Effect of Organic Fertilizers on Antioxidant Activity and Bioactive Compounds of Fenugreek Seeds in Intercropped Systems with Buckwheat

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Abstract: Antioxidants and flavonoids are beneficial compounds used in the pharmaceutical and food industries that are derived from natural sources. A two-year field experiment was undertaken to investigate the effect of agricultural management practices on the trigonelline content, antioxidant activity measured with DPPH (2,2-Diphenyl-1-Picrylhydrazyl) and FRAP (Fe+++-Reduction, Ferric reducing antioxidant power), total phenolic content, total flavonoids content, and specific flavonoid contents of fenugreek seeds. The treatments examined were sole fenugreek (Sole F) and three intercropping ratios with buckwheat (B) (F:B = 2:1, 1:1, and 1:2), each with three types of fertilizer (chemical fertilizer, integrated fertilizer, and broiler litter). The highest trigonelline content of fenugreek seeds was obtained in F:B = 2:1 fertilized with broiler litter. Compared to Sole F, the fenugreek seeds harvested in the intercropped treatments had higher antioxidant activity, total phenolic content, vitexin, isovitexin, orientin, and isoorientin. The highest trigonelline content of fenugreek seeds was obtained in F:B = 2:1 fertilized with broiler litter. Compared to Sole F, the fenugreek seeds harvested in the intercropped treatments had higher antioxidant activity, total phenolic content, vitexin, isovitexin, orientin, and isoorientin. The treatment F:B = 2:1 enhanced the antioxidant activity and the content of bioactive compounds. Overall, the addition of organic manure to fenugreek seeds was highly effective at promoting all compounds analyzed in both the sole and the intercrop systems. Our study found that harvested fenugreek seeds from plants that were intercropped with buckwheat and with the application of organic fertilizer enhanced the seed content of antioxidants and flavonoids.

Keywords: antioxidant activity; flavonoids; intercropping; organic manure; trigonelline; Trigonella foenum-graecum L.

1. Introduction

The interest in natural medicinal products, including legume seeds, for the pharmaceutical industry, is increasing worldwide [1,2]. Medicinal plant cultivation can increase the diversity of farming systems, improve their profitability, and make an important contribution to human health [3]. Fenugreek (Trigonella foenum-graecum L.) is an annual leguminous plant, widely cultivated as a traditional-medicinal plant, native to an area extending from Eastern Europe to Central Asia [4]. Recently, through both human and animal experiments, scientific evidence has shed light on the wide range of health benefits of fenugreek, including its positive anticarcinogenic, antidiabetic, antiatherogenic, antioxidant, antianorexic, galactagogue, antihyperlipidemic, anti-inflammatory,
antifungal, antibacterial, and neuroprotective effects [5]. Fenugreek has also been reported to exhibit strong antioxidant properties, which has led to an interest in using the inherent plant-based antioxidant for patients with heart disorders or cancer [6,7].

Fenugreek seeds contain several interesting biological and chemical active compounds including alkaloids (e.g., trigonelline), polyphenols (e.g., kaempferol, quercetin), antioxidants, flavonoids, carbohydrates (45%-60%), protein (20%-30%), lipids (7.5%), aromatic compounds, saponins, carotenoids, vitamins and minerals [7–10]. Trigonelline is a major and important alkaloid component with a high therapeutic potential, hypoglycemic effect, and low toxicity [11]. Numerous health benefits of fenugreek seeds have been primarily attributed to the powerful antioxidant activity, high amounts of flavonoids and alkaloids [7]. Kalinova and Vrchotova [12] reported on the amounts of bioactive phytochemicals and minerals, which depend not only on the environmental conditions of plant growth but also on crop management. A mixed cropping (i.e., an intercrop) system with organic fertilizer application can enhance fenugreek seed quality while producing an organic product [13–15]. An intercropped cultivation with legumes can increase the quality of seeds and yield productivity [15,16].

Another medicinal plant, the common buckwheat (Fagopyrum esculentum Moench), is an important crop in organic farming. Recently, buckwheat is gaining popularity for the development of new food products due to its nutritional and health-promoting composition [17]. Thus, common buckwheat can be one intercrop, which may affect the quality and productivity of its main crop.

Further environmental benefits of legume plants grown together with non-legume crops in intercropped systems include increased soil fertility through biological N₂ fixation [18] and improved soil phosphorus bioavailability by exuding H⁺ and various organic acids to enhance rhizosphere phosphatase levels [19]. The additional source of N from legumes is expected to (a) avoid inter-specific competition between the intercrops for N acquisition, and (b) increase soil N content for subsequent crops [20]. To ensure a high content of bioactive compounds in the plant, sufficient nutrients must be available in the soil for the plant needs [16,21]. Several experiments have been undertaken to examine different fertilizer types on the phenolic content of plants, such as chamomile [22] and chicory cultivars [23]. Aina et al. [24] reported a strong relationship between the level of soil macronutrients (i.e., nitrogen, phosphorus, and potassium) and the level of phenolics, flavonoids, and bioactive compounds. It has also been shown that organic fertilizers enhance antioxidant activity and bioactive compounds in Kacip Fatimah (Labisia pumila Benth) [25]. In a previous field study, we have shown the application of broiler litter or integrated fertilizer to increase the antioxidant activity and the content of bioactive compounds of buckwheat seeds harvested from an intercropped fenugreek–buckwheat system [15], and we have reported on the yields, biomass productivity and growth analysis of fenugreek intercropped with buckwheat under application of different fertilizer types [16,26,27].

