, Raychaudhuri U, Chakraborty R (2012) Role of nutraceuticals in human health. J Food Sci Technol 49(2):173–183

Donado-Pestana CM, Belchior T, Festuccia T, Genovese MI (2015) Phenolic compounds from cambuci (*Campomanesia phaea* O. Berg) fruit attenuate glucose intolerance and adipose tissue inflammation induced by a high-fat, high-sucrose diet. Food Res Int 69:170–178

Ince AE, Sahin S, Sumnu G (2014) Comparison of microwave and ultrasound-assisted extraction techniques for leaching of phenolic compounds from nettle. J Food Sci Technol 51:2776–2782

Joshi R, Rana A, Gulati A (2015) Studies on quality of orthodox teas made from anthocyanin-rich tea clones growing in Kangra valley, India. Food Chem 176:357–366

Lebot V, Michalet S, Legendre L (2016) Identification and quantification of phenolic compounds responsible for the antioxidant activity of sweet potatoes with different flesh colours using high performance thin layer chromatography (HPTLC). J Food Compos Anal 49:94–101

Lee DE, Shin GR, Lee S, Jang ES, Shin HW, Moon BS, Lee CH (2016) Metabolomics reveal that amino acids are the main contributors to antioxidant activity in wheat and rice *gochujangs* (Korean fermented red pepper paste). Food Res Int 87:10–17

Li N, Shi J, Wang K (2014) Profile and antioxidant activity of phenolic extracts from crab apples (*Malus* Wild Species). J Agric Food Chem 62:574–581

Li H, Deng Z, Liu R, Zhu H, Draves J, Marcone M, Tsao R (2015) Characterization of phenolics, betacyanins and antioxidant activities of the seed, leaf, sprout, flower and stalk extracts of three *Amaranthus* species. J Food Compos Anal 37:75–81

Liu Y, Ying Y, Yu H, Fu X (2006) Comparison of the HPLC method and FT-NIR analysis for quantification of glucose, fructose, and sucrose in intact apple fruits. J Agric Food Chem 54:2810–2815

Loizzo MR, Tundis R, Bonesi M, Menichini F, Mastellone V, Avallone L, Menichini F (2012) Radical scavenging, antioxidant and metal chelating activities of *Annona cherimola* Mill. (cherimoya) peel and pulp in relation to their total phenolic and total flavonoid contents. J Food Compos Anal 25(2):179–184

Maro AD, Dosi R, Ferrara L, Rocco M, Sepe J, Ferrari G, Parente A (2011) Free amino acid profile of Malus domestica Borkh cv. Annurca from the Campania region and other Italian vegetables. J. Am Chem Soc 5:154–161

Mc Lafferty FW (2000) Wiley registry of mass spectral data, 7th edn. Wiley, New York

Mulabagal V, Nocker SV, Dewitt DL, Nair GM (2007) Cultivars of apple fruits that are not marketed with potential for anthocyanin production. J Agric Food Chem 55:8165–8169

Pereira da Costa M, Conte-Junior CA (2015) Chromatographic methods for the determination of carbohydrates and organic acids in foods of animal origin. Compr Rev Food Sci Food Saf 14(5):586–600

Rahaiee S, Moini S, Hashemi M, Shojaosadati SA (2015) Evaluation of antioxidant activities of bioactive compounds and various extracts obtained from saffron (*Crocus sativus* L.): a review. J Food Sci Technol 52:1881–1888

Rana S, Bhushan S (2016) Apple phenolics as nutraceuticals: assessment, analysis and application. J Food Sci Technol 53(4):1727–1738

Rawat S, Bhatt ID, Rawal RS (2011) Total phenolic compounds and antioxidant potential of *Hedychium spicatum* Buch. Ham. ex D. Don in west Himalaya, India. J Food Compos Anal 24(4):574–579

Serce S, Ozgen M, Torun AA, Ercisli S (2010) Chemical composition, antioxidant activities and total phenolic content of *Arbutus andrachne* L. (Fam. Ericaceae) (the Greek strawberry tree) fruits from Turkey. J Food Compos Anal 23(6):619–623

Sharma R, Nath AK (2016) Antioxidant levels and activities of reactive oxygen-scavenging enzymes in crab apple fruits (*Malus baccata*). Proc Natl Acad Sci India Sect B: Biol Sci 86(4):877–885

Wang R, Xu S, Wang N, Xia B, Jiang Y, Wang R (2017) Transcriptome analysis of secondary metabolism pathway, transcription factors, and transporters in response to methyl jasmonate in *Lycoris aurea*. Front Plant Sci 7:1971

Zielinski AAF, Braga CM, Demiate IM, Beltrame FL, Nogueira A, Wosiacki G (2014) Development and optimization of a HPLC-RI method for the determination of major sugars in apple juice and evaluation of the effect of the ripening stage. Food Sci Technol 34(1):38–43