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Comparative Analysis of Bioactive Phenolic Compounds and Fatty Acids in Seeds and Seedlings of Canadian Alfalfa, Sainfoin, and Fenugreek

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Abstract: The interest in under-utilized crops as a functional food for animals and humans has been increasing recently with advancing research and the need for crop improvement. Canadian forage crops including alfalfa (*Medicago sativa L.*) and fenugreek (*Trigonella foenum-graecum* L.) are marketed in various forms due to their traditionally known health benefits. Sainfoin (*Onobrychis viciifolia* Scop.) is another forage crop with potential health benefits containing beneficial nutraceuticals. In this study, we assessed selected bioactive phenolic compounds and fatty acids in seeds and seedlings of Canadian-grown alfalfa, sainfoin, and fenugreek. Various phenolic compounds were detected in all three forage crop seeds and seedlings. In general, Sainfoin seeds were high in phenolic compounds relative to that of alfalfa and fenugreek. Chlorogenic acid, epigallo catechin, and gallic acid were at high concentrations at 56.6, 86.8, and 64.7 $\mu g.g^{-1}$, respectively, compared to other phenolic compounds in sainfoin seeds. The fatty acids content (%) was significantly affected by the seedling stage and crop type. Some of the bioactive compounds present in seeds were not detected in seedling stages. The comparative bioactive phenolic compounds and fatty acid assessments of these forage legumes could potentially be used as biomarkers for the selection and development of favorable cultivars for animal and human nutrition. In addition, these crops could be used for isolating these bioactive compounds, and thus increasing their agri-food value.

Keywords: phenolic compounds; fatty acids; forage legumes; sainfoin; alfalfa; fenugreek; apigenin; genistein; FAMEs; high-resolution mass spectrometry

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1. Introduction

The search for crops with high-nutritional ingredients has been a challenge, and the demand for functional foods in Canada and worldwide has been increasing over the years. In the last decade, the consumer market for functional foods in Canada was projected to double by 2025 [1]. There is a growing emphasis on exploring the potential of under-utilized crop varieties for food, especially those with potential bioactive phytonutrients [2]. Forage legumes are important for the livestock industry due to their high-nutrient quality and they also help to improve soil health when used in crop rotation with other food crops through improving soil nitrogen content [3,4]. Some of the forage legumes grown in Canada include alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus*), cicer milkvetch (*Astragalus cicer* L.), red clover (*Trifolium pratense* L.), fenugreek (*Trigonella foenum-graecum* L.) and sainfoin (*Onobrychis viciifolia* Scop.) (https://www.beefresearch.ca/topics/forage-species/ (accessed on 26 May 2023)). Out of these, sainfoin, fenugreek and alfalfa are important forage legumes grown in Western Canada

with high-economic potential. Alfalfa is a perennial legume considered as the queen of forages in the world due to its contribution to high-animal productivity [5,6], while sainfoin is popular as a drought resistant perennial legume used in Western Canada. Fenugreek is an annual legume not only used as a spice and a medicinal herb in many parts of the world, but also recently cultivated and introduced in Western Canada as a forage crop. Indeed, only one variety (Tristar) of fenugreek was developed in Western Canada, and thus more breeding studies may be needed [7–9]. These three forage legumes are highly nutritious and known to have various health benefits to livestock [10–12]. However, one of the important disadvantage of alfalfa as forage includes the induction of lethal bloat in ruminants [13]. Sainfoin and fenugreek both have bloat-free effects in ruminants, which may be due to the high content of condensed tannins and other bioactive nutrients [13–16]. Recent studies suggest that there is a need for future research on these under-utilized forage legume crops as a source of beneficial and health-promoting phytonutrients [17,18].

Alfalfa and fenugreek are found in different forms such as powdered herbs, capsules, tablets, and seeds in the market. Sainfoin, containing beneficial nutraceuticals, may also potentially contribute to food security and healthy living [18,19]. Recent studies suggested the potential use of alfalfa, sainfoin and fenugreek seeds and seedlings as a novel food source for humans and animals [12,17–19]. For example, both in alfalfa and sainfoin, germinated seeds and seedlings were low in calories but high in nutrients such as vitamins, minerals, carotene, chlorophyll, amino acids, antioxidants, and proteins, contributing to nutritional benefits [17,18]. Fenugreek seeds are a good source of nutrients and bioactive molecules [12,20]. For example, 4-hydroxyisoleucine from fenugreek seeds showed anti-diabetic properties [20,21]. Consumption of fenugreek seeds showed evidence of reduced systolic blood pressure in humans, attributed to the presence of proanthocyanidins [22,23]. In addition to seeds, fenugreek sprouts or seedlings are consumed as leafy green vegetables [7]. Previous studies investigated the seeds of five fenugreek genotypes, such as L3068, L3375, Tristar, PI143504 and Amber, which were grown in western Canada for their potential use as nutraceuticals [5].

Bioactive phenolic compounds have broader health benefits such as anti-inflammatory, anti-diabetic, anti-cancer, and antiatherogenic effects [8]. Antioxidant caffeic acid and sinapic acid are some other phenolic acids present in plants [24]. Phenolic acids, such as coumaric acid, ferulic acid, chlorogenic acid and gallic acid, as well as condensed tannins, flavanols, isoflavones, were previously reported in sainfoin [12]. A recent study showed the presence of eleven phenolic bioactive compounds in alfalfa, including phenolic acids and flavonoids [25]. The chemical composition analysis of fenugreek seedlings by Khole et al. [26] showed that vitexin is one of the abundant bioactive compounds that contributed to the antioxidant activity of the fenugreek extract. The beneficial indications of apigenin in human health, including its effects on different types of inflammatory and cancer associated diseases, were previously reported [27]. It was also suggested that cattle feed with a high lipid content can potentially help to reduce greenhouse gas emissions and thereby supplementing feed with oil may decrease methane production by cattle and ruminants [28–30]. However, few studies are available on profiling forage (seed and vegetative tissue) fatty acids [12,19]. It was also found that food sources rich in bioactive compounds, including phenolic compounds and polyunsaturated fatty acids, have demonstrated health benefits such as reducing hypertension [31].

Phytochemicals, such as phenolic acids, flavonoids, and fatty acids of Canadian-grown cultivars of alfalfa, sainfoin, and fenugreek, may contribute individually or synergistically to health benefits via preparations derived from these crops. This is true not only for the seeds, but also for the sprouts and seedlings, which could greatly improve the agri-food value of these crops. In this study, we investigate the phenolic compounds and fatty acid composition of different growth stages of Canadian forage legumes (alfalfa, sainfoin, and fenugreek) to use as potential functional food and nutraceutical for both animals and humans.