To the best of our knowledge, there is no information available on the bioactive compound composition of harvested fenugreek seeds that were grown with different crop management systems. Therefore, we hypothesize that intercropping legumes (fenugreek) with non-legumes (buckwheat) as well as the application of an organic fertilizer can increase the content of bioactive compounds and the seed quality of fenugreek, compared to fenugreek seeds grown alone and with chemical fertilizer application. The specific objectives of our study were to analyze: (a) trigonelline content (TC), (b) antioxidant activity, i.e., DPPH (2,2-Diphenyl-1-Picrylhydrazyl) and FRAP (Fe⁺⁺⁺⁻Reduction, Ferric reducing antioxidant power assay, (c) total phenolic content (TPC), (d) total flavonoids content (TFC), and (e) main flavonoid compounds content, i.e., vitexin, isovitexin, orientin, and isoorientin of fenugreek seeds harvested from field plots with different fertilizer applications in an intercrop system and in a sole crop system.

2. Material and Methods

2.1. Experimental Design, Set-Up, and Field Management

In 2014 and 2015, a two-factorial field experiment in a randomized complete block design was established with three replications at the research farm of Shahrekord University (32°21΄ N, 50°49΄ E;
2050 m a.s.l.), Iran. Each plot measured 2.5 × 3 m (7.5 m²). According to the Köppen climate classification system, the local climate is temperate and cold with dry and warm summers, and an average annual temperature of 10.5 °C and 280 mm precipitation. The soil type is a fine, carbonatic, mesic Calcixerupt with a clay loam soil texture.

Four cropping systems were established: a sole crop of fenugreek (Sole F); two rows of fenugreek + one row of buckwheat (F:B = 2:1); one row of fenugreek + one row of buckwheat (F:B = 1:1); and one row of fenugreek + two rows of buckwheat (F:B = 1:2). All systems were fertilized with the following three fertilizer types: chemical fertilizer (CF), broiler litter (BL) or integrated fertilizer (IF = 50% CF + 50% BL). Nitrogen was applied as urea at a rate of 80 kg N ha⁻¹ for fenugreek and 60 kg N ha⁻¹ for buckwheat, according to local farmers’ practices. Phosphorus was applied as triple superphosphate, and micronutrients (Cu, Fe, Mn, and Zn) were added to the urea-fertilized plots at a rate equivalent to the total amount added by the broiler litter treatments. Application of 10 and 7.5 Mg ha⁻¹ of broiler litter provided 80 and 60 kg N ha⁻¹, respectively (assuming 50% mineralization of broiler litter N during the first cropping season). The intercrop systems (F:B) received equivalent fertilizer amounts according to their species composition. Broiler litter was incorporated into the soil before sowing. The physical and chemical properties of the broiler litter and the soil were analyzed, and data are provided in Salehi et al. [15,16].

The seeds for the fenugreek (Isfahan landrace) plants were provided by Pakan Bazr Company (Isfahan, Iran). The seeds of both crops were sown by hand on 29 May 2014 and on 23 May 2015. Fenugreek and buckwheat were sown at 50 and 120 seeds m⁻², respectively. The sowing depth was 2 to 4 cm for fenugreek and 1 to 2 cm for buckwheat. The field experiment was managed according to organic agriculture guidelines, with no pesticide or herbicide applications. Plant shoots were hand-harvested by cutting the fully mature plants at the soil surface.

2.2. Seed Analysis, Reagents, and Chemicals

Mature fenugreek plants were hand-harvested on 12 September 2014 and 15 September 2015. After threshing, the seeds were oven-dried at 70 °C for 48 h until constant weight. To determine the bioactive compound content, fenugreek seed samples were transferred to the Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Austria.

Reagents and chemicals used in this study were acetonitrile HPLC grade (Chem-Lab NV, Belgium); FeCl₃·6H₂O, Folin–Ciocalteu reagent (Merck); AlCl₃·6H₂O, acetic acid p.a., TPTZ (2,4,6-tripyridyl-s-triazin, >99%) (Fluka); caffeic acid (>99%), HCl, ethanol (96%) p.a., isovitexin (ROTICHROM®, >99%), vitexin (ROTICHROM®, >99%), trigonelline hydrochloride >97.5%), methanol p.a., formic acid (ROTIPURAN, >98%), NaCH₃COOH·3H₂O, NaN₂, NaNO₂, and Na₂CO₃, were purchased from Roth; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl (>99%), orientin (>97%), isoorientin (HPLC-grade, >98%), trolox: (+)-6-Hydroxy-2,5,7,8-tetra-methylchromene-2-carboxylic acid (>98%), and rutin (>99%), were obtained from Sigma-Aldrich. More detailed information on the sample preparation and analysis is provided in Salehi et al. [15,16,27].

