VALIDATION OF A METHOD FOR DIOSGENIN EXTRACTION FROM FENUGREEK (TRIGONELLA FOENUM-GRAECUM L.)

Spandan A. Chaudhary1✉, Pooja S. Chaudhary1, Bakhtiyar A. Syed2, Richa Misra1, Prashanth G. Bagali1, Sara Vitalini3, Marcello Iriti3✉

1Diagnostics and Genomics Research Division, Xcelris Labs Limited, Ahmedabad, Gujarat, India
2Department of Biotechnology, Hemchandracharya North Gujarat University Patan-384265, Gujarat, India
3Department of Agricultural and Environmental Sciences, Milan State University G. Celoria 2, Milan 20133, Italy

ABSTRACT

Background. Diosgenin is a very important plant secondary metabolite and raw material for the drug industry. Plant sources rich in diosgenin include yam (Dioscorea spp.) and fenugreek (Trigonella foenum-graecum L.). A method for diosgenin extraction from yam extracts has previously been validated, but its extraction from fenugreek plants still requires validation. In addition, all available methods require time-consuming additional purification steps. The present study was aimed at developing a low cost, less time-consuming single-step method for diosgenin extraction from fenugreek.

Material and methods. This study represents a method developed for diosgenin extraction from fenugreek plants without any additional/supportive purification methods such as chromatography or thin-layer chromatography. Diosgenin yield estimation and purity analysis by HPLC method, along with accuracy and precision analysis, is presented.

Results. Five different fenugreek varieties were subjected to a newly developed diosgenin extraction method, and an HPLC chromatogram showed a single peak corresponding to diosgenin. Yield was determined by the standard curve method. Limit of detection (LOD) and limit of quantification (LOQ) for the assay were found to be 0.0312 and 0.102 μg, respectively; tcalc for slope and other statistical parameters were found to be significant (P value < 0.001) for this method.

Conclusion. We have developed a fast, accurate and low cost method for diosgenin extraction from fenugreek. Although the authors have studied this method only in fenugreek plants, it could be applied to the extraction of a few other plant secondary metabolites, which will help researchers to save time and effort.

Keywords: diosgenin, fenugreek, HPLC, LOD, quantification

INTRODUCTION

Diosgenin (25R-spirost-5-en-3β-ol) is a secondary metabolite produced by plants such as Dioscorea nipponoca Makino, Smilax china L., Smilax bockii Warb. ex Diels, Solanum incanum Scheff., Solanum xanthocarpum Schrad., Costus speciosus (J. Koenig) Sm. and Trigonella foenum-graecum L. (Lepage et al., 2011;
Patel et al., 2012; Xu et al., 2009). There are four types of steroids: (1) sterols such as sitosterol, sigmasterol, campesterol and cholesterols; (2) sex hormones such as estrone, which are insoluble in water; (3) steroidal saponins such as diosgenin and yamogenin; and (4) nitrogen-containing steroidal saponins.

Diosgenin deserves particular attention due to its biological activities and pharmaceutical applications. Indeed, the anticancer, anti-aging, cardioprotective and contraceptive properties have been reported in a number of studies (Agarwal et al., 2015; Dias et al., 2007; Gong et al., 2011; Lee et al., 2007; Tada et al., 2009; Yan et al., 2009), as well as its antiviral, antimicrobial, antifungal and insecticidal activities (Sautour et al., 2004; Wang et al., 2011).

Its antithrombosis effects were successfully demonstrated in animal models by Gong et al. (2011). Diosgenin reduced total cholesterol and LDL-cholesterol levels, and increased HDL-cholesterol to total cholesterol ratio by suppressing cholesterol absorption and increasing cholesterol secretion (Xu et al., 2009). In the isolated mouse aorta, diosgenin activated receptor-mediated calcium signaling and induced smooth muscle cell contraction, thus showing promising potential for the treatment of vascular disorders (Dias et al., 2007). The anticancer activity of diosgenin has also been investigated in a number of preclinical studies: S-180-, HepA- and U14-bearing mice, and L929, HeLa, MCF cell lines (Wang et al., 2002). Growth inhibition and apoptosis induction in HT-29 human colon cancer cells (Raju, 2004), inhibition of viability and proliferation of breast cancer cells (Li et al., 2005), p53 activation and cell cycle arrest in different cancer cell lines (Corbiere et al., 2004) have been documented.

