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Article

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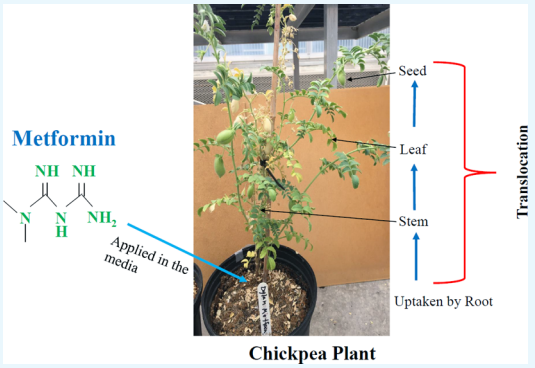
ABSTRACT: Multiple chronic conditions (MCCs) such as diabetes, hypertension, heart disease, arthritis, asthma, and common respiratory problems are prevalent in over one-fourth of Americans, and separate drugs are prescribed to manage each of the diseases. The nutritive crop seeds loaded with multiple drugs could be a cheap and sustainable alternative to drugs produced by pharmaceutical companies. Our long-term goal is to produce chickpea seeds containing comparable dosages of multiple drugs regularly prescribed for managing MCC. In this work, we conducted experiments to understand the uptake and translocation of metformin into the tissues of chickpea to demonstrate the applicability of LC−HR-ToF-MS in determining metformin concentration, and to investigate responses of increased dosage of metformin and it's accumulation into the chickpea seed. We treated the chickpea plants with 100 and 500 mg/L metformin chloride and analyzed its concentration in the leaf, stem, and seeds. We observed that metformin was successfully uptaken by chickpeas plant and translocated to stem, leaf, and seeds in both treatments. We also observed that the metformin concentration is responsive and as high as 349 times increase in seed when the dosage was increased from 100 to 500 mg/L.



INTRODUCTION

Over one-fourth of Americans suffer from multiple chronic conditions (MCCs), and whenever a person suffers from one chronic condition, it makes him or her vulnerable to another condition. According to the United States Center for Disease Control and Prevention, 75% of the health care budget is spent on managing chronic conditions.[1] A person with diabetes may also suffer from high blood pressure, which is linked to higher cholesterol levels[2] or any other chronic condition. One or more of the five chronic diseases, such as heart disease, cancer, stroke, chronic obstructive pulmonary disease, and diabetes cause more than two-thirds of the deaths in the US (United States Department of Health and Human Services, 2010). According to the CDC, over 100 million US adults are living either with diabetes or prediabetes (Center for disease control and prevention, 2017). The health care costs are increasing every year and the prices of the drugs by the pharmaceutical companies are unrealistically high. These are two major health care problems faced by us. The rising cost of medicine is not only true for new medicines but also for medicines which have been in the market for a long time. In 50 years, from 1965 to 2013, the monthly cost for cancer drug therapy has increased from $100 to $10 000,[3] which is also true for drugs that treat other diseases. In the United States, the price of medicine in the pharmacy, in many cases, is as high as 3000 times more than the manufacturing cost.[4] The success of disease control programs depends on the consistent use of prescribed medicines and adherence to the suggested therapy, which is especially important for the management of chronic diseases.[5] Over 75% of the adult nonadherent population are either not filling the medicines or taking lower dosages than the prescribed dosage.[6] The ever-increasing problem in health care management should be addressed in many different ways, starting from lifestyle, healthy diet, drug manufacturing procedure, and pricing, and the use of alternative, inexpensive, and sustainable sources of multiple drugs. A combination of inexpensive, healthy, and renewable carrier of more than one drug could be an effective and potential source of drugs for managing MCCs.

There have been plant improvement programs on the enhancement of health-related micronutrients, and researchers of these programs predicted that crop plants could be used as production factories (biofactories) of therapeutic and industrial products.[7] However, there is no such research on depositing active ingredients of medicine or other pharmaceuticals in the seed of crop plants, which could be used as

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alternative carriers of medicines. Also, there is often no reliable method for determining drugs in the plant tissues except the studies on the uptake and translocation of metformin and other pharmaceuticals in several crop species, including forages.[8,9] An antidiabetic II medicine, Metformin, is one of the most prescribed pharmaceuticals in many countries.[10] It is highly water-soluble and because of its ionic form, it is less likely to evaporate and thus uptake by the root, and translocation to the shoot is the dominating pathway for the movement of this compound in the plant tissue.[9] There are very few reports on uptake and translocation of some pharmaceuticals in crops, especially in the *Brassica* family.[11,12] However, all of these studies were focused mainly on the residual pharmaceuticals after human usages, which came to the agricultural soil and water body through human excretes and uptaken by different plant species.