2.3. Extraction Procedures

2.3.1. Preparation of Methanolic Extracts for Analysis of Trigonelline

The trigonelline content (TC) in the fenugreek seeds was analyzed as follows: 300 mg of milled, dried fenugreek seeds were dissolved in 1.5 mL of 80% MeOH and transferred to an ultrasonic bath (Sonorex RK 156H, Bandelin, Germany) for 15 min. The homogenates were centrifuged at 1000 rpm (10 min), and the supernatant was transferred to sealed vials. Six hundred microliters distilled water (AD) was added to 150 µL of each seed sample extract, and the sample was diluted 1:10 with AD. The extracts were filtered through membrane filters (Rotilabo® nylon, pore size 0.20 µm, Carl Roth, GmbH, Karlsruhe, Germany) to determine TC by HPLC. All extracts were stored at −20 °C.
2.3.2. Preparation Of Ethanolic Extracts for Antioxidant Activity and Flavonoids

The antioxidant activity analysis (evaluated by DPPH and FRAP assay), TPC, TFC, and the main flavonoid compound content (vitexin, isovitexin, orientin, and isoorientin) procedure was as follows: 400 mg of milled fenugreek seeds were weighed and extracted with 4 mL of 80% ethanol in an ultrasonic bath (Sonorex RK 156H, Bandelin, Germany) for 30 min. They were then filtered through Whatman filter paper and the extracts transferred to sealed vials [15]. All extracts were stored at –20 °C.

2.4. Measurements

2.4.1. Quantitative Determination of Trigonelline Content by High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA)

The chromatographic separation was performed on a Shimadzu Nexera HPLC system (Shimadzu, Kyoto, Japan) which consisted of a pump (LC-20ADXR), column oven (CTO-20AC), degasser (DGU-20A5R), autosampler (SIL-20AXR), controller (CBM-20A), photodiode array detector (SPD-M20A), and software LabSolution. Chromatographic analysis was performed on a Cogent Diamond HydrideTM TYPE-C silica column, 100 × 2.1 mm, MicroSolv Technology Corp., Leland, NC, USA; 4 µm, 100 Å, in combination with a guard column (security guard cartridge, RP-18, 4 × 3 mm, 5 µm, Phenomenex). The mobile phase consisted of 0.1% formic acid in acetonitrile (v/v) (solvent A) and 0.1% formic acid in water (solvent B). A linear gradient of the solvent B was applied from 5% to 35% for 20 min, followed by an isocratic washout phase of 50% B for 2 min before returning to the initial conditions. The flow rate was 0.4 mL min⁻¹, injection volume 10 µL. The column temperature was set at 30 °C, and trigonelline was detected at 264 nm. The identification of trigonelline was based on the retention time (12.6 min) and its UV-spectral data in comparison with an authentic commercially available reference substance (Figure 1A). For quantitative analysis with an external standard, trigonelline was prepared in methanol (80%) in duplicate at five concentrations. The trigonelline content in the 80% methanolic extract of each seed sample was expressed in milligrams per gram of dry weight (DW) of seed (mg/g DW).

![HPLC chromatograms of trigonelline (A) and flavonoid compounds (B) extracted from a sample of fenugreek seeds. The identified peaks (B) are orientin (1), isoorientin (2), vitexin (3), isovitexin (4), rutin as internal standard (5).](image-url)
2.4.2. Measurement of Antioxidant Activity

**DPPH Assay**

The DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay of each sample was conducted according to an improved procedure by Brand-Williams et al. [28]. Briefly, 3.8 mg DPPH was dissolved in 25 mL MeOH to prepare the DPPH-solution. Then 100 µL of that DPPH-solution was added into individual wells of 96-well plates and was mixed with 85 µL MeOH and 15 µL of each seed sample extract. Finally, the microplates were incubated in the dark, at room temperature, for 30 min. The absorbance of the resulting solution was measured at 515 nm using a microplate reader (iMark™, Bio-rad, Vienna, Austria). To obtain a calibration curve, Trolox was applied, the results (antioxidant activity for each seed sample) were expressed as milligram of trolox equivalents (TE) per gram of dry weight (DW) of seed (mg TE/g DW).

**FRAP Assay**

The FRAP (Fe³⁺-Reduction, Ferric reducing antioxidant power) assay was conducted on each sample according to a modified version of Benzie and Strain [29]. The FRAP reagent was provided by mixing 10 mM TPTZ in 40 mM/L HCl, 20 mM/L FeCl₃·6H₂O, and 300 mM/L acetate buffer (pH 3.6) using the ratio 1:1:10 (v/v/v). Then 180 µL of FRAP reagent was transferred into separate wells of 96-well plates and mixed with 9 µL of seed sample extract and 15 µL of distilled water. Microplates were incubated at room temperature in the dark for 5 min, and the absorbance of solutions was estimated at 490 nm using a microplate reader. Trolox was used to derive a calibration curve, and the results of antioxidant activity were expressed as milligram of trolox equivalents (TE) per gram of dry weight (DW) of seed (mg TE/g DW).

2.4.3. Measurement of Total Phenolic Content (TPC)

The Folin–Ciocalteu reagent (FCR) method was used according to a modified procedure of Singleton et al. [30]. In 96-well plates, 5 µL of FCR was mixed with 100 µL of distilled water (AD) and 10 µL of seed sample extract. After 3 min shaking, 125 µL AD and 10 µL of Na₂CO₃ (35 g in 100 mL AD) were added to each well. Microplates were incubated in the dark, at room temperature, for 60 min. The absorbance of each sample was measured at 750 nm. To obtain a calibration curve, caffeic acid was applied, and the TPC for the seed extracts was expressed as milligram of caffeic acid (CA) equivalents per gram of dry weight (DW) of seed (mg CA/g DW).