2. Materials and Methods

2.1. Seeds and Seedlings Growth

Viable seeds of one cultivar for each of the three forage crops were selected for seedling germination including alfalfa (*M. sativa*; *AC Bridgeview*), sainfoin (*O. viciifolia*; *Melrose*), and fenugreek (*T. foenum-graecum*; *Amber*). Seeds were supplied by Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada. Selected seeds' germination was carried out in replicates: in Petri-dishes with a wet paper towel until the embryonic root (radicle) emerged. The seedlings were then transplanted into individual pots containing potting mix (soil, Peatmoss, and Perlite) at the Canadian Centre for Agri-Food Research in Health and Medicine (Winnipeg, MB, Canada). Plants were grown under lab conditions with a light photoperiod (16 h) and dark (8 h) and with a light intensity of approximately 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. A sub-sample of seeds of these cultivars was also set aside for phenolic and fatty acid composition analyses. The germinating seedlings were sampled on Days 7, 14, and 30 representing 7-day, 14-day, and 1-month old seedlings, respectively (Figure 1). Each seedling germination experiment was carried out in replicates (n = 3, ~20 seeds each). Samples were stored at −80 °C until further processing for sample analysis. Replicate samples were pooled and the composite sample was used for phenolic compound analysis, whereas replicate samples were processed for fatty acid analysis.

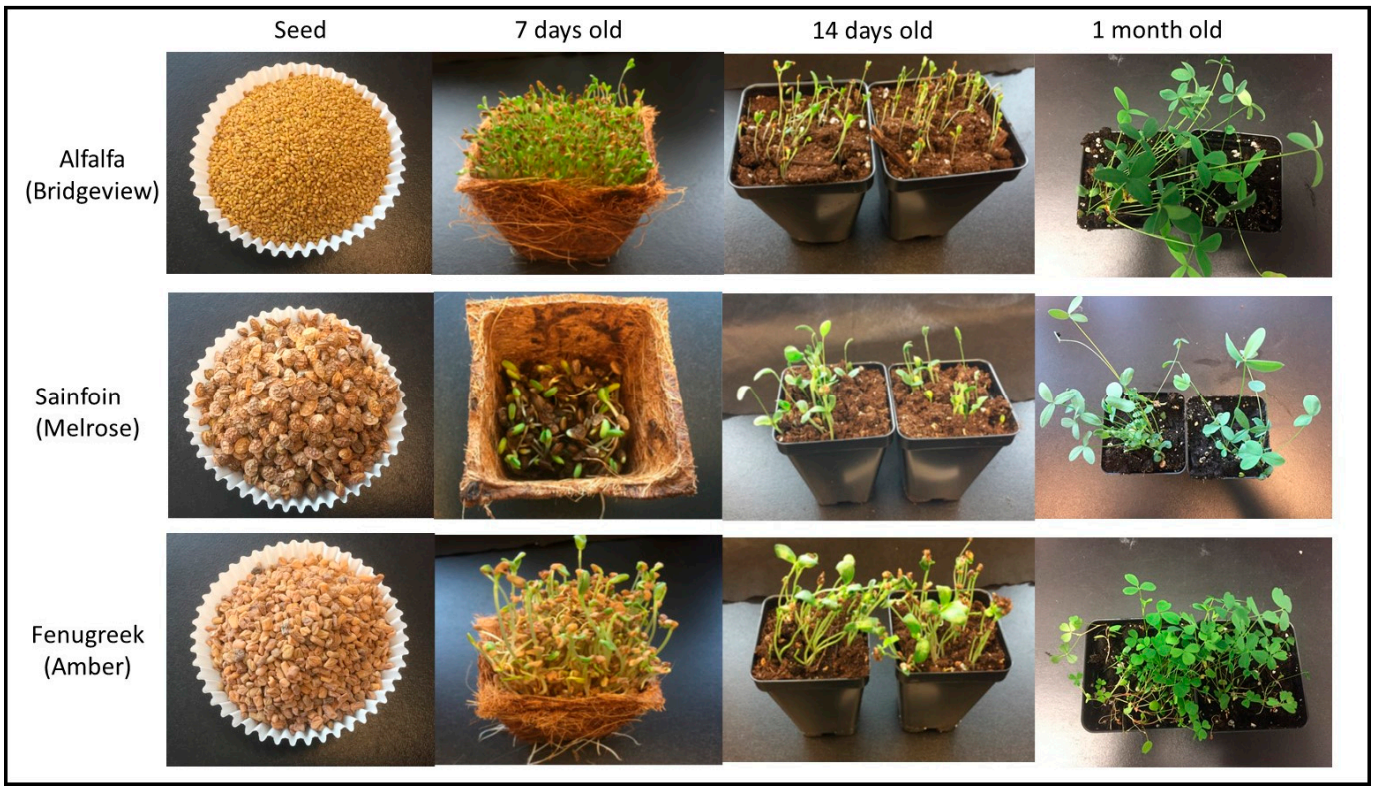

Figure 1. Seeds and different growth stages of seedlings of Alfalfa (Bridgeview variety), sainfoin (Melrose variety) and fenugreek (Amber variety) during germination at Days 7, 14, and 30 (1 month).

2.2. Sample Analysis for Phenolic Compounds and Fatty Acids

2.2.1. Extraction of Phenolic Acids and Flavonoids

All seeds and seedlings were extracted for 15 phenolic compounds including apigenin, caffeic acid, catechin, chlorogenic acid, epicatechin, epicatechin gallate, epigallocatechin, ferulic acid, gallic acid, genistein, iso-vitexin, resveratrol, rutin, sinapic acid, and vitexin (Table 1 and Figure 2). All samples (2.0 g each) were prepared for analysis by freeze-drying followed by defatting to remove oil content using petroleum ether and chloroform with sonication and maceration cycles. The macerated sample-solvent mixture was centrifuged,

the supernatant discarded, and the residue dried using a gentle stream of nitrogen gas. The dried residue material was quantitatively transferred into a 2-mL Eppendorf tube followed by the addition of 70% acetone (1.5 mL) and sonicated for 50 min in water-bath at 45 °C. The mixture was then centrifuged (3000 rpm) for 30 min and the supernatant was transferred quantitatively into a separate Eppendorf tube and dried under vacuum at 60 °C (Vacufuge Plus, Eppendorf AG, Hamburg, Germany). The dried extract was further extracted by acidic hydrolysis by addition of 10 µL of 12 M HCl, vortexing (30 s), followed by tubes placed in a dry bath (Boekel Scientific, PA, USA) at 70 °C for 105 min [32,33]. After hydrolysis of samples, ethyl acetate (1 mL) was added and thoroughly mixed by inverting and vortexing, followed by centrifugation (3000 rpm, 30 min) to separate the solvent. The supernatant layer of ethyl acetate was transferred into a clean Eppendorf tube using a Pasteur pipette and dried under vacuum at 60 °C for 30 min. The dried residue was re-suspended in methanol (1.2 mL) and filtered through a syringe filter (0.22 µm, Phenomenex, CA, USA) into LC vials for mass spectrometric analysis.