Like micronutrients, the contents of any other chemicals in plant products depend on their availability in the soil environment, acquisition by plant roots, and efficiency of transportation and storage mechanisms. The physiological basis of absorption and accumulation of chemicals in the plant seeds are not well understood.[13,14] There are many genes and regularity proteins such as transcription factors, protein kinase, and receptors that were determined to be involved in directing and controlling this network.[15] The uptake, translocation, and redistribution of chemicals also depends on the nature of the chemicals and their competition with others.[16] There are minimal concentrations of active ingredients of drugs present in the soil environment, which may or may not uptake and translocate into the plant seeds, thus the treatment of plants with active ingredients is only a viable option to concentrate into the seed, especially in functional food crops.

Functional foods are food crops that provide health benefits in addition to traditional nutrients. Functional foods are considered nutraceutical when used to prevent and treat diseases.[17] Thus, a functional food enriched with prescribed dosages of multiple drugs may bring an effective breakthrough in health care management and budgeting. The functional food components of some food crops are of increasing interest in the prevention and treatment of cancer, diabetes, cardiovascular disease, and hypertension, which are the four leading causes of death in the United States.[7] Among the food crops, legumes are gaining increasing interest because of their nutritional value and health benefits. Legumes are rich in protein, carbohydrates, dietary fibers and are sources of other nutritional components.[18,19] Dietary intake of legumes, such as different types of beans and peas, may help protect against numerous chronic conditions, such as coronary heart diseases, diabetes, high blood pressure, and inflammation.[20] Chickpeas (*Cicer arietinum* L.) are one of the most important legumes; they are a good source of carbohydrates and protein and contain important vitamins. Moreover, they have several potential health benefits in controlling human diseases, including cardiovascular disease, type 2 diabetes, digestive diseases, and some cancers.[21] Thus, the chickpea seeds loaded with multiple drugs may accelerate the treatment and management of MCC because chickpea seeds can be eaten raw, and a single seed is present in each of the chickpea pods, which is important in maintaining dosage.

A number of studies have reported the method for determining metformin using liquid chromatography (LC), often with mass spectrometry (MS) following solid-phase extraction (SPE). The majority of methods focus on plasma and wastewater using tandem MS with characteristic precursor and product ions.[22,23] Recently, low limits of quantification were achieved with LC and high resolution (HR) MS.[24,25] However, use of a broad range of matrices prevents direct comparison among different methods of determining metformin concentration. To our knowledge, few studies reported the determination of metformin in plant tissues employing LC−MS/MS.[8,9]

Our long-term goal is to develop chickpea seeds containing multiple drugs, which could be an effective alternative of treating multiple chronic conditions. The objectives of the present study were to (1) demonstrate applicability of LC coupled with HR time of flight (ToF) MS (i.e., LC−HR-ToF-MS) for determining metformin concentration in the tissues of chickpeas, (2) understand the uptake and translocation of metformin into the tissues of chickpeas, and (3) investigate the effect of a higher dosage metformin treatment on the increase of metformin deposition in the seeds.

MATERIALS AND METHODS

Plant Materials and Treatments. Three replications of three chickpea genotypes, Sawyer, Dylan, and Sierra were grown with control for metformin treatment at Mayville State University. Three seeds of each genotype were planted in 8.5″ × 11″ pots filled with Sunshine Mix, a professionally formulated growing mix containing complete fertilizer and trace elements at a normal rate. The sunshine mix was soaked with water until germination. Once germinated, a clear saucer beneath the treated plants was placed and filled with solutions (100 mg/L) of metformin chloride (1,1-dimethylbiguanide hydrochloride- $C_4H_{11}N_5$·HCl) until harvesting, while the control plants received only water. Two of the genotypes, Sawyer and Sierra were treated with 500 mg/L metformin chloride in similar ways, but only seed metformin chloride concentration was analyzed. Metformin chloride was purchased as a powder from Accela Bio-Pharmaceutical (http://www.accelachem.com/) and dissolved in water in required concentrations. At 50% leaf senescence, leaf and stem from treated and control plants, and after harvesting, seed samples were lyophilized and ground using mortar and pestle, and ∼100 mg of the ground samples were purified and analyzed for metformin contents (μg/g) using the protocol described below.