2.4.4. Measurement of Total Flavonoids Content (TFC)

The modified Leontowicz et al. [31] procedure was used. In short, 40 µL seed sample extract was transferred into individual wells of 96-well plates and mixed with 100 µL AD, 15 µL 10% AlCl₃·6H₂O-solution, and 15 µL 2.5% NaNO₂-solution. Microplates were then shaken for 5 min on the IKA MTS4 (IKA®-Werke GmbH, Staufen, Germany) and 50 µL 1M NaOH was added to each well, and the microplate was shaken for another 5 min. The absorbance of the resulting solutions at 490 nm was noted using a microplate reader. A calibration curve was obtained by using different concentrations of rutin following the same method, and the TFC for each sample extract was expressed as milligrams of rutin (RU) equivalents per gram of dry weight (DW) of seed (mg RU/g DW).

2.4.5. Quantitative Measurement of Specific Flavonoids by High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA)

The chromatographic separation of specific flavonoid compounds (vitexin, isovitexin, orientin, and isoorientin) in fenugreek seed extracts was performed using the Shimadzu Nexera HPLC system (as described in Section 2.4.1) according to the procedure described by Salehi et al. [15] with minor modifications, whereby rutin was the internal standard.
The identification of the compounds was based on their retention times and their UV-spectral data in comparison with authentical commercially available reference substances (Figure 1B). The flavonoid content in the 80% ethanolic extract of each seed sample was expressed in milligram per gram of dry weight (DW) of seed (mg/g DW).

2.5. Statistical Analysis

The data from each year was analyzed statistically using an analysis of variance procedure (PROC ANOVA in SAS (version 9.2)) that was applied to a two-factorial experiment in a randomized complete block design considering cropping system and fertilizer as the first and second factors, respectively. The means were compared by using least significant differences (LSD) at the 5% probability level. MSTAT-C was applied to calculate least significant differences (LSD) and letters were used for denoting significant mean differences of the treatments. The significance of the linear relationships between antioxidant activity and total phenolics, total flavonoids, and specific flavonoid compounds was analyzed using Pearson correlation coefficients.

3. Results and Discussion

3.1. Trigonelline Content (TC)

In both 2014 and 2015, the trigonelline content (TC) in the harvested fenugreek seeds was significantly affected by the interaction effect of cropping system and fertilizer (Table 1 and Figure 2A,B). The TC of the seeds varied from 5.64 to 7.86 mg/g DW in 2014 and from 5.10 to 8.0 mg/g DW in 2015 (Figure 2A,B). These values of TC are higher than those reported by Dadrasan et al. [32] and Hassanzadeh et al. [33] measured in domestic ecotypes of fenugreek seeds in Iran. Dadrasan et al. [32] reported TC values of 2.9 and 3.1 mg/g under full and severe deficit irrigation, respectively.

The TC in fenugreek seeds harvested from intercropped systems was, on average, 8.5% (2014) and 13.2% (2015) higher than from Sole F (Figure 2A,B). In both years, the TC was higher in the intercropped systems with a larger share of fenugreek, especially in treatments with BL application, so that in 2014, the maximum TC in fenugreek seeds was measured in F:B = 2:1 with BL and had a value of 7.86 mg/g DW, while in F:B = 1:1 the TC was 7.26 mg/g DW (Figure 2A). In 2015, the F:B = 2:1 values were 8.00 and 7.70 mg/g DW fertilized with IF and BL, respectively, and in F:B = 1:1 with BL the values were 7.80 mg/g DW (Figure 2B).

In Sole F treatments, when both IF or BL were applied, the TC was increased, on average, by 7.8% (2014) and 26.0% (2015) compared to the application of CF (Figure 2A,B). In intercropped treatments, the application of IF and BL showed the highest TC, which was, on average, 15.6% (2014) and 17.2% (2015) higher compared to the CF (Figure 2A,B). Baghbani-Arani et al. [34] also reported the TC in fenugreek to be enhanced after organic fertilizer (vermicompost) application. As well, Abdelkader and Hamad [35] measured a positive effect on the TC in the plants of intercropped safflower–fenugreek with foliar fertilization.

Overall, the application of BL and IF in intercropped systems increased the TC of the harvested fenugreek seeds. This can be explained by the availability of N due to the slower release of N from organic manure during the growing season and through additional soil N availability by the N-fixation of fenugreek [27]. Trigonelline is an alkaloid compound that is produced through methylation of nicotinic acid. Generally, N has a positive effect on increasing alkaloids content because N is a component of amino acids and metabolites [36].
Table 1. ANOVA results (p-values) for trigonelline, antioxidant activity estimated by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) assay, total phenolic, total flavonoids, vitexin, isovitexin, orientin, and isoorientin content of fenugreek seeds in 2014 and 2015.

