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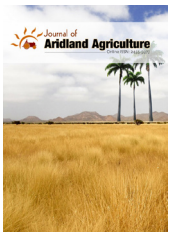

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Molecular characterization of diosgenin biosynthesis in different organs of *Trigonella foenum-graecum* L.

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ABSTRACT

Fenugreek, an annual herb, is a significant source of diosgenin, a triterpene with bioactive properties. This study aimed to molecularly characterize diosgenin biosynthesis in various organs of fenugreek, including flowers, immature leaves, developing leaves, mature leaves, stems, roots, and pods. The biosynthetic pathway involves vital enzymes such as squalene synthase, squalene monooxygenase, cycloartenol synthase, and Sterol-3-ß-glucosyl transferase. The study found that flowers and roots exhibited the highest gene expression levels, indicating their potential significance in diosgenin biosynthesis. Diosgenin content was quantified using high-performance liquid chromatography, with seeds being the primary source. The findings suggest that fenugreek offers a cost-effective and rapid alternative to yam for diosgenin production, and the differential accumulation of diosgenin in different plant organs underscores the importance of understanding organ-specific biosynthesis. This comprehensive analysis provides valuable insights into fenugreek's potential as a bioresource for diosgenin and its diverse applications in medicine and agriculture.

KEYWORDS: *Trigonella foenum*, Bioactive properties, Mature leaves, Diosgenin, Potential, Biosynthesis, Squalene

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INTRODUCTION

The annual herb fenugreek, or *Trigonella foenum-graecum* L. (Fabaceae), is widely grown from Iran to Northern India and also in Egypt, Southern Europe, China, and other places (Snehlata & Payal, 2012). Fenugreek leaves and seeds are significant reservoirs of steroidal sapogenins, particularly diosgenin, used in medicine. These sapogenins have been widely produced in extracts and powders (Acharya *et al.*, 2006; Mehrafarin *et al.*, 2010). Moreover, they show various beneficial effects, such as their antidiabetic effects and their nutritional and therapeutic properties (Mozaffari *et al.*, 2010). Fenugreek comprises secondary metabolites, including steroidal saponins, flavonoids, phenolic compounds, and alkaloids, among other chemicals (Laila & Murtaza, 2015; Wani & Kumar, 2016). The important bioactive chemical molecule diosgenin is found in fenugreek. It is a saponin with a structure similar to cholesterol and other steroids (Rahmati-Yamchi *et al.*, 2014).

Diosgenin is classified as a triterpene and is a significant bioactive compound. A Phyto steroid sapogenin that is naturally made and can be found in yams, fenugreek, and *Costus speciosus* (Liu *et al.*, 2012; Kumar *et al.*, 2014). Terpenes are made from the isoprene precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). This can be done through either the 2-C-methyl-d-erythritol 4-phosphate (MEP) or the mevalonate (MVA) pathway. An enzyme called squalene synthase (SQS) helps two molecules of farnesyl pyrophosphate (FPP) make squalene, a C30 compound. Following this, a squalene epoxidase (SQE) helps this compound undergo epoxidation, which creates 2,3-oxidosqualene. The cycloartenol synthase (CAS) enzyme catalyzes the conversion of 2,3-oxidosqualene into cycloartenol. Cycloartenol is the initial compound for synthesizing many plant steroids, such as diosgenin (Thimmappa *et al.*, 2014).

Yam is an essential source of diosgenin, but its production is expensive and time-consuming. In contrast, fenugreek has a

quick growth cycle, cheaper production costs, and consistent yields (De & De, 2003; Wang *et al.*, 2015). In addition, fenugreek is a highly significant plant with medicinal and agricultural significance. Many studies have shown evidence of the antioxidant and anti-inflammatory properties of fenugreek seeds and the impact of seed extracts on the development of aflatoxigenic fungi (El-Desouky *et al.*, 2013; George *et al.*, 2016; Khlifi *et al.*, 2016).

Researchers have already found that diosgenin builds up in different callus culture stages that come from leaf, shoot, and root apical meristems. The duplicate determinations have been carried out at different stages of growth in different organs, such as leaves, stems, roots, and pods, as well as the nutrient content and growth rate of different organs treated with NaCl (Rezaeian, 2011; Tunçtürk *et al.*, 2011; Ciura *et al.*, 2015). However, most reports have focused on the impact of fenugreek seed extracts on human cells or health. However, there is no study of all the many organs, including seeds, flowers, stalks, leaves (both young and adult), roots, and pods. So, the different parts of the Fenugreek plant were used to study the accumulation of diosgenin and the expression of genes related to biosynthesis.