The experiment for this study was conducted in the greenhouse following a randomized complete block design with three replications. Statistical analysis was conducted in SAS 9.4 following PROC GLM procedure.

Standards and Reagents. Standards of metformin hydrochloride and ammonium acetate (≥99%) were purchased from Sigma-Aldrich (St. Louis, Mo). Formic acid (puriss p.a., eluent additive for LC−MS) and high-performance liquid chromatography (HPLC)-MS grade methanol were obtained from Fluka (Steinheim, Germany) and Fisher Scientific (Fair Lawn, NJ, respectively). All chemicals were used as received without further purification. Deionized water was obtained using a Direct-Q 3 system, Millipore, Billerica, MA. For calibration, metformin standard was diluted using 2-fold serial dilution in a range between 0.08 and 500 ng/mL.

Sample Preparation. The sample preparation procedure was adapted from Hormazábal and Østensvik.[26] Briefly, 1.9 mL of an aqueous solution containing ammonium acetate and formic acid at a concentration of 450 and 50 mmol·L^{-1}, respectively, was added to a lyophilized plant matrix sample

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(0.10 g), followed by addition of dichloromethane (2 mL). The mixture was vortexed for 15 s, sonicated for 5 min, vortexed again for 3 s, and centrifuged for 5 min at 3600 rpm. A water-based supernatant was purified using SPE Bond Elut-LMS cartridges (Agilent Technologies, Santa Clara, CA) with a volume of 1 mL and a capacity of 25 mg. Each SPE cartridge was conditioned with 1 mL of methanol, followed by 2 mL of water and brought to dryness. Then, a 0.35 mL aliquot of the water-based supernatant was loaded on a cartridge, passed through (1 mL/min) and collected. An additional 0.35 mL of water was used, twice, to elute the remaining analyte, and the collected eluate was combined (total volume of ∼1.05 mL). The recovery of the method was validated using metformin-spiked sample (corresponding to concertation of 100 ng/mL), spiked onto controls showing recoveries of 97.1 ± 06%. The purified samples were diluted to fit linear calibration range (e.g., seed treated with 500 mg were diluted to 5 μL in the total volume of 10.000 mL); the control seeds (untreated) were not diluted.

Analysis. Reverse-phase HPLC was performed using an Agilent 1100 Series HPLC system equipped with a Restek Allure PFP Propyl column (length: 100 mm; internal diameter: 3.2 mm; particle size: 5 μm) with a guard column Ultra PFP (20 mm × 2.1 mm). Agilent 6210 electrospray ionization (ESI) HR-ToF-MS system was utilized for detection. The column was thermostated at 25 °C. The mobile phase consisted of 26.5 mmol/L formic acid in water with 1.5% methanol (solvent A), and 26.5 mmol/L formic acid in methanol (solvent B). The gradient program used for analysis started with isocratic elution at 0% B for 2 min, followed by a linear gradient to 75% B from 2 to 2.1 min with a hold until 4 min and then a linear gradient to 90% B from 4 to 5 min and hold for 1 min. The last step was 6 to 7 min to 0% B followed by a 5 min hold. The flow rate was set to 0.5 mL/min. The injection volume was 60 μL.

For HR-ToF-MS analysis, an ESI source was operated in the positive mode at 350 °C with the optimized settings: electrospray (e.g., capillary) and collision-induced dissociation (e.g., fragmentor) potentials of 5000 and 125 V, respectively; drying gas (nitrogen) 12 L/min; nebulizer gas (nitrogen) 25 psi.

Mass Hunter package B.02.00 software was used for LC−HR-ToF-MS data processing. For metformin determination, an external standard calibration was performed using metformin protonated molecular ion of 130.11 ± 0.03 m/z, and qualifier ions of 131.11 (6.6 ± 0.6% of the abundance of 130.11) and 71.06 (0.7 ± 0.1%) m/z. As shown in Figures 1 and 2 qualifier ions were less abundant, yet their consistent ratio to quantifier/qualifier ions/ was used as criteria allowing for quantification. Instrumental limits of detection (LODs) and lower limits of quantitation (LLOQs) were calculated from calibration curves of metformin using the equations LOD = 3.3 × s_y/k and LOQ = 5 × s_y/k, where k is the slope of the calibration curve and s_y is the standard error of the predicted y-value for each x-value. These values were obtained using a linear least-squares regression within 1 order of magnitude of LLOQ.