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<th>DPPH</th>
<th>FRAP</th>
<th>Total Phenolic</th>
<th>Total Flavonoids</th>
<th>Vitexin</th>
<th>Isovitexin</th>
<th>Orientin</th>
<th>Isoorientin</th>
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Figure 2. Trigonelline content (A,B), and antioxidant activity estimated by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) (C,D) and Ferric reducing antioxidant power (FRAP) (E,F) assays of fenugreek seeds as affected by cropping system × fertilizer types interaction in 2014 and 2015. Chemical fertilizer (CF), integrated fertilizer (IF), broiler litter (BL), sole fenugreek (Sole F), F:B = 2:1, F:B = 1:1 and F:B = 1:2 are chemical fertilizer, integrated fertilizer, broiler litter, sole fenugreek, two rows of fenugreek + one row of buckwheat, one row of fenugreek + one row of buckwheat, and one row of fenugreek + two rows of buckwheat, respectively. Different letters indicate significant differences at p < 0.05 by the least significant differences (LSD) test.

3.2. Antioxidant Activity

3.2.1. DPPH Assay

In our experiment, the antioxidant activity was assessed using two different in vitro antioxidant assays (DPPH radical and FRAP). In the DPPH assay, the reaction between DPPH radicals and antioxidant is based on an electron transfer mechanism and hydrogen-atom absorption [37]. In both years, a significant interaction of cropping system and fertilizer was found on the antioxidant activity measured by DPPH radical assay in the harvested fenugreek seeds (Table 1 and Figure 2C,D). The DPPH values varied from 3.37 to 5.42 mg TE/g DW (2014) and 3.26 to 5.48 mg TE/g DW (2015). The seeds harvested from the intercropped plots had higher DPPH levels, on average, by 12.3% (2014) and 12.5% (2015) compared to Sole F, and the antioxidant activity increased with an increasing share of fenugreek plants in the intercrop system (Figure 2C,D). The highest DPPH level in fenugreek seeds was measured with BL application, in 2014 in the F:B = 2:1 with 5.42 mg TE/g DW, and in 2015 in the F:B = 2:1 with 5.48
and in the F:B = 1:1 with 5.10 mg TE/g DW. The increase in antioxidant activity could be caused by the overall promotion of organic manure in supplying macro- and micro-nutrients that are responsible for antioxidant activity [38]. In connection with the effect of intercropping, Moghbeli et al. [39] reported ascorbic acid content (one of the most powerful antioxidants) in a fenugreek–onion intercropped system to increase with decreasing onion density so that the peak levels of antioxidants in fenugreek were in treatments with a higher fenugreek density.

The seeds harvested from Sole F treated with IF and BL had higher DPPH concentrations, which were, on average, 14.6% (2014) and 9.22% (2015) higher compared to the CF (Figure 2C,D). In the intercropped system, the antioxidant activity was significantly higher with IF and BL treatments. On average, it exceeded the CF treatment by 16.8% (2014) and 33.1% (2015), respectively (Figure 2C,D). Similar to our results, Pandey et al. [40] found higher antioxidant levels in basil with the application of poultry manure compared to chemical fertilizer. Overall, organic manures are reportedly less concentrated nutrient sources compared to chemical fertilizers and have slower nutrient mineralization rates, leading to an overall lower nutrient bioavailability especially during the demanding plant development and growth stages [24,41]. This condition causes organically fertilized treatments to experience (i) oxidative stress from superoxide dismutase, which is a key enzyme in plant defense and development, and (ii) abiotic stress which causes an accumulation of reactive oxygen species (ROS) that inhibits enzymatic activity, disturbs cellular homeostasis and ruptures membranes with subsequent deleterious effects on plant growth [24,42]. Consequently, organically grown seeds react to this condition by activating their own defense mechanisms, which leads to the synthesizing of more bioactive and antioxidant compounds. They activate the signaling pathway for the detoxification of ROS by synthesizing antioxidants that scavenge ROS [24].

3.2.2. FRAP Assay

The FRAP assay is based on an electron transfer mechanism without the involvement of free radicals [37]. In both 2014 and 2015, a significant interaction of cropping system and fertilizer application was determined on the FRAP assay of fenugreek seeds (Table 1 and Figure 2E,F), so that the FRAP in fenugreek seeds varied from 2.63 to 5.46 mg TE/g DW in 2014 and from 2.73 to 5.78 mg TE/g DW in 2015. The results showed, seeds harvested from the intercropped plots had the highest FRAP levels, which was, on average, 20.3% (2014) and 42.5% (2015) above that of the Sole F. In the intercropped systems, the highest antioxidant activity was measured in the F:B = 2:1 plots with 4.25 (2014) and 4.90 (2015) mg TE/g DW (Figure 2E,F).

The seeds harvested from Sole F with IF and BL applications had improved FRAP levels that were, on average, 29.0% (2014) and 11.6% (2015) higher than for CF (Figure 2E,F). As well, intercropping with IF and BL had superior FRAP concentrations, on average, by 37.3% (2014) and 27.6% (2015) more, respectively, than with CF (Figure 2E,F). The maximum antioxidant activity measured with FRAP assay in the harvested fenugreek seeds occurred in the treatment with the highest share of fenugreek plants (F:B = 2:1) with BL application, with FRAP values of 5.46 (2014) and 5.78 (2015) mg TE/g DW (Figure 2E,F). Our results are corroborated by Rostaei et al. [38] who reported the application of organic manure on intercropped soybean–dill to result in higher antioxidant activity in the plants, especially when a larger proportion of soybean was in the intercropped system. Pandey et al. [40] also determined the beneficial effects of organic manure application on the secondary metabolic pathways to increase the antioxidant activity.