In plants, diosgenin is produced from squalene 2,3-oxide by two routes: (1) from lanosterol, via the formation of cholesterol; and (2) from cycloartenol via sitosterol (Chaudhary et al., 2015). Diosgenin is extracted by the acid hydrolysis of sugars present in some plants. Traditionally, Dioscorea zingiberensis C. H. Wright is used as source for diosgenin production (Dong et al., 2010). Diosgenin is mainly used as a precursor for the production of many steroidal drugs, oral contraceptives and sex hormones such as testosterone, glucocorticoids and progesterone (Raghu ram et al., 1994). Diosgenin extraction from yams and high-performance liquid chromatography (HPLC) analysis (Li et al., 2012) are well established and commonly used methods, although extraction from fenugreek plants still needs validation.

A number of standard methods have been developed to detect diosgenin including thin-layer chromatography (TLC) (Brain and Hardman, 1968), HPLC (Huang et al., 2008; Zhang and Miller, 1992), the gravimetric method (Morris et al., 1958), the spectrometric methods (Chen et al., 2010; Sánchez et al., 1972), gas chromatography (Šavikin-Fodulović et al., 1998) and enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2010). HPLC-based quantitative determination has been successfully used for some drugs such as nimesulide (Hanif et al., 2011), levobupivacaine (Liu, 2011) and sumatriptan (Sheshala et al., 2012). In addition, Li Peiqin et al. reported analysis of diosgenin by microplate-spectrophotometry with perchloric acid as a color-developing reagent. It is noteworthy that all available methods require additional time-consuming purification steps (Li et al., 2012). Therefore, in the present study, a low-cost, less time-consuming single-step method for diosgenin extraction from fenugreek was developed and validated.

MATERIALS AND METHODS

Plant material

Five fenugreek varieties, namely Gujarat Methi-2 (GM-2), Kasuri, Pusa early branching (PEB), Rajasthan Methi-1 (RMT-1) and Maharashtra Methi-5 (MMT-5), were used in the present study. All of the fenugreek varieties were collected from different states in India, according to their names; GM-2 was collected from Gujarat, Kasuri and PEB from Punjab, RMT-1 from Rajasthan and MMT-5 from Maharashtra. The fenugreek varieties were collected from the respective state agricultural universities. Protocol was initially standardized on fenugreek seeds (data not shown), followed by validation of the same for plant leaves and seedlings.

Diosgenin extraction

Diosgenin extraction from fenugreek seeds has been described previously by Li Peiqin et al., and their method has been further modified for extraction from seedlings (Li et al., 2012). The seedlings were grown
Chaudhary, S. A., Chaudhary, P. S., Syed, B. A., Misra, R., Bagali, P. G., Vitalini, S., Iriti, M. (2018). Validation of a method for diosgenin extraction from fenugreek (Trigonella foenum-graecum L.). Acta Sci. Pol. Technol. Aliment., 17(4), 377–385. http://dx.doi.org/10.17306/J.AFS.2018.0606

for 15 days and dried for 5 days at room temperature. Dried seedlings were powdered with liquid nitrogen using a mortar and pestle. Two grams of powdered fenugreek were transferred to 50 ml round-bottom flask. Fenugreek powder was refluxed with 50 mL of 2.5 M ethanol sulfuric acid at 80°C for 4 h. The resulting solution was filtered using Whatman® filter paper no. 1 to remove debris. The filtered solution was diluted with 50 mL ultra-distilled water and extracted with 50 mL of n-hexane 3 times. Extracts were then pooled and evaporated to dryness at room temperature. Dry residues were dissolved in 25 mL acetonitrile:water (90:10) and filtered through a 0.22 μm filter before HPLC analysis.