RESULTS AND DISCUSSION

Quantification Using LC−HR-ToF-MS Method. The LC−HR-ToF-MS quantification in chickpea plant matrices was based on a protonated molecular ion of metformin extracted within the narrow mass range of ± 0.03 m/z to increase the selectivity of the method (Figure 1). This mass extraction window allowed for achieving the best signal-to-noise ratio without compromising the abundance, wherein a total ion current data (no ion extraction) metformin could not be easily discerned even at a concentration of 6 ng/mL (Supporting Information Figure S1). The instrumental LOD and LLOQ were 6.1 and 11 pg injected on the column. This was sufficient to directly analyze untreated seeds as shown in Figure 2 with final concentration as low as 70 ng/g. The lowest concentration found in untreated seeds was above LLOQ of 30 ng/g previously determined in plant materials with LC−MS/MS with sufficient signal-to-noise ratio.[9,26] For treated samples, the abundance of metformin was so high that significant dilution (1000-fold) for samples treated with metformin was essential (Figure 2c−f). The need for dilution was further confirmed by a linear range limited up to 100 ng/mL (i.e., 6 ng injected) from LLQ. Yet, even with sufficient sensitivity, as shown for comparison of total ion current (TIC) to extracted ion chromatograms (EICs), the selectivity of MS analysis is essential (Figure 2). At higher concentrations, dynamic regression between concentrations was observed but deemed unsuitable for quantification (Supporting Information Figure S2). The repeatability of the analysis, sample preparation, and seed variability are supported by RSD of 1, 5, and 18%, respectively, showing the higher variance for different seed batches. The interday repeatability of the same sample batch corresponded to 7% RSD (Table 1).

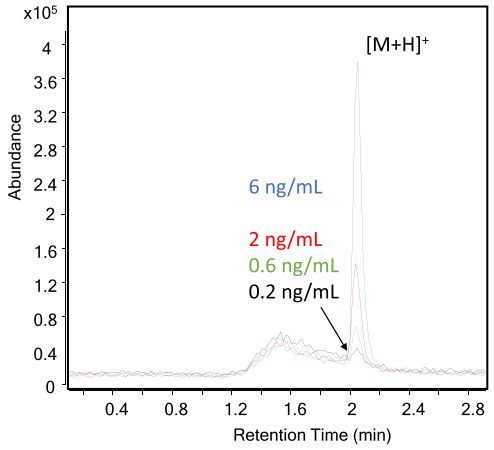

Figure 1. LC−HR-ToF-MS extracted ion chromatogram (EIC) of ion 130.11 m/z for metformin standards showing its protonated ion with 60 μL injected.

Uptake and Translocation of Metformin in Chickpea Tissues. The mean concentrations of metformin in all tissues resulted from various treatments are presented in Table 2. As evident from Table 2, metformin concentration was observed in the stems of Dylan (15.502 μg/g), Sawyer (44.420 μg/g), and in the stem of Sierra (and 30.086 μg/g) compared to the respective controls (untreated) while the chickpeas were treated with 100 mg/L metformin chloride (Figure 3a). In the leaf of the genotype Dylan, 1.486 μg/gm metformin concentration was observed, in the leaf of the genotype Sawyer, 1.339 μg/g metformin concentration was found whereas in the leaf of Sierra metformin concentration of 0.765 μg/g was observed compared to the untreated leaf (Figure 3b). The highest seed metformin concentration was observed in the genotype Dylan (5.865 μg/g) followed by Sawyer (5.786 μg/