Overall, in this study, increased antioxidant activity in harvested fenugreek seeds (determined by both DPPH radical and FRAP) was obtained with the application of IF and BL manures compared to the CF. Previous research supports our findings that the antioxidant activity as estimated by the DPPH and FRAP assays increased due to the application of organic fertilizer [43], while inorganic fertilizer was proven to reduce the antioxidant content in plants [44]. Indeed, plants tend to generate antioxidants as a protective or preventive measure against abiotic and oxidative stress, which may be a result of the low but constant availability of macronutrients, such as N, in organic amendments [24].
3.3. Total Phenolic Content (TPC)

The TPC in the harvested fenugreek seeds was significantly affected by the cropping system and fertilizer interaction in both years (Table 1 and Figure 3A,B). The TPC ranged from 3.11 to 4.05 mg CA/g DW in 2014 and from 3.08 to 3.96 mg CA/g DW in 2015 (Figure 3A,B). Our data showed that for seeds harvested from intercropped systems, the TPC was, on average, 8.00% (2014) and 3.33% (2015) higher compared to the Sole F, with the most TPC present in treatments with a higher share of fenugreek (F:B = 2:1) (Figure 3A,B). The highest TPC concentrations of 4.05 (2014) and 3.96 (2015) mg CA/g DW were measured in F:B = 2:1 treated with IF and BL fertilizers, respectively (Figure 3A,B). In both years, the TPC was higher with IF and BL application in the intercropped systems and in the sole plots compared with CF application. Several studies have linked the accumulation of secondary metabolites to the level of available N in the soil. For example, Oliveira et al. [45] indicated that organically produced tomatoes accumulated more bioactive compounds, such as phenolics and flavonoids, compared to inorganically grown tomatoes. Other researchers have also reported a decrease in the total phenolic content of, e.g., potatoes with mineral fertilization application [46].

![Figure 3](image)

Figure 3. Total phenolic content (A,B) and total flavonoids content (C,D) of fenugreek seeds as affected by cropping system × fertilizer type interaction in 2014 and 2015. Different letters indicate significant differences at p < 0.05 by LSD test. See Figure 2 for treatments.

Our results show that fenugreek seeds harvested in Sole F fertilized with either IF or BL had higher TPC, on average, by 10.7% (2014) and 3.30% (2015), compared with CF application. In fenugreek seeds harvested from intercropped systems when IF or BL was applied, the TPC was increased by 14.4% (2014) and 14.9% (2015) compared to CF (Figure 3A,B). Similarly, Salama et al. [47] reported that a higher total flavonoid content was achieved when fennel plants were treated with 50% mineral fertilizer + 50% organic fertilizer compared with a control treatment. The higher amounts of phenolics can be explained by the role of organic fertilizers in the biosynthesis of secondary metabolites, which induces the acetate shikimate pathway, resulting in the highest production of flavonoids and phenolics [48].

3.4. Total Flavonoid Content (TFC)

A significant interaction of cropping system and fertilizer was found on the TFC in fenugreek seeds in both years (Table 1 and Figure 3C,D). The TFC in fenugreek seeds varied from 3.40 to 6.90 mg RU/g DW in 2014 and from 4.0 to 7.25 mg RU/g DW in 2015. A higher TFC was found in
fenugreek seeds harvested from intercropped systems, on average, 32.4% (2014) and 23.8% (2015) higher than in seeds harvested from Sole F (Figure 3C,D). In Sole F, the highest TFC value was measured with BL application of 4.82 (2014) and 5.10 (2015) mg RU/g DW, while for intercropping, the TFC was significantly higher with IF and BL by, on average, 24.7% (2014) and 41.8% (2015) than for CF (Figure 3C,D). Naguib et al. [49] also indicated that organically fertilized broccoli generally had elevated levels of flavonoids.

In the current study, the TFC was significantly enhanced in the fenugreek seeds harvested with BL application from F:B = 2:1 with 6.90 mg RU/g DW (2014), and in F:B = 2:1 with 7.21 mg RU/g DW and in F:B = 1:1 with 7.02 mg RU/g DW (2015) (Figure 3C,D).

Overall, intercropping combined with the application of IF or BL promoted the TFC in fenugreek seeds. This research and others show that organically produced plant foods may be more health-promoting than conventional foods, due to their higher amounts of secondary compound metabolites [50]. The combination of intercropping with the application of organic fertilizer beneficially influences the flavonoid content. Previous research indicates the increase of these compounds is caused by the interaction among rhizobium bacteria, AMF (arbuscular mycorrhizal fungi), plant growth promoting rhizobacteria, and nematodes. Organically fertilized intercropped systems supply organic materials, which enrich the soil microbe population (such as bacteria, fungi, and nematodes) that are beneficial to plant roots. The interaction between soil microbes and plant roots increase the synthesis of flavonoids [38,51,52]

3.5. Flavonoids Compounds Content

3.5.1. Vitexin and Isovitexin

The vitexin and isovitexin contents of fenugreek seeds were significantly affected by cropping system and fertilizer interaction in both years (Tables 1 and 2). The vitexin content in fenugreek seeds ranged from 7.15 to 14.45 mg/g DW (2014) and from 8.30 to 15.0 mg/g DW (2015). Overall, higher vitexin content was measured in seeds harvested from the intercropped systems by, on average, 40.2% (2014) and 17.5% (2015) than for seeds from Sole F (Table 2). The vitexin content improved with an increasing share of fenugreek in intercrops in both years (Table 2). The highest vitexin content was obtained in the F:B = 2:1 with both IF and BL and in the F:B = 1:1 with BL (in 2014 and 2015).