HPLC analysis

Standard preparation for HPLC. A stock diosgenin (CAS Number 512-04-9) solution at 1 mg mL⁻¹ was used as a standard for HPLC analysis. 10 mg of diosgenin were transferred to a 10 mL volumetric flask with 5 mL of acetonitrile. To ensure complete solubilization, the solution was sonicated for 10 to 15 min. The solution volume was diluted to constant volume (10 mL) with acetonitrile followed by filtration through a membrane filter (pore size 0.22 μm).

Instrument validation. The HPLC method was validated according to the procedures described in ICH guidelines Q2 (R1) for the validation of analytical methods (Hubert et al., 2004; 2007).

HPLC run. HPLC analysis was performed on high performance liquid electrograph LC-2010HT (Shimadzu, Japan) which consists of two high-pressure solvent delivery pumps, SPD-M20A photodiode array detector (PAD), SIL-20AC auto sampler, CTO-10AS column oven, and CBM-20Alite system controller. For an HPLC run, Hypersil ODS C18, 5 μm, 250×4.6 mm (Thermo scientific) was used at flow rate of 1 mL per min. Acetonitrile:water (90:10) was used as a mobile phase as well as a diluent for preparing the sample extract. Column temperature was maintained at 35°C temperature for 30 min. The flow rate of the mobile phase was kept at 1 mL per min and the wavelength used for detection was 194 nm with UV detector. All the samples were analyzed in triplicate and the standard was run 5 times initially, as well immediately and twice after completion of all the samples. The run time used for each sample was 30 min.

Standard curve preparation. Aliquots of standard solution containing 1 mg mL⁻¹ of diosgenin were diluted with acetonitrile:water (90:10) solution to 7 different concentrations, corresponding to 750, 500, 375, 250, 125, 62.5 and 31.2 μg mL⁻¹ of diosgenin solutions. The injection volume was 10 μL, resulting in 7.5, 5.0, 3.75, 2.50, 1.25, 0.625 and 0.312 μg diosgenin in HPLC analysis. A calibration curve was constructed by plotting the peak area versus the respective diosgenin quantity [μg] and the obtained data were subjected to regression analysis.

Method accuracy analysis. The accuracy of the method was determined by analyzing the crude extracts spiked with diosgenin. The latter was added at low, medium and high levels, i.e. 0.1, 0.4 and 0.7 μg mL⁻¹ in each tube for HPLC. Average peak area (calculated from the standard curve method from the HPLC results) was similar to the actual amount used (Table 4).

Precision analysis of the assay. Three concentrations of standard diosgenin – 0.1 μg, 0.3 μg and 0.8 μg – were used for intra-day precision analysis. Each concentration of diosgenin was subjected to HPLC detection for three repetitions (n = 3) on the same day. Similarly, inter-day precision was also analyzed by the same three concentrations of diosgenin on three different days within the same week (n = 9). Diosgenin quantity [μg] and the relative standard deviations (RSD) were provided (supplementary Table 1).

RESULTS

HPLC analysis of diosgenin

In HPLC analysis, a detection wavelength of 194 nm was determined by spectrum scanning from 190 to 400 nm. In all of the fenugreek varieties, HPLC analysis revealed a single peak of diosgenin. The chromatogram obtained from the diosgenin standard run showed a single peak at a retention time of 14.088 seconds (Fig. 1A). Chromatograms of all the fenugreek samples showed a single peak similar to the diosgenin standard, indicating purity of the extracts (Fig. 1B–1F).
Quantification of the extracted diosgenin

Linear regression curve analysis was used for calculating the concentration of diosgenin obtained by extraction from all fenugreek varieties, similar to Trivedi et al. (2007). All samples analyzed by HPLC were quantified using the standard curve method. Diosgenin standard (1 mg mL⁻¹) was diluted using acetonitrile solution to different dilutions, i.e. 0.75, 0.5, 0.375, 0.25, 0.125, 0.0625 and 0.0312 mg mL⁻¹, and analyzed by HPLC in triplicate; the standard solution was analyzed five times. The average area of each dilution of diosgenin standard is summarized in Table 1. Diosgenin quantity was calculated and used for linear regression analysis.