Table 1. Phenolic compounds detected and quantified along with method validation parameters.

Phenolic Compound	*m/z* of Precursor & Product Ions	Retention Time (min)	LOD [1] ($ng \cdot mL^{-1}$)	LOQ [2] ($ng \cdot mL^{-1}$)	Calibration Curve Range ($ng \cdot mL^{-1}$)	Linearity (R^2)
Apigenin [3]	269.0456 > 225.0553 269.0456 > 201.0551	10.2	1.4	4.2	0.5–250	0.9987
Caffeic acid	179.0347 > 135.0449	3.80	2.1	6.4	0.5–250	0.9981
Catechin	289.0708 > 245.0811 289.0708 > 205.0499 289.0708 > 179.0343	5.21	1.1	3.4	0.5–250	0.9977
Chlorogenic acid	353.0872 > 191.0559	4.59	1.7	5.2	0.5–250	0.9969
Epicatechin	289.0708 > 245.0811 289.0708 > 205.0499 289.0708 > 173.4917	5.44	1.0	2.9	0.5–250	0.9997
Epicatechin gallate	441.0817 > 289.0712 441.0817 > 169.0138	7.13	2.5	7.5	0.5–250	0.9997
Epigallocatechin	305.0662 > 221.0453 305.0662 > 179.0347	3.98	41.9	127	0.5–250	0.9996
Ferulic acid	193.0504 > 149.0603	6.80	49.6	150	0.5–250	0.9906
Gallic acid	169.014 > 125.0241	1.98	3.3	10	0.5–250	0.9973
Genistein [3]	269.0449 > 159.0446 269.0449 > 133.0291	10.1	2.9	8.8	0.5–250	0.9973
Iso-vitexin	431.0972 > 311.0552	6.55	1.0	3.0	0.5–250	0.9991
Resveratrol	227.0711 > 185.0605	6.61	9.7	29.4	0.5–250	0.9981
Rutin	609.1450 > 301.0349	6.26	2.1	6.3	0.5–250	0.9969
Sinapic acid	223.0609 > 179.0711 223.0609 > 164.0475	6.92	33.0	99.9	0.5–250	0.9938
Vitexin	431.0972 > 311.0552	8.09	1.0	3	0.5–250	0.9991

[1] LOD: Limit of detection; [2] LOQ: Limit of quantification; [3] Apigenin and Genistein: Chromatographic separation of these isomers was not achieved in the method.

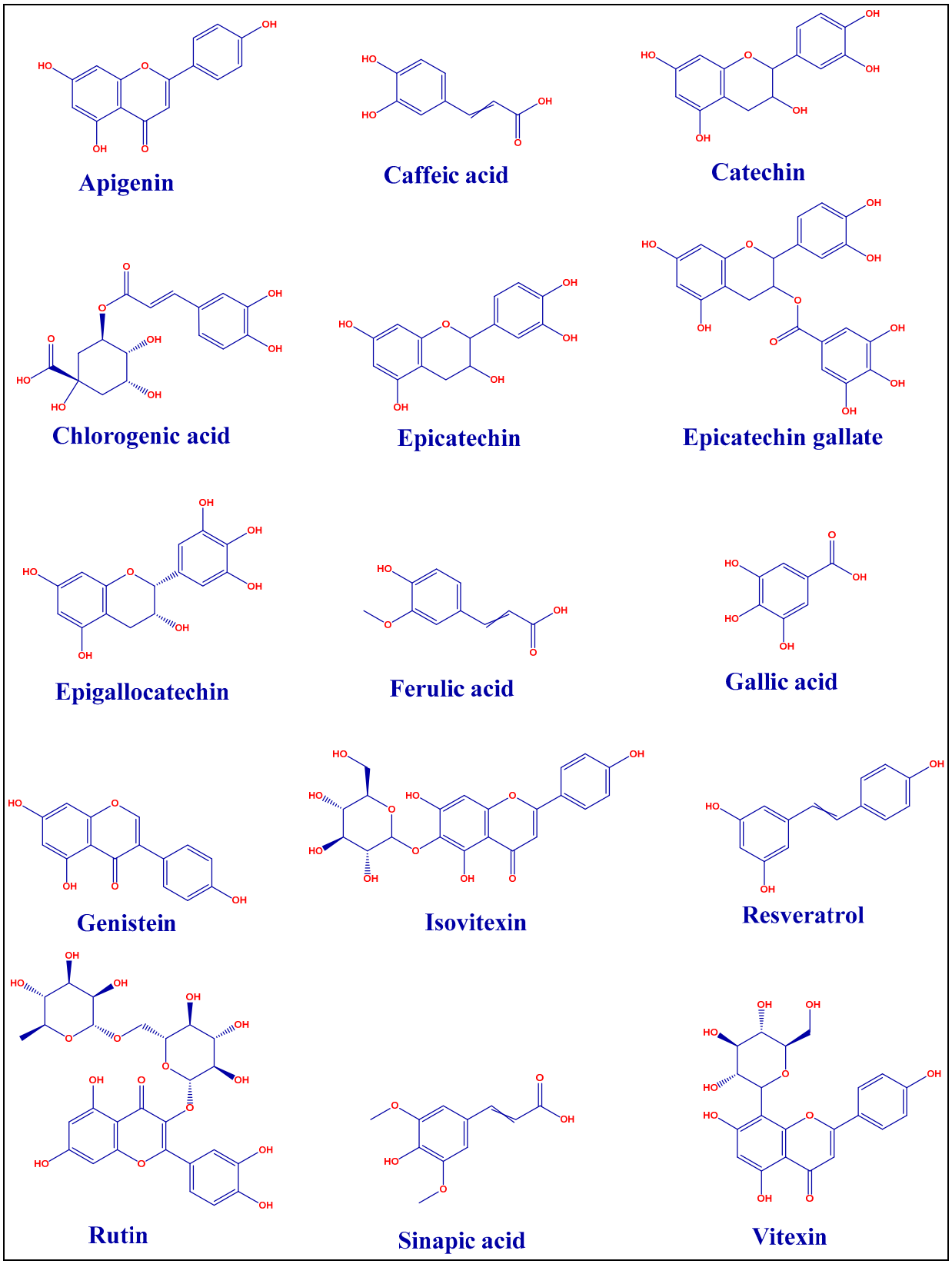

Figure 2. Chemical structures of phenolic compounds investigated in this study. Chemical structures were drawn using ChemDraw Professional software v.19., PerkinElmer Inc. (Waltham, MA, USA).