<table>
<thead>
<tr>
<th>Table 2. Vitexin, isovitexin, orientin, and isoorientin content of fenugreek seeds (mg/g DW) as affected by cropping system × fertilizer types interaction in 2014 and 2015.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sole F</strong></td>
</tr>
<tr>
<td><strong>2014</strong></td>
</tr>
<tr>
<td>Vitexin</td>
</tr>
<tr>
<td>CF</td>
</tr>
<tr>
<td>IF</td>
</tr>
<tr>
<td>BL</td>
</tr>
<tr>
<td>Isovitexin</td>
</tr>
<tr>
<td>CF</td>
</tr>
<tr>
<td>IF</td>
</tr>
<tr>
<td>BL</td>
</tr>
<tr>
<td>Orientin</td>
</tr>
<tr>
<td>CF</td>
</tr>
<tr>
<td>IF</td>
</tr>
<tr>
<td>BL</td>
</tr>
<tr>
<td>Isoorientin</td>
</tr>
<tr>
<td>CF</td>
</tr>
<tr>
<td>IF</td>
</tr>
<tr>
<td>BL</td>
</tr>
</tbody>
</table>

CF, IF, BL, Sole F, F:B = 2:1, F:B = 1:1, and F:B = 1:2 are chemical fertilizer, integrated fertilizer, broiler litter, sole fenugreek, two rows of fenugreek + one row of buckwheat, one row of fenugreek + one row of buckwheat, and one row of fenugreek + two rows of buckwheat, respectively. Different letters indicate significant differences at p < 0.05 by LSD test.
The seeds harvested from Sole F fertilized with BL and IF had higher vitexin contents of 9.82 (2014) and 11.8 (2015) mg/g DW, respectively (Table 2). The fenugreek seeds harvested in the IF and BL fertilized intercrops had greater vitexin contents by, on average, 30.5% (2014) and 31.5% (2015) compared to the CF (Table 2).

The isovitexin content of fenugreek seeds varied from 0.29 to 0.47 mg/g DW (2014) and from 0.33 to 0.50 mg/g DW (2015) (Table 2). In the intercropped treatments, the isovitexin content of seeds was, on average, 14.9% (2014) and 9.88% (2015) more than in Sole F (Table 2). In the seeds harvested from intercropped systems, the highest isovitexin content was obtained in the F:B = 2:1 with, on average, 0.40 (2014) and 0.41 (2015) mg/g DW followed by the F:B = 1:1 with, on average, 0.39 mg/g DW (in both years). Sole F had significantly higher isovitexin contents when BL and IF were applied. The effect was more pronounced with the application of IF with values of 0.37 (2014) and 0.40 (2015) mg/g DW (Table 2). The IF and BL fertilized intercrops had higher isovitexin contents, on average, by 15.1% (2014) and 24.3% (2015) compared to the CF, and the effect was more pronounced with BL (Table 2).

These findings confirm that the fertilizer type can influence the phytochemical status of seeds and subsequently of foods [47]. These results support our previous findings [15] in which buckwheat seeds harvested from the IF and BL treated fenugreek–buckwheat intercrop had higher vitexin and isovitexin contents than with chemical amendments. Ibrahim et al. [25] also reported that organic fertilizers have positive effects on the enhancement of secondary metabolites.

The accumulation of macronutrients (such as nitrogen phosphorous, sulfur, and potassium) is increased by nutrient complementarity in intercrop systems and application of organic fertilizer [38]. These nutrients are readily available to the crops and that are then utilized in secondary metabolite synthesis, and subsequently increase the concentration of main flavonoids [52].

### 3.5.2. Orientin and Isoorientin

The contents of orientin and isoorientin in fenugreek seeds were significantly positively affected by the cropping system and fertilizer interaction in both years (Tables 1 and 2). The orientin levels in seed samples ranged from 0.33 to 0.56 mg/g DW (2014) and from 0.34 to 0.56 mg/g DW (2015). Overall, the seeds harvested from the intercropped treatments had a higher orientin content by, on average, 23.1% (2014) and 15.5% (2015) compared to Sole F (Table 2). The highest orientin content in both years was found with BL application and the highest share of fenugreek (F:B = 2:1) of 0.56 mg/g DW, while the lowest orientin content was obtained with CF in Sole F and F:B = 1:1; 1:2 intercropped treatments. This result could be attributed to the positive N and P supply in intercropped systems with organic fertilizers. These systems have been reported to play an important role in the biosynthesis of plant metabolic processes [15,38].

The isoorientin content of the harvested fenugreek seeds varied from 0.70 to 1.20 mg/g DW (2014) and from 0.69 to 1.30 mg/g DW (2015) (Table 2). The highest isoorientin content was measured in the F:B = 2:1 with BL, with levels of 1.20 (2014) and 1.30 (2015) mg/g DW. The seeds harvested from Sole F with IF and BL had higher isoorientin contents by, on average, 18.4% (2014) and 20.3% (2015) than with CF. The seeds harvested from intercropped systems with IF and BL had higher isoorientin contents by, on average, 16.3% (2014) and 17.2% (2015) than with CF (Table 2).