Analytical parameters obtained from the linear regression analysis were summarized in Table 2. The equation used for the calculation is described below.

Diosgenin yield calculation

Diosgenin obtained from the extraction process was subjected to HPLC analysis. Diosgenin yield was calculated on the basis of retention time obtained from HPLC analysis reported in Table 3. Finally, the diosgenin linear regression curve (Fig. 2) was used for calculating the concentration of diosgenin obtained by extraction from all fenugreek varieties. Extracted diosgenin from all the samples was analyzed in triplicate.
Table 1. Average area of dilutions of diosgenin standards analyzed on HPLC

| Sample   | Diosgenin concentration mg mL⁻¹ | Average area*         | Quantity detected |
|----------|---------------------------------|-----------------------|-------------------|
| Standard 1 | 1                               | 14 096 474.6          | 0.92 ±0.03        |
| Dilution 1 | 0.75                            | 7 749 869.5           | 0.72 ±0.06        |
| Dilution 2 | 0.5                             | 7 158 630             | 0.48 ±0.02        |
| Dilution 3 | 0.375                           | 5 124 731             | 0.362 ±0.09       |
| Dilution 4 | 0.25                            | 2 552 776             | 0.241 ±0.03       |
| Dilution 5 | 0.125                           | 2 589 161             | 0.122 ±0.08       |
| Dilution 6 | 0.0625                          | 1 323 964             | 0.0598 ±0.09      |
| Dilution 7 | 0.0312                          | 680 844               | 0.0255 ±0.11      |

*n = 5 for standards, n = 3 for dilutions.

Table 2. Analytical parameters for the calibration curve data obtained for diosgenin by HPLC method

| Parameter                          | HPLC                  |
|------------------------------------|-----------------------|
| Detection wavelength, nm           | 194                   |
| Linearity range, mg mL⁻¹           | 0.0312–1.000          |
| Regression equation                | \( Y = 549456 + 1.3 \times 10^7 (X) \) |
| S.D. of slope (n = 5)              | 1967102               |
| Intercept (a)                      | 549456                |
| Sy/x                               | 1803000               |
| \( t_{\text{calculated}} \) for slope | 6.583 (significant***; \( P = 0.00059 \) *** < 0.001) |
| \( t_{\text{calculated}} \) for intercept | 0.554 (not significant; \( P = 0.559 \)) |
| LOD, μg                           | 0.0312                |
| LOQ, μg                           | 0.102                 |
| Coefficient of determination \( R^2 \) | 0.878                 |
| Adjusted \( R^2 \)                | 0.86                  |

Fig. 2. Linear regression curve for diosgenin
Detection and quantification limits

The limit of detection (LOD) and limit of quantification (LOQ) are presented in Table 2 (0.0312 and 0.102 μg respectively).

DISCUSSION

Qualitative analysis of diosgenin was performed using the HPLC method. Chromatograms of diosgenin extracted from all of the fenugreek varieties showed a single peak similar to the diosgenin standard, thus proving no residual components or contamination in the extracted diosgenin. The method was assessed for linearity using the regression analysis method. The linearity of the calibration curve was validated by the high value of the correlation coefficient of the regression equation and by the low value of the intercept on the ordinate. The \( t_{\text{calculated}} \) for slope was found to be significant \( (P = 0.00059) \) whereas \( t_{\text{calculated}} \) for the intercept was found not to be significant \( (P = 0.559) \). Coefficient of determination: \( R^2 \) obtained from the curve and adjusted \( R^2 \) were 0.878 and 0.87 respectively, indicating that the regression line perfectly fits the data.