MATERIALS AND METHODS

Plant Materials

The seeds were sown in pots with specific dimensions (top diameter: 116 mm, height: 105 mm, bottom diameter: 85.5 mm) that were filled with vermiculite. These pots were placed in a growth chamber with controlled conditions, including a 16 hour photoperiod with a light intensity of 300 μmol $m^{-2}s^{-1}$ and a temperature of 25 °C. Each pot contained 20 seeds. Following 2-3 weeks of seed germination, the seedlings were transplanted to a field plot at the experimental farm of Chungnam National University in Daejeon, Korea. During the flower blossoming period, the plant's various components, including the flowers, stems, immature leaves, developing leaves, mature leaves, roots, and pods, were cut off. The samples were promptly cryopreserved in liquid nitrogen and kept at a temperature of -80 °C until they were used for analysis.

RNA Isolation and cDNA Synthesis

The materials were crushed in liquid nitrogen to get a fine powder, which was then transferred to a 1.5 mL microcentrifuge tube. Following the manufacturer's instructions, a Plant Total Mini Kit (Generaid, Taiwan) was used to isolate total RNA from the frozen tissues. Following the initial stage, cellular homogenization occurred by using Trizol. Subsequently, chloroform was used to facilitate phase separation, and then cold isopropanol was added for the precipitation of RNA. The measurements and purity of total RNA were assessed using a NanoVue Plus Spectrophotometer (GE Healthcare Bio-Science Crop, USA) at a wavelength ratio of 260:280 nm. The quality of the RNA was evaluated by running it on 1% formaldehyde RNA agarose gels. For synthesizing complementary DNA (cDNA), one μg of total RNA was used and subjected to reverse transcription using a ReverTra Ace-α kit (Toyobo, Japan). After that, an oligo primer was employed, and a 20 fold dilution of the resultant 20 μL cDNA products was utilized as the template for accurate real-time PCR analysis.

Real-time PCR

The qRT-PCR analysis was conducted using BIO-RAD CFX96 real-time PCR equipment manufactured by Bio-Rad Laboratories in the United States. Squalene synthase (SQS), squalene monooxygenase (SQLE), and cycloartenol synthase (CAS) primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). The Sterol-3-ß-glucosyl transferase (STRL) enzyme was purchased from Bioneer based on the transcriptome data of the GujaratMethi-1 variety reported by Chaudhary *et al.* (2015). The 18S gene was used as a reference gene for normalizing gene expression levels. The PCR products obtained from cDNA were purified using a Gel Extraction Kit (ELPIS Biotech, Korea) to quantify the standard. The concentration of the purified products was determined to estimate the number of cDNA copies. The SYBR Green quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiment was conducted using a total volume of 20 μL. The reaction mixture consisted of 10 μL of 2× Real-Time PCR Smart mix (Biofact Korea), 1 μL (10 pmol/μL) of each primer, 5 μL of template cDNA, and 3 μL of distilled water (D.W.). The conditions were as stated: The process involves a pre-denaturation step at 95 °C for 15 seconds, followed by a step at 95 °C for 20 seconds. This is followed by an annealing step at 55 °C for 40 seconds and, finally, 40 elongation cycles at 72 °C for 20 seconds each. The process was completed by extending the time at a temperature of 72 °C for 10 minutes. The trials were conducted thrice, and the findings were expressed as means ± standard deviation (S.D.).