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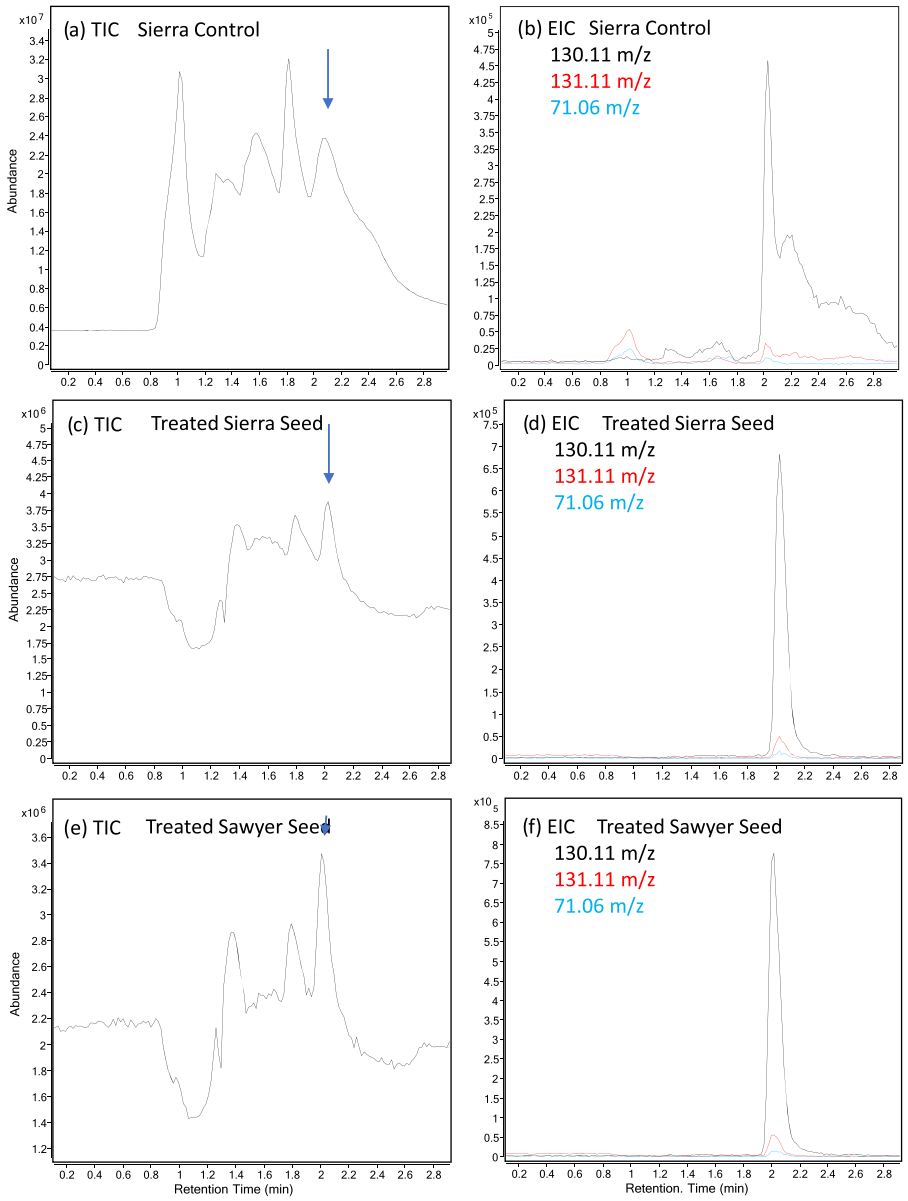

Figure 2. LC−HR-ToF-MS of metformin in chickpea seeds (a) TIC of Sierra control seed (b) EICs of Sierra control seed (c) TIC of treated Sierra seed (d) EIC of treated Sierra seed m/z (e) TIC of treated Sawyer seed (f) EIC of treated Sawyer seed. The EICs were obtained for ions of 130.11, 131.11, and 71.06 ± 0.03 m/z.

Table 1. Concentration of Metformin in Treated Plants with 500 mg/L[a]

intraday		mean (μg/g)	SD	RSD (%)
LC injection		1535	20	1.3
sample prep. Sierra	batch 1	1582	75	4.8
	batch 2	1061	53	5.0
	batch 3	1137	44	3.9
variation between Sierra batches		1359	246	18
sample prep. Sawyer		1513	54	4
interday		mean (μg/g)	SD	RSD (%)
sample prep. Sierra	batch 1	1586	112	7.1

[a]Intrada and interday repeatability of LC injection, sample preparation, and between batches.

g) and Sierra (4.133 μg/g), respectively, when the plants were treated with 100 mg/L metformin (Figure 3c).