Accuracy and precision analysis

Accuracy of the assay was analyzed using the spiking of diosgenin standard at concentrations of 0.1, 0.4 and 0.7 μg mL\(^{-1}\). The difference between the amount spiked and diosgenin detected by HPLC method was in the acceptable range (refer Table 4).

Methods for the determination of diosgenin extracted from various sources, for instance yams, fenugreek leaves, stems and seeds, have been previously developed and reported, including spectrophotometry, TLC, HPTLC, HPLC and gas chromatography. In the spectrophotometric method presented by Baccou et al. (1977) and Uematsu et al. (2000) and further modified by Chapagain and Wiesman (2005), diosgenin extracts were treated with anisaldehyde, sulfuric acid and ethyl acetate to be measured at 430 nm. In the present study, we analyzed diosgenin at 194 nm, which was obtained by spectrum scanning from 190 to 400 nm.

Trivedi et al. used the calibration curve method for quantifying the extracted diosgenin. These authors found the green color spot on the TLC plate corresponding to diosgenin at \( R_f = 0.30 \pm 0.04 \) and visualized the same in scanner III at 428 nm. They found diosgenin at 0.087% (w/w) in fenugreek leaf powder, 0.015 and 1.27% (w/w) in fenugreek stem powder and extract respectively, and 0.586% (w/w) in a formulation containing fenugreek seed powder (Trivedi et al., 2007), with few other compounds present alongside diosgenin. In the present study, we obtained 0.43 μg to 0.7 μg of pure diosgenin from different varieties of fenugreek. Dangi et al. (2014) used the method described by Trivedi and colleagues to extract diosgenin from 15 different varieties, but a chromatogram of the sample run on HPTLC showed multiple peaks, indicative of crude extract or presence of other similar secondary metabolites. Li Peiqin et al. (2012) developed a spectrophotometric method to quantify diosgenin in which they dissolved the diosgenin in acetonitrile and evaporated the solution until dryness at room temperature, before dissolving the diosgenin in perchloric acid and measuring it at 410 nm wavelength in spectrophotometer. They also performed the HPLC analysis.

Table 3. Diosgenin yield calculated on the basis of retention time obtained by HPLC

| Fenugreek variety | Area of samples obtained by HPLC | Diosgenin yield using standard curve method*, % |
|-------------------|---------------------------------|-----------------------------------------------|
| GM-2              | 9 435 220                       | 0.65 ±0.01                                    |
| Kasuri            | 6 200 574                       | 0.43 ±0.02                                    |
| PEB               | 10 238 472                      | 0.7 ±0.02                                     |
| RMT-1             | 11 208 472                      | 0.77 ±0.01                                    |
| MMT-5             | 10 156 334                      | 0.7 ±0.02                                     |

*\(n = 3\).

Table 4. Diosgenin yield obtained by HPLC from the crude extracts spiked with diosgenin

| Amount of diosgenin spiked, μg | Average area of samples obtained by HPLC* | Quantity detected μg |
|--------------------------------|-------------------------------------------|----------------------|
| 0.1                            | 2 434 035.4                               | 0.17 ±0.02           |
| 0.4                            | 5 177 635.6                               | 0.39 ±0.08           |
| 0.7                            | 7 326 356.2                               | 0.69 ±0.02           |

*\(n = 3\) dilutions.
of diosgenin and reported a retention time (RT) of 18.064 ±0.096 (Li et al., 2012). The amount of diosgenin in fenugreek, yam or any other plant source is influenced by both genetic and environmental factors. In western Canada, 10 accessions of fenugreek varieties were studied to assess the influence of genetic and environmental factors on seed diosgenin yield. Four fenugreek accessions, namely CN 19062, CN 19067, CN 19070 and CN 19071, yielded 0.70, 0.98, 0.84, and 0.87% diosgenin respectively, as analyzed by capillary gas chromatography (Taylor et al., 2002). These data are in agreement with our results showing yields ranging from 0.54% (in the Kasuri variety) to 0.83% (in RMT-1 variety).

