Isolation and Characterization of Stigmasterol from *Fritillaria roylei*

Gunpreet Kaur¹,*, Vikas Gupta¹, R. G. Singhal², Parveen Bansal¹

¹Baba Farid University of Health Sciences, Faridkot, India
²Shobhit University, Meerut, India

 Corresponding author*
reetjattan21@gmail.com

Manuscript received: 26 June, 2020. Revision accepted: 06 November, 2020. Published: 16 November, 2020.

Abstract

*Fritillaria roylei* (Kshirakakoli) is the threatened species of “Ashtwarga” group suffers lot of confusion for identification & authentification in Ayurvedic system of medicine. Due to lack of natural sources and insufficient availability of kshirakakoli, chances of adulteration and substitution increases which in turn leads to loss of faith of people in herbal drugs. Thus for identification and differentiation, quality standardization and quality assurance of kshirakakoli containing herbal formulations there is a need to isolate chemical marker compound using advanced analytical techniques. The methanol extract of root samples of plant was prepared and phytochemical screening was performed. Marker compound was isolated from the extract using column chromatography. Single compound having *Rt* value 0.31 was isolated with TLC by using mobile phase n-hexane: ethyl acetate: formic acid (8:2:0.1 *v/v/v*) and purified by re-crystallization with methanol. Isolated compound was further characterized by using melting point and spectral analysis. The methanol extract was dark brown in color and showed the presence of steroids, amino acids and flavonoids. The isolated compound was found to be white crystalline powder with melting point range of 167-169°C. Spectral analysis confirmed the presence of Stigmasterol. In present study stigmasterol was isolated for the first time and can be used as chemical marker for identification and differentiation of the plant from its substitutes.

Keywords: Isolation; Kshirakakoli; Marker; Standardization; Stigmasterol.

INTRODUCTION

Ayurveda is one of the most distinctive systems of medicine known to man (Jaiswal and Williams, 2017; Sreena et al., 2011). It is regarded as the oldest divine knowledge in the humankind which is based on the principle of maintaining a balance between the interconnected relations within the body and mind. Ayurvedic medicines include herbs, herbal materials, herbal preparations, minerals and finished herbal products. Ayurveda is blessed with plentiful speculate medicinal plants including Ashtawarga group of eight medicinal plants i.e. Kakoli (*Roscoea purpurea*), Kshirakakoli (*Fritillaria roylei*), Jeevaka (*Microstylis muscifera*), Rishabhbhaka (*Malaxis acuminate*), Meda (*Polygonatum cirrhifolium*), Mahameda (*Polygonatum verticillatum*), Riddhi (*Habenaria edgeworthii*) and Vridhi (*Habenaria internedia*) (Balkrishna et al., 2012; Saha D et al., 2015). In recent days, herbal medicines are getting more popular with the comprehensive movement of people towards natural therapies. This increasing demand of the population towards herbal medicines results in shortage of authentic raw materials leading to increase in chance of adulteration and substitution because the regulatory authorities lack the strict quality control measures of herbal medicines (Shukla and Dhanya, 2017). This same situation happens with plants of Ashtawarga group. According to International Union of Conservation of Nature (IUCN) and Conservation Assessment and Management Plan (CAMP), *Fritillaria roylei* (Kshirakakoli) is considered as threatened medicinal plant (Saha et al., 2015; IUCN 2001; Kuniyal et al., 2015). From various studies, it has been found that due to lack of natural sources and insufficient availability to meet the requirements of market for the raw material the Department of AYUSH, Govt. of India, permitted the use of available substitutes in place of original plant. The substitution of *Fritillaria roylei* is done with Ashwagandha (root) (*Withania somnifera*) or Safed musli root (*Chlorophyllum arundinaceum* Boker) (Balkrishan et al., 2012; Sagar, 2014). However, literature survey reveals that Ayurvedic parameters as well as pharmacological actions of the *Ashtawarga* plants do not match with their substitutes. The substitute of *F. roylei* that is *W. somnifera* or *C. arundinaceum* shows 33% or 16% similarity which ultimately results in reduced efficacy of the drugs along with loss of faith of people towards use of herbal drugs (Virk et al., 2015). Thus, drug standardization and quality assurance is essential to assess the safety, efficacy and quality of herbal drugs. Lack of chemical markers is a major problem for the
regulatory authorities for enforcement of quality regulations. Therefore, the study was designed to isolate chemical markers from *Fritillaria rolei* (Kshirakakoli) using advanced analytical techniques.

**MATERIALS AND METHODS**

**Chemicals**
In the present study all the reagents and solvents used were of Analytical grade. For isolation of marker compound, precoated aluminum-backed TLC plates with 0.2 mm layer of silica gel 60 F<sub>254</sub> (20 cm × 10 cm) manufactured by E. Merck (Germany) were purchased from local authorized dealer.