2.2.2. Liquid Chromatography–Mass Spectrometric Analysis of Extracts for Phenolic Compounds

All sample extracts in LC vials were analyzed using a high-performance liquid chromatograph (UHPLC; Vanquish, Thermo Fisher Scientific, Mississauga, ON, Canada) with a high-resolution mass spectrometer (HRMS; ID-X Tribrid Orbitrap, Thermo Fisher Scientific, Mississauga, ON, Canada). Separation and elution of various phenolic compound analytes were achieved using a reverse-phased biphenyl column (100 × 2.1 mm, particle size 2.6 µm, Kinetex, Phenomenex, Torrance, CA, USA) with a column temperature held at 35 °C using a pre-column heater and column heater compartment. Gradient elution with mobile phase A (100% water containing 0.1% formic acid) and mobile phase B (100% acetonitrile containing 0.1% formic acid) at a flow rate of 0.25 mL·min^{-1} was used to separate analytes on a total run cycle time of 20 min. Subsequently separated analytes in the mobile phase were subsequently passed through heated electrospray ionization (HESI–II, Thermo Fisher Scientific, Mississauga, ON, Canada) to achieve a steady state of electrospray to HRMS for detection and quantification. Data acquisition was carried out in negative mode and pure analytical standard stock solutions in acetonitrile or methanol were infused to optimize HRMS acquisition parameters, including collision energy and product-ion identification. An HRMS acquisition method consisted of a full scan for mass-to-charge ratio (m/z) values of 150 to 800 at 120,000 resolution with a mass tolerance of ± 5 ppm, encompassing all analytes investigated in this study followed by an intensity and data-dependent precursor ion fragmentation to the acquired product ions (MS2 spectra, Figure 3) at an orbitrap resolution of 30,000. Fragmentation of the precursor ions was through the collision-induced dissociation (CID) cell, which allows for softer fragmentation with stepped collision energies of 20, 30, and 40%. Method validation parameters, including precursor and product ion m/z, column retention times, method detection and quantification limits, and calibration curve range and linearity, are listed in Table 1. Chromatographic separation of two isomers, apigenin and genistein, was not achieved in this method with the same accurate mass and m/z values, quantified together (Table 2). Quantification of analytes in the sample extracts was achieved using 8-level (0.5 to 250 ng·mL^{-1}) calibration curves. Calibration curves for each compound were prepared with a mixture containing pure analytical standards at pre-determined concentrations. Analysis sequence included sample extracts sandwiched between calibration standards. Solvent blanks were also included at frequent intervals to assess and account for any analyte carryover. Peak area integration, quantification using an accurate mass of the precursor ion, and analyte conformation using fragment ions for each analyte (Figure 3) were performed using TraceFinder software *v4.1*, Thermo Fisher Scientific, Mississauga, ON, Canada).