Table 2. Mean Concentrations (μg/g) of Metformin in Different Tissues of Chickpea in Different Treatment Conditions (T1-Treated 100 mg/L), (T2- Treated 500 mg/L)

treatment	genotype	stem	leaf	seed
T1	Dylan	15.37	1.38	5.80
T1	Sawyer	44.20	1.12	5.56
T1	Sierra	27.64	0.46	3.78
T2	Sawyer			1587.41
T2	Sierra			1318.70

To analyze the significance of differences of mean values of metformin contents in different chickpea tissues resulted from 100 mg/L treatments (T1), one-way analysis of variance (ANOVA) was performed, and F statistics are presented from the analysis of variance (Table 3). Genotypes showed significant variation in stem translocation. However, leaf and

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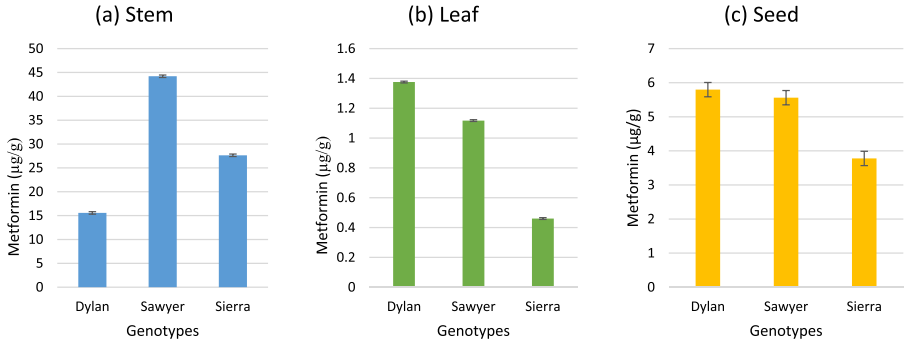

Figure 3. Uptake and translocation of Metformin in different tissues of chickpea when treated with 100 mg/L of metformin. (a) Uptake and translocation in the stem of different genotypes of chickpea. (b) Uptake and translocation in the leaf of different genotypes of chickpea. (c) Uptake and translocation in the seed of different genotypes of chickpea.

Table 3. Analysis of Variance Showing Treatment Effect (100 mg/L) on Stem, Leaf, and Seed across Three Genotypes[a]

		F value		
sources of variance	DF	stem	leaf	seed
rep	2	2.3	0.19	1.86
genotype	2	99.45***	2.59	3.19

***= $p < 0.001$

[a]*** Significantly different at $P \leq 0.001$.

seed translocation were not significant for genotypes. This suggests that genotypes may not differ when the metformin translocated towards the sink tissue. There was no significant difference in metformin content between treated stem, leaf, and seeds observed among the replications.

Effect of a Higher Dosage of Metformin on Its Deposition in Seeds. To understand the response of higher dosage metformin chloride on the seed metformin concentration, seeds of two chickpea genotypes were treated with 500 mg/L metformin chloride. In the seed of the genotype Sawyer, the metformin concentration was increased to 1587.41 μg/g that is 274 times higher than the metformin concentration (5.786 μg/g) when the seeds were treated with 100 mg/L of metformin chloride (Table 2). In the genotype Sierra, the concentration was increased to 1318.70 μg/gm, which is 349 times increase of the metformin content when the seed was treated with 100 mg/L metformin chloride (Table 1 and Figure 4).

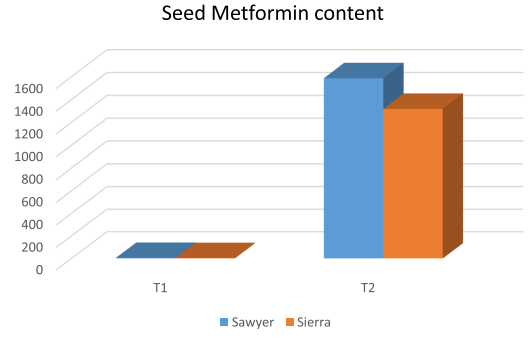

Figure 4. Seed metformin concentrations of chickpea seeds under different treatment T1- 100 mg/L and T2- 500 mg/L metformin chloride treatment.