**Plant Material**
The root samples of *Fritillaria roylei* were procured from cultivar source of Himachal Pradesh and authenticated by National Botanical Research Institute, Lucknow wide authentication letter no. NBRI/CIF/535/2017 dated 04/01/2017. Root samples of the plant were washed, shade dried and stored in air tight container.

**Extract Preparation**
The shade dried roots of *Fritillaria roylei* (500gm) were coarsely powdered and defatted with petroleum ether followed by continuous hot extraction process with methanol. The methanol extract thus obtained was filtered and then evaporated to obtain a concentrated semisolid mass. The final extract thus obtained was then stored in vacuum desiccator for further use.

**Phytochemical Screening**
Preliminary phytochemical screening of the extract were performed for the detection of phytoconstituents like alkaloids, flavonoids, steroids, proteins, tannins, saponins, phenolics, carbohydrates and amino acids (Banu and Cathrine, 2015; Kokate et al., 2000; Tiwari et al., 2011).

**Isolation of Chemical Marker**
About 8.4g of methanol extract was mixed with methanol and then silica gel having pore size 60–120 mesh was added to form slurry which was then dried on water bath to form a free flowing powder. Silica gel (675g) suspended in n-hexane was poured into the glass column having dimensions 1000mm x 50 mm to give rise to silica bed. Saturated silica bed was allowed to stand overnight for uniform bed packing. After 12hrs, elution was started with non-polar solvent n-hexane followed by an increase in polarity of the solvents and fractions were collected with optimum flow rate of 10ml/min. Thin layer chromatography (TLC) of collected fractions were performed using different solvents selected by hit and trial method and on the basis of TLC profile similar fractions were pooled together. Elution with the solvent system n-hexane: chloroform: ethyl acetate 60:20:20 yielded a pool of two compounds with R<sub>t</sub> 0.31 and 0.42 on TLC plates by the use of mobile phase n-hexane: ethyl acetate: formic acid (8:2:0.1 v/v/v) along with mixture of other compounds on TLC plates. Single compound was isolated by cutting and pooling of TLC plate of compound having R<sub>f</sub> value 0.31 and purified by re-crystallization with methanol. The fraction was kept in a refrigerator to get the crystallized compound (Virk et al., 2016; Cannell, 1998).

**Characterization of Isolated Compound**
Isolated crystallized compound was characterized by using different chemical test, melting point and spectral analysis (IR, NMR, Mass, and UV spectroscopy).

---

Figure 1. NMR spectra of isolated compound Stigmasterol from *Fritillaria roylei* (Kshirakakoli).
RESULT AND DISCUSSION

Physical Evaluation and Percentage Yield of Extract
The methanol extract of *Fritillaria roylei* was dark brown in color, and the percentage yield of extract was 7.48%.

Phytochemical Screening of Extract
Preliminary phytochemical analysis confirmed the presence of steroids, amino acids, flavonoids and proteins

Characterization and Identification of Isolated Compound
Isolated compound was found as white crystalline powder after crystallization from CHCl₃-MeOH. The compound was shown positive test of steroids. Melting point of the compound was found to be 167-169°C (lit. 164-171°C).

Spectroscopic Data
IR: The Infra-red spectra of isolated compound, showed very intensely broad peak at 3428 cm⁻¹ and moderately intense peak at 1192 and 699 cm⁻¹ were observed for the O-H bond vibrations of hydroxyl group. The unsaturated part of C-H and C=C vibrations was observed at 881 cm⁻¹ and 1642 cm⁻¹. The vibrations of =CH₃ were observed at 2937 cm⁻¹ and at 1465 cm⁻¹ and vibrations of =CH₂ were observed at 2852 cm⁻¹ and at 1460 cm⁻¹ respectively. The C-C vibration peak was shown at 1053 cm⁻¹ which confirm the structure of stigmasterol.

NMR: In the ¹H-NMR spectrum of isolated compound showed two olefinic protons appeared downfield at δ 4.57 (m) and δ 4.14 (m) which were identical with the chemical shift of H-22 and H-23, respectively of stigmasterol. Six methyl protons at δ 1.23 (s, 3H), δ 1.19 (s, 3H), δ 1.06 (s, 3H), δ 1.00, δ 0.98 (s, 3H) and δ 0.91 (s, 3H); [(3H each, s, CH₃)] confirms the structure of stigmasterol.

Mass spectra: Mass spectrum of isolated compound showed parent molecular ion [M⁺] peak at m/z 412 which was being in agreement with the proposed structure of stigmasterol.