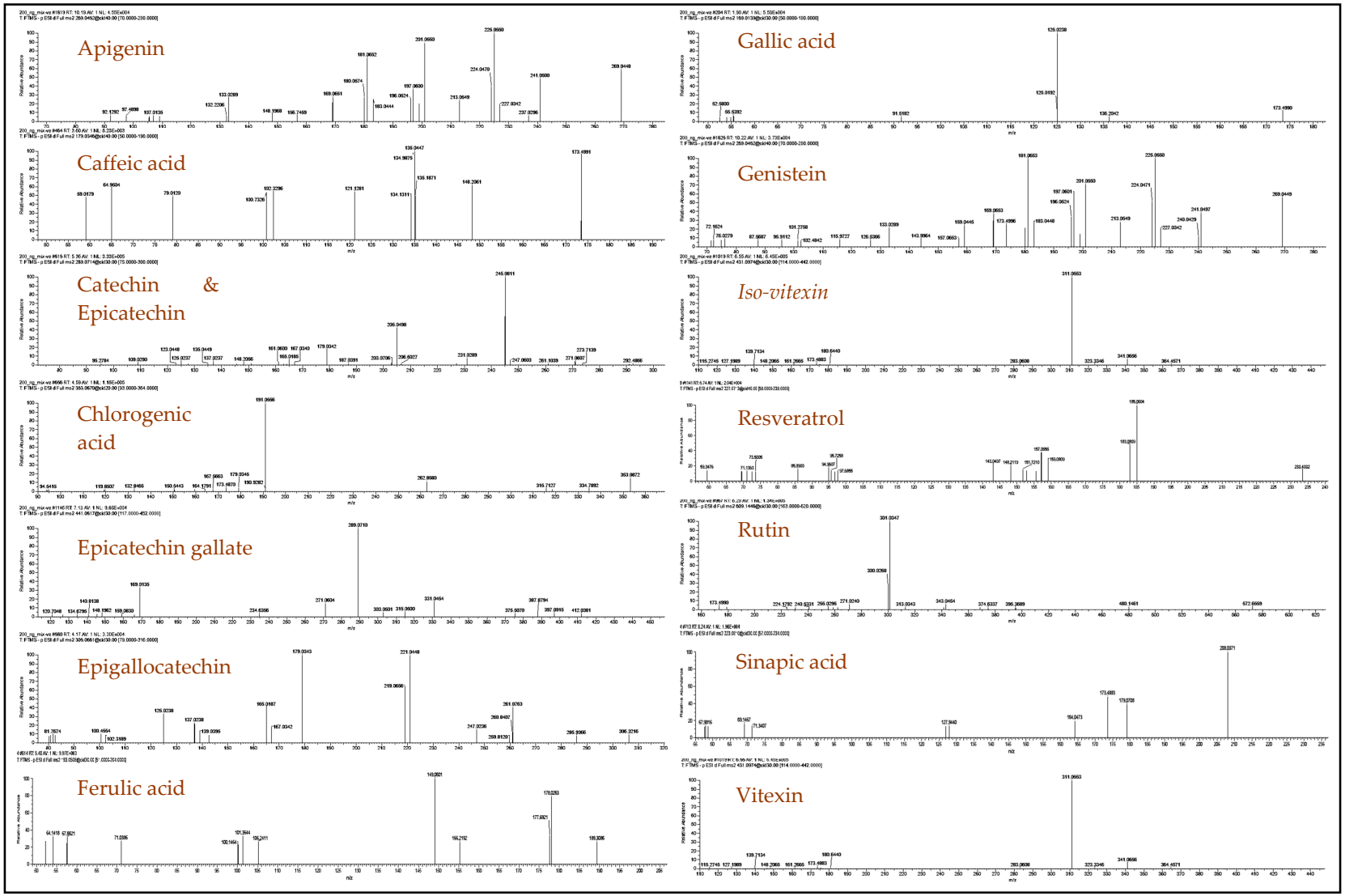

Figure 3. MS2 mass spectra of phenolic compounds showing product ions used as confirmatory fragments for each of the phenolic compounds.

Table 2. Concentrations of phenolic compounds detected and quantified in forage crops seeds and seedlings at various growth stages.

Forage Crop (Cultivar)	Seedling Growth Stage	Apigenin + Genistein [1]	Caffeic Acid	Catechin	Chlorogenic Acid	Epi-Catechin	Epicatechin Gallate	Epigallocatechin	Ferulic Acid	Gallic Acid	*Iso*-Vitexin	Sinapic Acid	Resveratrol	Rutin	Vitexin
		<µg g^{-1} dry wt.>													
Alfalfa (Bridgeview)	Seed	0.85	0.11	4.98	ND [2]	5.00	ND	0.24	6.11	ND	1.27	0.32	ND	0.27	1.27
	Day 7	12.56	0.01	0.01	ND	0.01	ND	<LOQ [3]	<LOQ	ND	<LOQ	0.25	<LOQ	0.02	<LOQ
	Day 14	11.64	0.01	ND	ND	ND	ND	<LOQ	<LOQ	ND	0.02	ND	<LOQ	0.01	0.02
	1 Month	27.14	ND	0.003	ND	0.003	ND	<LOQ	<LOQ	ND	0.01	ND	<LOQ	0.01	0.02
Sainfoi (Melrose)	Seed	0.14	0.27	1.38	56.57	1.39	0.05	86.77	4.89	64.73	ND	0.39	1.39	7.05	0.04
	Day 7	0.03	0.89	0.01	0.34	0.01	ND	ND	1.88	0.16	ND	0.10	<LOQ	1.37	<LOQ
	Day 14	0.01	0.12	ND	0.32	ND	ND	ND	0.65	0.14	ND	ND	<LOQ	0.05	ND
	1 Month	0.08	0.18	ND	0.66	ND	ND	<LOQ	3.27	0.04	ND	0.57	<LOQ	25.79	ND
Fenugreek (Amber)	Seed	0.17	0.02	0.003	0.04	0.01	0.01	0.13	0.17	0.30	0.78	0.09	14.01	0.20	1.80
	Day 7	0.34	0.01	0.003	ND	0.01	ND	0.15	ND	ND	0.13	ND	0.28	0.12	ND
	Day 14	0.02	0.01	ND	ND	ND	ND	<LOQ	ND	ND	0.01	0.18	<LOQ	0.36	ND
	1 Month	0.39	0.08	ND	ND	ND	ND	ND	2.24	ND	0.03	ND	0.03	28.05	ND

[1] Apigenin and genistein are isomers and could not be chromatographically separated in the chromatographic method. The concentrations reported are a sum of both compounds, apigenin and genistein. [2] ND: Not detected. [3] LOQ: Limit of Quantification. LOQ values for each phenolic compound are listed in Table 1.

2.2.3. Fatty Acid Composition Analysis

All three varieties of seeds and seedlings harvested at Days 7, 14, and 30 (1 month) were processed for fatty acid composition using methods adapted from the published literature [34,35]. Seeds and harvested seedlings were washed in distilled water, transferred into centrifuge tubes (50 mL) and stored in freezer at −80 °C until further processing. All samples were homogenized in liquid nitrogen with the help of a mortar and pestle and the resulting powder was used for fatty acid analysis. Quantitatively, powdered material (200 mg) was weighed into a 12-mL Pyrex tube with Teflon tape. Methanolic-HCl (3 N, 2 mL) was added to each sample tube and mixed thoroughly by vortexing (30 s). These Pyrex tubes were then incubated at 80 °C for 45 min in a dry bath (Digital, Boekel Scientific, PA, USA) to digest the seeds and seedlings material and simultaneously extract and convert fatty acids to the corresponding methyl esters. An aliquot of hexane (4 mL) was added to each tube using a repeater pipette and vortexed for 30 s. The top layer of hexane containing fatty acid methyl esters (FAMEs) was transferred to a clean glass tube and evaporated to dryness using a Vacufuge Plus sample concentrator (Eppendorf AG, Hamburg, Germany) at 60 °C for 40 min. The dried extract was resuspended in hexane (1 mL) by vortexing for 30 s, and transferred to GC vials for analysis. Analysis of FAMEs was carried out using gas chromatography (Bruker 436-GC, Bruker Daltonics, Germany) equipped with a capillary column (Rt-2560, 100 m × 250 µm × 0.20, Restek, Bellefonte, PA, USA). Separation of FAMEs was achieved using a gradient elution and the oven was programmed with an initial temperature of 100 °C for 4 min and ramped up to 250 °C at a rate of 3 °C.min^{-1} and holding for 8 min. The total run time for each sample was 62 min. High-purity helium was used as the carrier gas at a flow rate of 0.8 mL/min. A standard mixture of FAMEs (GLC Reference standard:463, Nu-Chek-Prep Inc, Elysian, MN, USA) was used to acquire retention times of all fatty acid methyl esters. The FAMEs in the samples were identified by comparing the retention times of each FAME with those of the standard mix, and the quantity of each FAME was presented as a percentage relative to total FAMEs.