CONCLUSIONS

The method described in this study provides pure diosgenin, with the same yield obtained by other reported methods. Moreover, this method does not require the use of thin layer chromatography or other purification methods, which, in turn, helps to reduce production costs and is less time-consuming. However, validation of the method for large scale applications is still pending.

ACKNOWLEDGMENT

The authors are grateful to the management of Xcelris Labs Ltd. for providing financial support and facilities for carrying out the work.

REFERENCES

Agarwal, M., Agarwal, M., Jain, S. C. (2015). In vitro regulation of bioactive compounds in Trigonella species by mutagenic treatments. J. Plant Sci., 3, 40–44.

Baccou, J. C., Lambert, F., Sauvaire, Y. (1977). Spectrophotometric method for the determination of total steroidal sapogenin. The Analyst, 102(1215), 458–465. https://doi.org/10.1039/an9770200458

Brain, K. R., Hardman, R. (1968). An improved method of densitometric thin layer chromatography as applied to the determination of sapogenin in Dioscorea tubers. J. Chromat., A, 38, 355–363.

Chapagain, B., Wiesman, Z. (2005). Variation in diosgenin level in seed kernels among different provenances of Balanites aegyptiaca Del (Zygophyllaceae) and its correlation with oil content. Afr. J. Biotechnol., 4, 1209–1213. https://doi.org/10.3920/aob.mcn125

Chaudhary, S., Chikara, S. K., Sharma, M. C., Chaudhary, A., Syed, B. A., Chaudhary, P. S., …, Iriti, M. (2015). Elicitation of diosgenin production in Trigonella foenum-graecum (fenugreek) seedlings by methyl jasmonate. Int. J. Mol. Sci., 16(12), 29889–29899. https://doi.org/10.3390/ijms161226208

Chen, H. G., Jin, F. Y., Zhao, C., Zhou, X., Gong, X. J. (2010). Content determination of total saponins in Trigonella foenum-graecum. Chinese J. Spectr. Lab., 27, 580–582.

Corbiere, C., Liagre, B., Terro, F., Beneytout, J.-L. (2004). Induction of antiproliferative effect by diosgenin through activation of p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells. Cell Res., 14(3), 188–196. https://doi.org/10.1038/sj.cr.7290219

Dangi, R., Misar, A., Tamhankar, S., Rao, S. (2014). Diosgenin content in some Trigonella species. Indian J. Adv. Plant Res., 1(2), 47–51. Retrieved from http://ijapronline.com/PDFUpload/V1_I2_A18.pdf

Dias, K. L. G., Correia, N. de A., Pereira, K. K. G., Barbosa-Filho, J. M., Cavalcante, K. V. M., Araújo, I. G. A., …, Meadeiros, I. A. (2007). Mechanisms involved in the vasodilator effect induced by diosgenin in rat superior mesenteric artery. Euro. J. Pharmacol., 574(2–3), 172–178. https://doi.org/10.1016/j.ejphar.2007.07.017

Dong, Y., Teng, H., Qi, S., Liu, L., Wang, H., Zhao, Y., Xu, Z. (2010). Pathways and kinetics analysis of biotransformation of Dioscorea zingiberensis by Aspergillus oryzae. Biochem. Eng. J., 52(2–3), 123–130. https://doi.org/10.1016/j.bej.2010.07.011

Gong, G., Qin, Y., Huang, W. (2011). Anti-thrombosis effect of diosgenin extract from Dioscorea zingiberensis C.H. Wright in vitro and in vivo. Phytomedicine, 18(6), 458–463. https://doi.org/10.1016/j.phymed.2010.08.015

Sánchez, G. L., Acevedo, J. C. M., Soto, R. R. (1972). Spectrophotometric determination of diosgenin in Dioscorea composita following thin-layer chromatography. Analyst, 97(1161), 973–976.