High Performance Liquid Chromatography Analysis (HPLC)

All specimens were dried in a freeze-dryer at a temperature of 80 °C for about 48 hours. The dried samples (100 mg) were incubated with 5 mL of 20% H_2SO_4 in 70% isopropanol. This process was carried out in a water bath for 8 hours at a temperature of 80 °C. Subsequently, the samples were thoroughly mixed every 30 minutes while undergoing incubation. Following extraction, the samples were centrifugated at 12,000 revolutions per minute at a temperature of 4 °C for 10 minutes. After that, the supernatants were moved to fresh 15 mL conical tubes and supplemented with 5 mL of n-hexane. The mixture was then forcefully vortexed and agitated. After vortexing, the samples were centrifuged at 12,000 revolutions per minute, at a temperature of 4 °C, for 10 minutes. Only the remaining liquid portion was transferred to the fresh 15 mL conical tubes after sedimentation. The process was repeated thrice to get optimal extraction. Subsequently, the samples were dried by volatilizing the solvent at a temperature of 40 °C using a Rotovac evaporator. The dried raw extracts were dissolved in 1 mL of HPLC-grade acetonitrile.

After solubilization, the samples were filtered using 0.45 µm PTFE hydrophilic syringe filters (Advantec DISMIC-13HP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for subsequent HPLC analysis. Diosgenin was separated based on its retention time using an Agilent 1100HPLC system. The system was equipped with a C18 reverse-phase column (250 mm x 4.6 mm, five µm; RStech, Daejeon, Korea), and diosgenin was detected using a UV detector set at 203 nm. The solvent A used in the experiment was water, whereas solvent B was Acetonitrile. The retention time of diosgenin was 20 minutes. The original ratio of the mixture consisted of 10% solvent A and 90% solvent B. The solvent combination was consistently maintained at a 1.0 mL/min flow rate. We determined the presence of diosgenin by comparing its retention time and spectrum features to those obtained from a single HPLC analysis of a confirmed diosgenin standard.

RESULTS

Expression Analysis of Diosgenin Biosynthesis-related Genes

For gene expression analysis, the study involved sampling various parts of fenugreek plants, including flowers, immature leaves, developing leaves, mature leaves, stems, roots, and pods (Figure 1). The bar diagram represents the expression levels of genes (presumably) in different parts of a fenugreek plant, measured across multiple samples (Figure 2). Overall gene expression studied flowers have the highest gene expression, followed by roots, immature leaves, developing leaves, mature leaves, stems, and pods (Figure 2). *TfSQS* showed high expression in flowers (0.95) and roots (0.86), while immature, developing, mature, and stems showed almost similar expression levels. Pods consistently exhibit the lowest gene expression levels among all plant parts. The standard deviation measures the variability or spread of the data around the mean. Higher standard deviations indicate more significant variability in gene expression levels for a particular plant part. In the case of *SQLE*, all plant parts showed a shallow expression level except for flowers. Flowers have the highest average value (1), indicating they might be the most prevalent or have the highest measurement among the plant parts; mature leaves have the lowest average value (0.12), Stems and roots have relatively high average values (0.21, and 0.3, respectively), and pods have the lowest average value (0.1). Standard deviation indicates the spread or variability of the data. For instance, the standard deviation for flowers is approximately 0.05, for immature leaves is approximately 0.02, and so on. CAS showed a pattern of expression similar to *SQS*. Flowers have an average value of approximately 0.1, indicating a relatively high prevalence of measurement among the plant parts; developing leaves have the lowest average value (0.39), mature leaves have an average value of 0.5, whereas stem, and roots have average values of 0.24, and 0.6, respectively. Pods have a minimum average value of 0.18, and mature leaves have a relatively high standard deviation (0.09), indicating more significant variability in measurements for this part. On the other hand, the expression level of *STRL* in flowers and roots was almost the same and higher than in any other organs, which showed low levels of expression. After examining the overall gene expression pattern, it was determined that flowers had the most significant expression level for all genes analyzed, followed by roots. Moreover, no discernible gene expression disparity between juvenile and fully developed leaves was observed.