2.3. Statistical Analysis

A two-way ANOVA (OriginPro 2022, OriginLab Corporation, Northampton, MA, USA) was performed on the data to look for significant differences, including the effect of the developmental stages, species and their interaction for each fatty acid methyl esters. A comparison of the average values of treatments was carried out using Tukey's test. Significantly different ($p < 0.05$) concentrations are denoted with a different lower-case alphabet (Table 3). Phenolic compound data was generated using a composite sample of replicates ($n = 3$), and thus, statistical analysis was not possible on phenolic compound data. Limit of detection (LOD) and limit of quantification (LOQ) values for each phenolic compound were calculated using the calibration curves acquired for each analyte using HRMS. The value of LOD for each analyte was calculated as 3.3 times the ratio of the standard deviation of 6 single measurements of the lowest concentration of the respective analyte, detected above blank and square root of number of measurements, i.e., $n = 6$. Similarly, LOQ was calculated as 10 times the ratio of the standard deviation of 6 single measurements of the lowest concentration of the respective analyte, detected above blank and square root of number of measurements, i.e., $n = 6$ [36]. Less than equal to half of the LOD values are designated as not detected (ND).

Table 3. Fatty acid content as fatty acid methyl esters (FAMEs, %) in seeds and various developmental stages of seedling of forage crops. Values are reported as mean of replicates ± standard deviation, $n = 3$.

Fatty Acid [1]	Seeds			Seedlings Day 7			Seedlings Day 14			Seedlings 1 Month		
	Alfalfa Bridgeview	Fenugreek Amber	Sainfoin Melrose	Alfalfa Bridgeview	Fenugreek Amber	Sainfoin Melrose	Alfalfa Bridgeview	Fenugreek Amber	Sainfoin Melrose	Alfalfa Bridgeview	Fenugreek Amber	Sainfoin Melrose
C14:0	0.26 ± 0.04 b	0.18 ± 0.00 e	0.24 ± 0.01 c	0.31 ± 0.01 a	0.23 ± 0.01 d	ND [1]	ND	ND	ND	ND	ND	ND
C15:0	0.19 ± 0.03 d	0.23 ± 0.01 c	0.17 ± 0.01 e	0.3 ± 0.02 a	0.29 ± 0.00 b	ND	ND	ND	ND	ND	ND	ND
C16:0	14.9 ± 2.33 b–d	16.2 ± 0.55 a–c	11.4 ± 0.22 e	16.9 ± 0.53 ab	15.9 ± 0.41 a–d	13.5 ± 0.34 c–e	15.9 ± 0.50 a–d	15.7 ± 0.54 a–d	18.3 ± 0.61 a	15.4 ± 0.30 b–d	13.9 ± 1.82 c–e	13.3 ± 0.35 de
C16:1	0.0.09 ± 0.02 e	0.07 ± 0.00 f	0.1 ± 0.00 d	0.3 ± 0.04 c	0.6 ± 0.06 a	0.4 ± 0.01 b	ND	ND	ND	ND	ND	ND
C18:0	2.4 ± 0.38 e	3.9 ± 0.07 a–c	2.9 ± 0.03 de	3.2 ± 0.11 b–e	4.2 ± 0.36 a	3.3 ± 0.10 a–e	3.1 ± 0.03 c–e	4.2 ± 0.06 a	4.1 ± 0.22 ab	2.6 ± 0.04 de	3.4 ± 0.39 a–d	2.8 ± 0.83 de
C18:1 n9	8.0 ± 0.73 e	14.9 ± 0.41 c	25.7 ± 0.48 a	7.8 ± 0.08 e	10.6 ± 0.89 d	20.4 ± 0.70 b	3.9 ± 0.20 f	3.4 ± 0.19 f	6.9 ± 0.65 e	2.9 ± 0.14 f	3.6 ± 0.51 f	2.9 ± 0.87 f
C18:2	34.8 ± 4.07 a	38.3 ± 0.26 a	17.4 ± 0.13 de	36.5 ± 0.33 a	37.3 ± 1.73 a	21.8 ± 0.43 cd	27.6 ± 1.61 b	27.3 ± 1.28 bc	25.8 ± 1.03 bc	19.0 ± 0.87 de	16.8 ± 2.09 de	15.8 ± 3.67 e
C18:3 n3	27.0 ± 1.35 c	19.3 ± 1.27 d	35.2 ± 0.24 b	26.8 ± 1.53 c	23.4 ± 0.90 cd	34.8 ± 0.28 b	36.9 ± 1.31 b	37.0 ± 0.85 b	34.5 ± 0.42 b	35.9 ± 0.79 b	50.6 ± 6.52 a	45.5 ± 1.41 a
C20:0	0.5 ± 0.10 fg	0.9 ± 0.05 de	0.4 ± 0.02 g	1.1 ± 0.11 cd	1.0 ± 0.13 cd	0.5 ± 0.4 fg	1.1 ± 0.10 cd	1.5 ± 0.07 a	1.2 ± 0.12 bc	0.7 ± 0.03 ef	1.1 ± 0.11 bc	1.4 ± 0.03 ab
C20:1 n9	0.1 ± 0.03 e	0.2 ± 0.01 d	0.3 ± 0.02 b	ND	0.2 ± 0.01 c	0.4 ± 0.02 a	ND	ND	ND	ND	ND	ND
C20:2	0.11 ± 0.04 e	0.13 ± 0.01 c	0.1 ± 0.01 d	0.36 ± 0.03 b	0.4 ± 0.06 a	ND	ND	ND	ND	ND	ND	ND
C20:3 n3	0.4 ± 0.09 f	0.4 ± 0.02 f	0.6 ± 0.03 ef	1.1 ± 0.16 cd	0.6 ± 0.08 ef	0.8 ± 0.08 d–f	1.7 ± 0.02 ab	1.7 ± 0.22 a	1.8 ± 0.18 a	1.2 ± 0.03 b–d	1.0 ± 0.08 c–e	1.4 ± 0.40 a–c
C20:3 n6	0.1 ± 0.03 e	0.12 ± 0.01 d	0.15 ± 0.01 c	0.3 ± 0.01 a	0.23 ± 0.01 b	ND	ND	ND	ND	ND	ND	ND
C24:0	0.1 ± 0.03 c	0.3 ± 0.02 a	0.07 ± 0.02 d	0.3 ± 0.01 b	ND	ND	ND	ND	ND	ND	ND	ND

[1] C14:0, Methyl myristate; C15:0, Methyl pentadecanoate; C16:0, Methyl palmitate; C16:1, Methyl palmitoleate; C18:0, Methyl stearate; C18:1n9 cis, cis-9-Oleic acid methyl ester; C18:2, Methyl linoleate; C18:3n3, Methyl linolenate; C20:0, Methyl arachidate; C20:1n9, Methyl cis-11-eicosenoate; C20:2, cis-11,14-Eicosadienoic acid methyl ester; C20:3n3, cis-11,14,17-Eicosatrienoic; C20:3n6, cis-8,11,14-Eicosatrienoic; C24:0, Methyl lignocerate; [1] ND: Not detected. Values accompanied with different letters in the same row are significantly different at level 0.05 probability using Tukey's HSD test.