Hanif, M., Shaob, M. H., Yousuf, R. I., Khan, A., Anwer, S., Rasul, A., …, Arshad, H. M. (2011). Reverse phase high performance liquid chromatographic (HPLC) method for nimesulide tablets dosage form prepared for in vivo in vitro correlation (IVIVC) studies. Afr. J. Pharm. Pharmacol., 5(20), 2342–2348. https://doi.org/10.5897/AJPP11.545
Huang, W., Zhao, H., Ni, J., Zuo, H., Qiu, L., Li, H., Li, H. (2008). The best utilization of D. zingiberensis C.H. Wright by an eco-friendly process. Biore. Technol., 99(15), 7407–7411. https://doi.org/10.1016/j.biortech.2008.01.015
Hubert, P., Nguyen-Huu, J. J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., …, Laurentie, M. (2004). Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal – part I. J. Pharm. Biomed. Anal., 36(3), 579–586.
Hubert, P., Nguyen-Huu, J. J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., …, Laurentie, M. (2007). Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal – part II. J. Pharm. Biomed. Anal., 45(1), 70–81.
Lee, J., Jung, K., Kim, Y. S., Park, D. (2007). Diosgenin inhibits melanogenesis through the activation of phosphatidylinositol-3-kinase pathway (PI3K) signaling. Life Sci., 81(3), 249–254. https://doi.org/10.1016/j.lfs.2007.05.009
Lepage, C., Léger, D. Y., Bertrand, J., Martin, F., Beneytout, J. L., Liagre, B. (2011). Diosgenin induces death receptor-5 through activation of p38 pathway and promotes TRAIL-induced apoptosis in colon cancer cells. Cancer Lett., 301(2), 193–202.
Li, J., Liu, X., Guo, M., Liu, Y., Liu, S., Yao, S. (2005). Electrochemical study of breast cancer cells MCF-7 and its application in evaluating the effect of diosgenin. Anal. Sci., 21(5), 561–564. https://doi.org/10.2116/analsci.21.561
Li, P., Mou, Y., Lu, S., Sun, W., Lou, J., Yin, C., Zhou, L. (2012). Quantitative determination of diosgenin in Dioscorea zingiberensis cell cultures by microplate-spectrophotometry and high-performance liquid chromatography. Afr. J. Pharm. Pharmacol., 6(15), 1186–1193. https://doi.org/10.5897/AJPP11.440
Liu, J., Zhou, X., Jiang, J. (2011). A validated HPLC method for the determination of levobupivacaine in plasma and its application to pharmacokinetic studies in Chinese people. Afr. J. Pharm. Pharmacol., 5(20), 2232–2237. https://doi.org/10.5897/AJPP11.399
Morris, M. P., Roark, B. A., Cancel, B. (1958). Tuberculosis, simple procedure for the routine assay of Dioscorea tubers. J. Agrie. Food Chem., 6(11), 856–858.
Patel, K., Gadewar, M., Tahilyani, V., Patel Kumar, D. (2012). A review on pharmacological and analytical aspects of diosgenin: a concise report. Nat. Prod. Bioprospect., 2(2), 46–52. https://doi.org/10.21040/npbi/2012004
Raghuram, T. C., Sharma, R. D., Sivakumar, B., Sahay, B. K. (1994). Effect of fenugreek seeds on intravenous glucose disposition in non-insulin dependent diabetic patients. Phytother. Res., 8(2), 83–86.
Raju, J., Patlolla, J. M., Swamy, M. V., Rao, C. V. (2004). Diosgenin, a steroid saponin of Trigonella foenum-graecum (Fenugreek), inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. Cancer Epidem. Prev. Biomark., 13(8), 1392–1398.
Sánchez, G. L., Acevedo, J. C. M., Soto, R. R. (1972). Spectrophotometric determination of diosgenin in Dioscorea composita following thin-layer chromatography. Analyst, 97(1161), 973–976.
Sautour, M., Mitaine-Offer, A. C., Miyamoto, T., Dongmo, A., Lacaille-Dubois, M. A. (2004). Antifungal steroid saponins from Dioscorea cayenensis. Planta Med., 70(1), 90–92. https://doi.org/10.1055/s-2004-815467
Šavikin-Fodulović, K., Grubišić, D., Ćulafić, L., Menković, N., Ristić, M. (1998). Diosgenin and phytosterols content in five callus lines of Dioscorea balcanica. Plant Sci., 135(1), 63–67.
Sheshala, R., Khan, N., Darwis, Y. (2012). Validated high performance liquid chromatography (HPLC) method for the determination of sumatriptan in rabbit plasma: Application to pharmacokinetic study. Afr. J. Pharm. Pharmacol., 6(2), 98–107. https://doi.org/10.5897/AJPP11.436
Tada, Y., Kanda, N., Haratake, A., Tobiishi, M., Uchiwa, H., Watanabe, S. (2009). Novel effects of diosgenin on skin aging. Steroids, 74(6), 504–511. https://doi.org/10.1016/j.steroids.2009.01.006
Taylor, W. G., Zulyniak, H. J., Richards, K. W., Acharya, S. N., Bittman, S., Elder, J. L. (2002). Variation in diosgenin levels among 10 accessions of fenugreek seeds produced in western Canada. J. Agric. Food Chem., 50(21), 5994–5997.
Trivedi, P. D., Pandurikakshudu, K., Rathnam, S., Shah, K. S. (2007). A validated quantitative thin-layer chromatographic method for estimation of diosgenin in various plant samples, extract, and market formulation. J. AOAC Int., 90(2), 358–363.
Uematsu, Y., Hirata, K., Saito, K., Kudo, I. (2000). Spectrophotometric determination of saponin in Yucca extract used as food additive. J. AOAC Int., 83(6), 1451–1454.
Wang, L., Zhang, Y., Gao, X., Duan, Z., Wang, S. (2010). Determination of chloramphenicol residues in milk by enzyme-linked immunosorbent assay: improvement by biotin–streptavidin-amplified system. J. Agric. Food Chem., 58(6), 3265–3270.
Wang, Y. J., Pan, K. L., Hsieh, T. C., Chang, T. Y., Lin, W. H., Hsu, J. T. A. (2011). Diosgenin, a plant-derived
Chaudhary, S. A., Chaudhary, P. S., Syed, B. A., Misra, R., Bagali, P. G., Vitalini, S., Iriti, M. (2018). Validation of a method for diosgenin extraction from fenugreek (*Trigonella foenum-graecum* L.). *Acta Sci. Pol. Technol. Aliment.*, 17(4), 377–385. http://dx.doi.org/10.17306/J.AFS.2018.0606