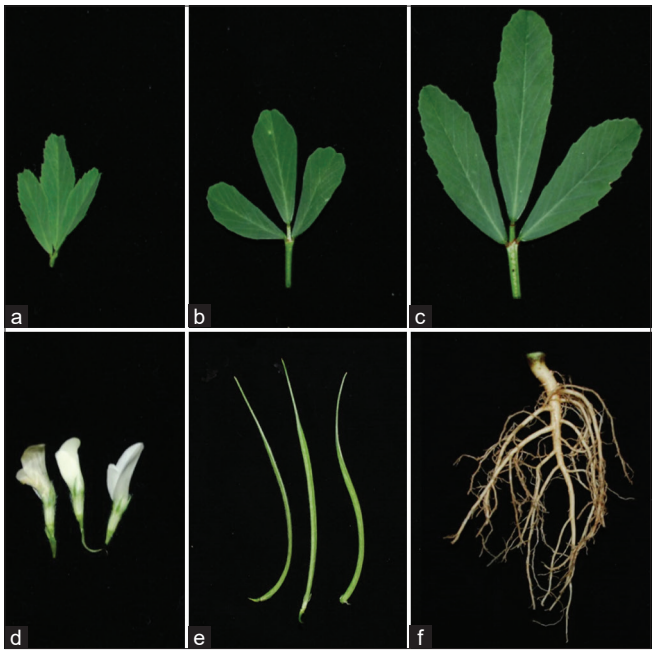

Figure 1: Different organs of *Trigonella foenum-graecum* L.: (a) immature leaves, (b) developing leaves, (c) mature leaves, (d) flowers, (e) pods, and (f) roots

Analysis of Diosgenin by HPLC

Seeds have the highest average Diosgenin content (approximately 2.6833 mg/g), indicating that they might be a significant source of Diosgenin. Flowers have an average Diosgenin content of approximately 0.7767 mg/g. Developing leaves have the highest average Diosgenin content among leaves (2.1267 mg/g). Stems and roots have lower average Diosgenin content (0.2533 mg/g and 0.4767 mg/g, respectively), and pods have an average Diosgenin content of approximately 0.7967 mg/g (Figure 3). Standard deviation values provide insights into the variability of Diosgenin content measurements for each part. For example, seeds have a relatively higher standard deviation (0.249 mg/g), indicating more variability in Diosgenin content among samples.

DISCUSSION

The fenugreek plant undergoes a life cycle that spans around 4-5 months, starting from germination and ending with the development of seeds. Therefore, the process of producing diosgenin may be completed in a faster timeframe compared to yam. Fenugreek is suitable for cultivation in a controlled environment such as a greenhouse. The expression and accumulation of genes linked to diosgenin production were investigated in several organs of fenugreek in this study. Trigonella species produce natural diosgenin found in the aerial parts and seeds. The quantity of diosgenin in these