Based on the two-way ANOVA, the F ratio is presented in Table 4. The seed metformin concentrations resulted from 100 to 500 mg/L metformin chloride dosages varied significantly (Table 4). The analysis also showed that there was no significant influence of genotype or replication on seed metformin concentration while seeds were treated with different dosages of metformin chloride. This supports our earlier conclusion that metformin translocation towards the sink tissue may not be genotype-dependent.

Table 4. F Ratio of Metformin Uptake by Seeds of Two Chickpea Varieties under Two Different Treatments (100 and 500 mg/L)[a]

		F value
sources of variance	DF	seed
treatment	1	350.94***
rep (treatment)	6	1.84
genotype	1	3.06
treatment × genotype	2	2.98

***= $p < 0.001$

[a]*** Significantly different at $P \leq 0.001$.

Our ultimate goal is to concentrate nutritional crop plant seeds with different drugs, which are usually prescribed for maintaining MCC. We believe that if nutritious crop plant seeds are concentrated with comparable dosages of drugs, they could be an important alternative in human health management and will help to reduce the dependency on pharmaceutical companies. However, there is no work conducted with this goal; only there have been reports on uptake and translocation of metformin into oilseed, cereal, and vegetables, which were grown on agricultural soil contaminated with residual metformin resulted from human excretes into the soil and water bodies.[9] There was a study on the uptake and translocation of the antibiotic agent ciprofloxacin and the anticoccidial narasin along with antidiabetic compound metformin.[8] They selected these compounds based on their presence in environmental matrixes and to cover various chemical properties. In their study, they found a higher root concentration of all of these chemicals compared to their corresponding leaf concentration. They also found higher uptake of metformin compared to others in all plant tissues they analyzed. In our study, we studied the uptake and translocation of metformin in chickpea and found a significantly higher concentration of metformin in the stem than the leaf and seed, and the leaf concentration was lower

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than that of seed (Table 2); this trend was also observed among the genotypes. In our study, we did not quantify root metformin concentration; however, in the immediate tissue (stem), we observed a significantly higher concentration of metformin compared to the control as well as other tissues. In the studies,[8,9] they used contaminated agricultural soil and conducted their experiments in the greenhouse. In their studies, they used different plant species and pharmaceuticals including metformin. In all tissues including root, they found higher uptake and accumulation of metformin; however, their seed metformin concentrations varied from species to species and the location of the fruits in tomato.[9] We observed a higher concentration of metformin in the seed compared to the leaf, which is consistent with previous findings;[8] however, the metformin concentrations in the stem varied from genotype to genotype while treated with 100 mg/L metformin chloride.

We observed 286−349 times increase of seed metformin concentration when the treatment dosage was increased from 100 to 500 mg/L in the genotypes Sawyer and Sierra, respectively. The uptake and translocation of nutrients and other solutes from the soil to storage tissues depend on the transpiration rate, which is increased due to higher growth temperature.[9] In our work, we maintained a greenhouse temperature range of 60−70 °C throughout the growing period with a continuous supply of water mixed with metformin chloride, which may have resulted in a higher concentration of metformin in the seed. Plants uptake nutrients and other solutes from soil environment through the root and translocate through xylem vessel and at leaf senescence, the nutrients and solutes remobilize to the storage tissue (seed).[27] In our work, we also found a higher concentration of metformin in the seed compared to the leaf. Higher allocation nitrogen from vegetative tissue to the seed has been reported in some plant species,[28] and the mimicking effect of metformin with nitrogen was reported earlier.[9] Thus in this study, metformin may have induced the nitrogen transporters, which resulted in higher translocation of metformin in the storage tissue. Like other biofactory-based pharmaceutical production, the concentration of the active ingredient of metformin in chickpea is a major hurdle towards achieving the goals. With five times increase in treatment dosage, we observed over 300 times increase in seed metformin concentration. However, to achieve a comparable prescribed dosage of metformin, we need a further increase in seed metformin concentration. To achieve this goal, we are growing chickpeas in the aquaponic method using plastic tubs. Our work clearly demonstrated that chickpeas could be used as an alternative carrier of metformin for millions of people who are suffering from MCC-related diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02783.

LC−HR-ToF-MS TIC of metformin standards with 60 μL injection volume; LC−HR-ToF-MS analysis of metformin standards in dynamic and linear range with 60 μL injection volume (PDF)

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Notes

The authors declare no competing financial interest.

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