3. Results

3.1. Comparison of Bioactive Phenolic Compounds in Three Forage Crop Cultivars

This study aimed to investigate bioactive phenolic compounds in seeds and seedling stages of three forage crops including alfalfa, sainfoin, and fenugreek. The targeted phenolic compounds were detected at various concentrations in seeds and seedlings (Table 2). In alfalfa, of the 15 phenolic compounds investigated, 3 phenolic compounds, including chlorogenic acid, epicatechin gallate, and gallic acid, were not detected. The remaining 12 targeted phenolic compounds were observed at various concentrations (Table 2).

In sainfoin, only iso-vitexin was not detected, while the other 14 phenolic compounds were detected. In fenugreek, all 15 targeted phenolic compounds were detected at various concentrations. Total catechins, including catechin, epi-catechin, epicatechin gallate, and epigallo catechin, were notably present at higher concentrations in alfalfa seeds compared to three seedling growth stages of alfalfa. A similar trend was observed with total catechins in seeds of sainfoin and fenugreek. Caffeic acid, ferulic acid, iso-vitexin, rutin and vitexin were detected at higher concentrations in seeds compared to seedling growth stages in alfalfa, whereas a similar trend was not observed in sainfoin and fenugreek. For example, rutin concentrations were significantly higher in the 1-month old growth stage in both sainfoin and fenugreek. Apigenin and/or genistein were the two phenolic compounds that increased in seedlings compared to seeds in alfalfa. Chlorogenic acid was notably at higher concentration in seeds of sainfoin while at low concentrations in sainfoin seedlings. However, chlorogenic acid was not detected in seeds or seedling growth stages of alfalfa. In fenugreek, chlorogenic acid was detected at a low concentration, only in the seeds. Resveratrol was present at higher concentration in seeds compared to seedlings in both fenugreek and sainfoin, and notably not detected in seed of alfalfa. Fenugreek seeds have a comparatively higher concentration of resveratrol than sainfoin seeds. Ferulic acid content in alfalfa and sainfoin seeds were higher compared to the fenugreek seeds, while by the 1-month growth stage, fenugreek increased its ferulic acid content. Overall, sainfoin seeds had the highest concentrations of catechins, chlorogenic acid, and gallic acid, compared to those in alfalfa and fenugreek seeds.

3.2. Fatty Acid Composition in Seeds and Seedling Growth Stages of Three Forage Crops

The development of forage from seeds to seedling growth stages significantly affected the myristic acid (C14:0) content. Generally, Day 7 seedlings (7 days old) had significantly higher C14:0 content (%) than that in seeds (Table 3). Alfalfa contained a higher % of C14:0 compared to those in fenugreek and sainfoin. At Day 14 and 1-month growth stages, myristic acid was not detected in any of the three forage crops.

Pentadecanoic acid (C15:0) was not detected at Day 14 and 1-month growth stage seedlings and generally the highest content was at Day 7 growth stage of seedlings. Sainfoin had significantly lower C15:0 (%) compared to alfalfa and fenugreek. Similarly, palmitoleic acid (C16:1) was not detected at Day 14 and 1-month growth stage of seedlings and palmitoleic content (%) was similar in three forage crops.

In addition, seedlings at Day 14 growth stage showed the highest content of palmitic acid (C16:0), followed by Day 7 seedlings. Alfalfa and sainfoin seedlings contained higher levels of palmitic acid (%) than sainfoin. However, the highest value (18.33%) was reported in sainfoin at Day 14 growth stage seedlings. Generally, Day 7 and 14 growth stage seedlings contained higher stearic acid (C18:0) compared to those in seeds and 1-month seedlings, whereas fenugreek contained the highest content of stearic acid.

In terms of unsaturated fatty acids, 1-month seedlings contained the highest α- linolenic acid (C18:3n3) (%) compared to those in seedling growth stages and seeds. Sainfoin, in particular, had the highest content (%) of C18:3n3. Fenugreek and sainfoin seedlings at 1-month showed the highest observed content of C18:3n3 at 50.6% and 45.5%, respectively. Seeds and Day 7 seedlings had significantly higher linoleic acid (C18:2) content, particularly in alfalfa and fenugreek, whereas sainfoin contained the lowest content. In general, all three forage crop seeds contained significantly higher oleic acid (C18:1n9),

and sainfoin, in particular, had the highest content (%) compared to fenugreek and alfalfa. The highest value at 25.74% was observed in the seeds of sainfoin (Table 3).

Arachidic acid (C20:0) was significantly affected by both the seed growth stage and forage crop type. Generally, C20:0 reached the highest content (%) in Day 14 seedlings and then declined at 1-month growth stage. This fatty acid methyl ester (FAME) was observed at a relatively higher content (%) in fenugreek than those in alfalfa and sainfoin.

In general, eicosenoic acid (C20:1n9) was not detected in forage crop seedlings at later seedling growth stages (Day 14 and 1 month). Sainfoin seedlings on Day 7 had the highest content at 0.392%. On the other hand, cis-11, 14-eicosadienoic acid (C20:2) was not detected in seedlings at Day 14 and 1 month, however, it was observed at the highest content (0.368%) in Day 7 seedlings of fenugreek. Similarly, lignoceric acid (C24:0) was not detected in Day 14 and 1-month seedlings, but the highest content (%) was found in the seeds of fenugreek. In comparison, forage seedlings at Day 14 and 1-month growth stages did not accumulate cis-8,11,14 eicosatrienoic acid (C20:3n6) in their tissues, while the highest level (0.3435%) was observed on Day 7 seedling of alfalfa.