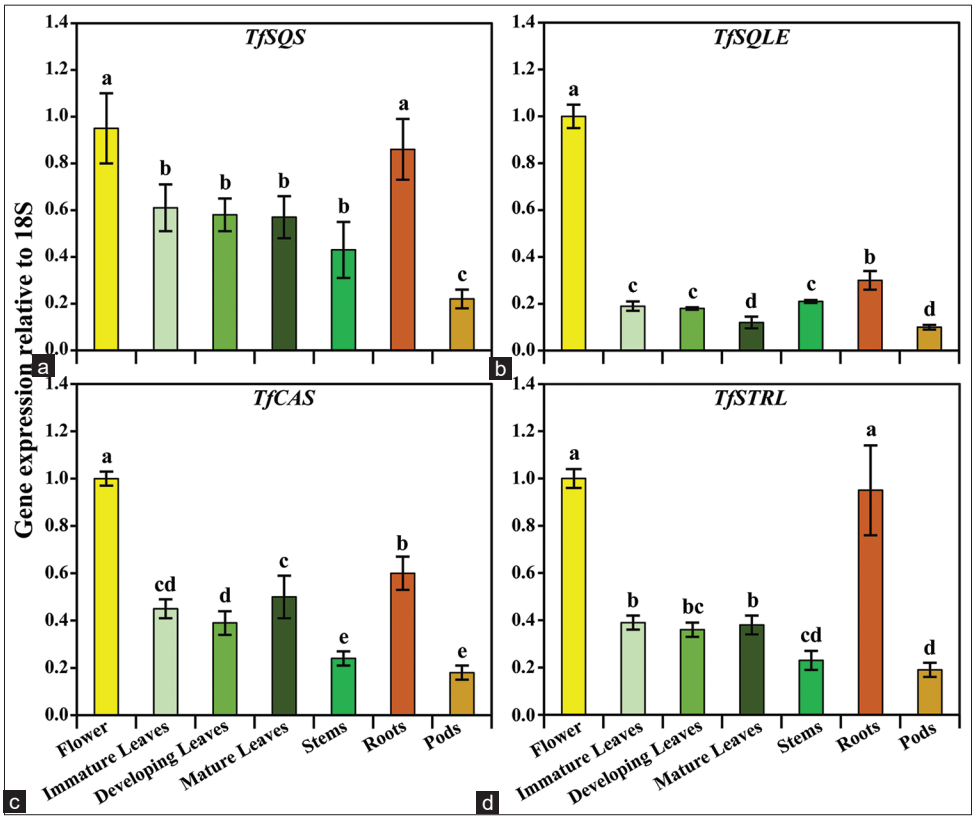

Figure 2: Expression (*n*=3) of diosgenin biosynthesis-related genes flowers, young leaves, mature leaves, stems, roots, and pods of 3-month-old fenugreek plants. Transcript levels from three biological replicates were analyzed relative to that of 18S. (a) *TfSQS*: *T. foenum-graecum* Squalene synthase, (b) *TfSQLE*: *T. foenum-graecum* Squalene monooxygenase, (c) *TfCAS T. foenum-graecum* Cycloartenol synthase and (d) *TfSTRL*: *T. foenum-graecum* Sterol-3-*β*-glucosyl transferase

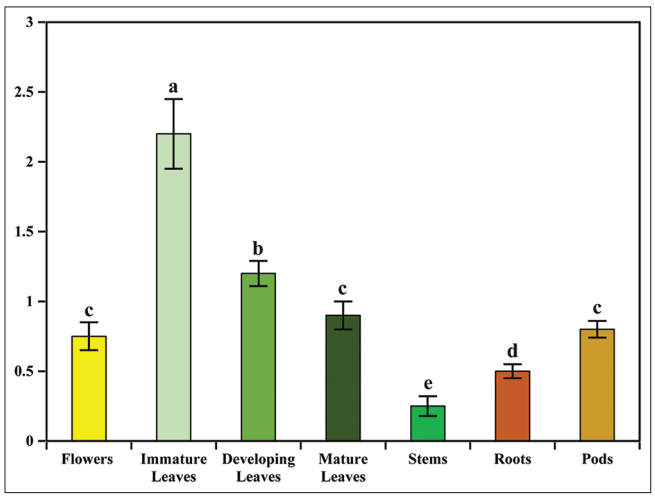

Figure 3: Diosgenin content in different organs of *T. foenum-graecum* L. (n=3). R^2=0.9992; *bars* labeled with different letters are significantly different from each other at $P \leq 0.05$ according to Tukey's Multiple Range Test

components ranges from 0.01% to 0.5% (Sharma *et al*., 2016). The various organs of the fenugreek plant exhibited varying levels of diosgenin accumulation, distinct from those observed in *Lycium chinense*. In *L. chinense*, the highest diosgenin content was found in green fruits, followed by stems and flowers.

On the other hand, in fenugreek, the seeds contained the highest concentration of diosgenin. The Diosgenin concentration in fenugreek flowers and stems was comparatively lower than in the corresponding organs of *L. chinense*. In contrast, the fenugreek plant exhibited more significant diosgenin levels in its leaves and roots than *L. chinense*. Earlier research has shown that the stems of fenugreek include two furastanol glycosides, which may serve as precursors for diosgenin production by opening the F-ring. On the other hand, fenugreek leaves contain seven saponins that are glycosides of diosgenin (Snehlata & Payal, 2012; Wani & Kumar, 2016). Legumes' seeds and leaves are recognized for their significant diosgenin accumulation (Mehrafarin *et al*., 2010). In addition, the study conducted by Rezaeian (2011) revealed that calluses produced from leaf segments, shoot apical meristem (SAM), and root apical meristem (RAM) had comparable findings in terms of diosgenin content. Specifically, the diosgenin level in the leaf callus was found to be the greatest, while SAM and RAM callus had lower diosgenin content. The outcome suggests that the plants synthesized diosgenin through the diosgenin biosynthetic route and exhibited distinct functionalities.

CONCLUSIONS

From this study, we found that flowers and roots showed the highest diosgenin gene expression levels, whereas the diosgenin

content was highest in immature leaves. From this result, we conclude that differential accumulation of diosgenin in different plant organs showed that they are organ-specific biosynthesis. Among the different organs, we found that immature leaves of *T. foenum-graecum* L. have a rich source of diosgenin content, which can be widely consumed by humans.

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