We previously demonstrated in harvested buckwheat seeds that the highest amount of orientin and isoorientin content in an intercropped fenugreek–buckwheat system was related to a high share of the legume and organic fertilizer [15]. Applications of even partial amounts of organic fertilizer contribute to a positive soil microbe population, leading to a higher interaction between crop roots and the microbes, which leads to the synthesis of chemical compounds including flavonoids [52].

### 3.6. Correlations among Antioxidant Activity, Total Phenolic, Total Flavonoids, and Specific Flavonoid Compounds Content

The correlations among antioxidant activity (estimated by DPPH and FRAP), TPC, TFC, and specific flavonoid compounds content were tested by means of Pearson correlation coefficient...
In 2014 and 2015, significant positive correlations were determined between the DPPH assay with total phenolics and total flavonoids. The antioxidant activity estimated by FRAP significantly and positively correlated with total phenolics and total flavonoids. The antioxidant activity estimated by both DPPH and FRAP assay were significantly and positively correlated with vitexin, isovitexin, orientin, and isoorientin.

Similarly, Salehi et al. [15] also found significant and positive correlations of antioxidant activity (both DPPH and FRAP assay) with total phenolic, total flavonoids, and flavonoid compounds (vitexin, isovitexin, orientin, and isoorientin) in seeds of common buckwheat intercropped with fenugreek that was fertilized with organic amendments. They concluded that a high antioxidant activity is caused by a high phenolic content.

Table 3. Pearson’s correlation coefficients ($r$) among antioxidant activity (estimated by DPPH and FRAP) and total phenolic content, total flavonoids content, and flavonoid compounds in fenugreek seeds in 2014 and 2015 ($n = 36$).

<table>
<thead>
<tr>
<th>Antioxidant Activity</th>
<th>DPPH 2014</th>
<th>DPPH 2015</th>
<th>FRAP 2014</th>
<th>FRAP 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolic content (TPC)</td>
<td>0.44 **</td>
<td>0.74 ***</td>
<td>0.72 ***</td>
<td>0.60 ***</td>
</tr>
<tr>
<td>Total flavonoids content (TFC)</td>
<td>0.78 ***</td>
<td>0.82 ***</td>
<td>0.81 ***</td>
<td>0.59 **</td>
</tr>
<tr>
<td>Flavonoid compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitexin</td>
<td>0.72 ***</td>
<td>0.77 ***</td>
<td>0.70 ***</td>
<td>0.57 ***</td>
</tr>
<tr>
<td>Isovitexin</td>
<td>0.70 ***</td>
<td>0.70 ***</td>
<td>0.65 ***</td>
<td>0.47 **</td>
</tr>
<tr>
<td>Orientin</td>
<td>0.80 ***</td>
<td>0.79 ***</td>
<td>0.80 ***</td>
<td>0.48 **</td>
</tr>
<tr>
<td>Isoorientin</td>
<td>0.75 ***</td>
<td>0.65 ***</td>
<td>0.69 ***</td>
<td>0.46 **</td>
</tr>
</tbody>
</table>

** and *** Significant effect at $p < 0.01$ and $0.001$.  

4. Conclusions

The findings of the present study demonstrate that the bioactive compounds of harvested fenugreek seeds and their antioxidant activity can be significantly affected by agronomic and environmental conditions, including the cropping system and the management type including the fertilizer type applied. The interaction of intercropping (2:1 for fenugreek:buckwheat) and the addition of an organic fertilizer amendment was found to be the treatment with the most significant positive effects on the levels of bioactive compounds analyzed.

The use of organic fertilizer either as broiler litter alone or in combination with chemical fertilizer (as an integrated fertilizer) significantly promoted the trigonelline content (TC), antioxidant activity estimated by DPPH and FRAP assay, total phenolic content, total flavonoids content, and flavonoid compounds content (vitexin, isovitexin, orientin, and isoorientin) of harvested fenugreek seeds compared to the chemical fertilizer treatment. The highest levels of TC, antioxidant activity (DPPH and FRAP) and all of the bioactive compounds tested in this study were measured in the intercrop system F:B = 2:1 fertilized with either broiler litter alone or with 50% broiler litter and 50% mineral fertilizer.

These findings are of great interest for the pharmaceutical industry and for fenugreek producers, especially in semiarid areas. Fenugreek grown in intercropped systems with buckwheat using broiler litter or integrated fertilizer can produce seeds with significantly higher contents of bioactive compounds, than under conventional practices, making the seeds a more valuable source of functional and medicinal food products with great potential advantages for health and nutrition. Intercropping systems, such as those outlined in this study, are already widespread throughout the world and can easily be adopted by farmers for the production of medicinal plants, which subsequently can contain higher amounts of bioactive compounds in harvested plants.
Author Contributions: A.S. and S.F. conceived of the presented idea, designed the methodological framework, advised and contributed to the entire strategic and conceptual framework of the study. A.A.S. supported the field experiment in Iran. K.Z.-E. provided laboratory equipment and materials in Austria. K.Z.-E., and H.-P.-K., supervised the lab experiment and verified the analytical methods. A.S. analyzed the results and contributed to the final manuscript and contributed to the scientific rigor. B.M. also assisted with the overall scientific communication.

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Conflicts of Interest: The authors declare no conflict of interest.

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