Diosgenin, exhibits antiviral activity in vitro against hepatitis C virus. *J. Nat. Prod.*, 74(4), 580–584.

Wang, L. J., Wang, Y., Chen, S. W., Ma, J. S., Fu, Q., Wang, B. X. (2002). The antitumor activity of Diosgenin in vivo and in vitro. *Zhongguo Zhong Yao za zhi Zhongguo zhongyao zazhi* [China J. Chinese Mat. Med.], 27(10), 777–779.

Xu, L., Liu, Y., Wang, T., Qi, Y., Han, X., Xu, Y., ... Tang, X. (2009). Development and validation of a sensitive and rapid non-aqueous LC-ESI-MS/MS method for measurement of diosgenin in the plasma of normal and hyperlipidemic rats: A comparative study. *J. Chromat. B: Anal. Technol. Biomed. Life Sci.*, 877(14–15), 1530–1536. https://doi.org/10.1016/j.jchromb.2009.03.039

Yan, L. L., Zhang, Y. J., Gao, W. Y., Man, S. L., Wang, Y. (2009). In vitro and in vivo anticancer activity of steroid saponins of *Paris polyphylla* var. *yunnanensis*. *Experim. Oncol.*, 31(1), 27–32.

Zhang, Y., Miller, R. M. (1992). Enhanced octadecane dispersion and biodegradation by a *Pseudomonas rhamnolipid surfactant* (biosurfactant). *Appl. Environ. Microbiol.*, 58(10), 3276–3282.