Overall, Day 14 seedlings showed the highest % of cis-11,14,17 eicosatrienoic acid (C20:3n3) compared to other growth stages, i.e., Days 7 and 1 month. Within Day 14 seedlings, fenugreek contained less % of cis-11,14,17 eicosatrienoic acid (C20:3n3) than those in alfalfa and sainfoin. Both the growth stages (Day 7, 14, and 1 month) and forage crop type significantly affected FAMEs showing significant interactions. The major fatty acids identified in forage crop tissues were C18:2, C18:3n3, C18:1n9, and C16:0. However, the content (%) of each compound was significantly affected by the growth stage and the forage type and their interactions. Some fatty acids disappeared at the later growth stages, for example, C14;0, C15:0, C16:1, C20:1n9, C20:2, C20:3n6, and C24:0 in all forage crops investigated in this study (Table 3).

4. Discussion

The current study aimed to investigate the bioactive phenolic compounds and fatty acids in three forage legume crops, namely alfalfa, fenugreek and sainfoin including seeds and three seedling growth stages (Days 7, 14, and 1 month). It was observed that the 15 targeted phenolic compounds, including apigenin, caffeic acid, catechin, chlorogenic acid, api-catechin, epicatechin gallate, epigallo catechin, ferulic acid, gallic acid, genistein, iso-vitexin, sinapic acid, resveratrol, rutin, and vitexin, were present at different concentrations in the three forage crops at seed and seedling growth stages. Recent research on alfalfa [17,18], fenugreek [12], and sainfoin [12,17] seeds, sprouted seeds and microgreens suggests the presence of potential bioactive compounds contributing to nutritional and health benefits. Microgreens are tender petite leafy plants that fall between the sprouts and are baby green 1–3 inches tall, and can be consumed fresh on salads, sandwiches, smoothies, etc., [37]. Such delicate plants are considered a significant source of vitamins, mineral elements, flavors, carotenoids, tocopherols, phenolic antioxidant compounds compared to mature counterparts [38]. Nowadays, there is more interest in growing various types of microgreens, including fenugreek, alfalfa, cabbage, arugula, beet, beans, lentils, etc., to fulfill the daily nutritional requirements. These microgreens can grow indoor, outdoor, and in greenhouses in rural and urban cultivation system to boost the human nutrient uptake and can be even a source of income to small businesses and emerging entrepreneurs. Numerous in vivo and in vitro studies showed the association between microgreen consumption and the reduced risk of chronic diseases, such as obesity, inflammation, diabetes, and certain types of cancers [39–41]. In the current study, we investigated the chemical composition of fenugreek, alfalfa, and sainfoin at different developmental stages in attempt to identify characteristic bioactive compounds that possess health benefits and improve the well-being of consumers. In this regard, apigenin is a flavonoid present in forage legumes and is considered one of the phytoestrogens that has beneficial effects in the prevention and treatment of many diseases [42]. In addition, apigenin has therapeutic potentials for diabetes, amnesia, Alzheimer's disease, depression, insomnia and cancer [27]. We

observed that alfalfa seedlings have the highest content of apigenin compared to the tested forage crops, and thus having alfalfa as a forage for animals and as a microgreen in the human diet has many potential health benefits. Vitexin belongs to the group of flavonoid C-monoglycosides that are absorbed in the intestine and distributed to other tissues showing significant antioxidant activity, anticancer and antitumor activity, hepatoprotective activity, anti-inflammatory activity, anti-diabetes activity, antiviral activity, antibacterial and antifungal activity, and other biological effects [43]. In this study, vitexin was the highly available polyphenol in the fenugreek seeds and present in all three tested forage legume seedlings that may be used as a functional food for both animals and humans. A wide array of phenolic compounds were previously identified in seeds and the fenugreek herb but, generally, the fenugreek herb contains predominantly di- and triglycosides of quercetin and kaempferol [44]. For instance, pyrogallol, chlorogenic acid, oleoropine, ellagic acid, catechin, apigenin-6-O-arabinose-8-O-galactose, hesperidin, apigenin-7-O-glucoside, and kaempferol-3,2-p-coumaroyl glucose were the major phenolic compounds found in air dried fenugreek leaves [45]. In another recent study, quercetin, gallic acid, caffeic acid, vanillic acid, syringic acid and m-coumeric acid were identified in fenugreek leaves [46], while in our study, quercetin was not detected. Similarly, in the current study, alfalfa seedlings did not contain a measurable amounts of ferulic acid (FA), while in a previous study, FA was the most abundant compound in both soluble and bound extracts of alfalfa leaves [25]. This variation could be attributed to differences in the extraction method, origin of plant material or environmental conditions.

In the lipophilic fraction, α-linolenic acid, linoleic acid, and palmitic acid were the three major FAMEs and constituted more than 80% of the total fatty acids in the seeds and vegetative tissues of forage crops in this study. This is in agreement with previous reports on several traditional and novel forages, such as white clover, chicory, and borage [47]; blue fenugreek [44]; sainfoin [48]; alfalfa and sainfoin [28]. Previous evidence showed that sainfoin seeds had a better ratio of unsaturated fatty acids to saturated fatty acids [12]. Recent breeding studies of sainfoin and fenugreek led to improving these crops as bloat-free forage legumes by enhancing their fatty acid composition in vegetative parts [28]. In this study, in both sainfoin and fenugreek, the major fatty acids of seeds and Day 7 seedlings were C18:2 and C18:1n9, while the later-stage seedlings were higher in C18:3n3, suggesting the use of forage sprouts as a good source of lipophilic bioactive compounds that promote health and prevent certain diseases.

In conclusion, variation in the level and composition of phenolic compounds and fatty acids in the three forage crop varieties was dependent on the growth stage and genotype. Apigenin and genistein may be a good candidate for exerting health benefits of alfalfa and fenugreek seedlings, while sainfoin seeds can be considered a good source of bioactive compounds, such as chlorogenic acid, catechin, epigallocatechin, ferulic acid, gallic acid, which may suggest the suitability of sainfoin seeds as a functional ingredient in nutraceutical products and functional foods. In addition, at all stages, sainfoin seeds and seedlings were characterized by a higher abundance of oleic acid: an unsaturated fatty acid with health benefits. Assessment of total and/or individual phenolic compounds and fatty acids in these crops could potentially be used as biomarkers for the selection and development of these forage cultivars to contribute to animal and human nutrition and health. While this study provides a good understanding of phenolic compounds and fatty acid content in controlled conditions, further studies are warranted for investigating the molecular and biochemical mechanisms for the accumulation of these bioactive compounds as a function of developmental stages and environmental conditions under field conditions.

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