Structure and Molecular Formula of Isolated Compound
The molecular formula of isolated molecule stigmasterol is C₃₆H₄₈O that is confirmed by IR, mass spectra and NMR data. Its IUPAC name is (3S,8S,9S,10R,13R,14S,17R)-17-{(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl}-10,13-dimethyl,2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.

As phytosterols are not synthesized by humans and animals so they are needed to be included in the diet. Stigmasterol is present in various naturally occurring items of plant origin like vegetables, nuts, seeds and unpasteurized milk. Edible oils contain higher amount of stigmasterol than vegetables. It is widely used as food additives in food and beverage industries for production of sausages, yogurt, cold cuts, margarines, bakery products, milk and spreads (Aboobucker et al., 2019; Jun-Hua et al., 2008; Siddiqui et al., 1990). Stigmasterol is also used as a precursor or intermediate for synthesis of various human steroid hormones like progesterone, androgen corticoids, estrogens etc (Sundaramaran and Djerassi, 1977; Hogg, 1992). Stigmasterol is used as raw material in pharmaceutical industries for commercial synthesis of vitamin D3 (Kametani and Furuyama, 1987). In addition to this various studies showed that stigmasterol possesses various pharmacological properties like cytotoxic activity, antioxidant activity, anti-hypercholesterolemic, anti-osteoarthritic activity, anti-inflammatory activity, hypoglycemic activity etc. High therapeutic value and regulatory approval status as Generally Recognized As Safe (GRAS) in the U.S., followed by an approval from the FDA and by the EU Scientific Committee on Food (SFC) increases the demand of phytosterol significantly (Ghosh et al., 2011; Chandler et al., 1979; Batta et al., 2006; Gabay et al., 2010; Panda et al., 2009; Navarro et al., 2001).

As the market price of Stigmasterol is 32,334/10g (approximately) and it will be difficult for commercial manufacturers to replace *Fritillaria roylei* plant with stigmasterol just to claim the presence of *Fritillaria roylei* (Kshirakakoli). Presence of stigmasterol has not been ever reported in its substitutes/adulterants. It has been reported first time from root of Kshirakakoli plant. Thus, for quality standardization of Kshirakakoli containing formulations isolated compound i.e. stigmasterol may be used as chemical markers for identification and differentiation of authentic plants from its substitutes and common adulterants.

CONCLUSION

In the present study, stigmasterol was isolated from roots of *Fritillaria roylei* using column chromatography and TLC. As per the knowledge, this is first report on chromatographic method of isolation of stigmasterol from natural source that is *Fritillaria roylei* plant. This compound can be used as a chemical marker by regulatory authorities for identification of kshirakakoli plant from other substitutes.

Conflict of interest: The author declares that there are no conflicts of interest concerning the publication of this article.

Source of Funding: The authors gratefully acknowledge authorities of Baba Farid University of Health Sciences, Faridkot for necessary permission and providing all research facilities. This work was supported by the National Medicinal Plants Board, Ministry of AYUSH, Govt. of India, New Delhi, India [Grant numbers R&D/CH-01/2012].
REFERENCES

Aboobucker, S. I., Suzu, W. P. (2019). Why do plants convert sitosterol to stigmasterol?. *Frontiers in plant science*, 10 354-362. DOI: 10.3389/fpls.2019.00354.

Balkrishna, A., Srivastava, A., Mishra, R. K., Patel, S. P., Vashistha, R. K., Singh, A., et al. (2012). Astavarga plants-threatened medicinal herbs of the North-West Himalaya. *Int. J. Med. Arom. Plants*, 2(4) 661-676. ISSN: 2249-4340.

Banu, K. S., Cathrine, L. (2015). General techniques involved in phytochemical analysis. *Journal of Advanced Research in Chemical Science*, 2(4) 25-32. ISSN 2349-0403

Batta, A. K., Xu, G., Honda, A., Miyazaki, T., Salen, G. (2006). Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. *Metabolism*, 55(3) 292-299. DOI: 10.1016/j.metabol.2005.08.024.

Cannell RJP (1998) How to Approach the Isolation of a Natural Product. In: Cannell RJP (eds) Natural Products Isolation. Methods in Biotechnology, Vol 4. Humana Press, Totowa, NJ. DOI: 10.1007/978-1-59295-256-2_1.

Chandler, R. F., Hooper, S. N., Ismail, H. A. (1979). Antihypercholesterolemic studies with sterols: β-sitosterol and stigmasterol. *Journal of pharmaceutical sciences*, 68(2) 245-247. DOI: 10.1002/jps.2600680235.

Gabay, O., Sanchez, C., Salvat, C., Chevy, F., Breton, M., Nourissat, G., E et al. (2010). Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis and cartilage*, 18(1) 106-116. DOI: 10.1016/j.joca.2009.08.019.

Ghosh, T., Maity, T. K., Singh, J. (2011). Evaluation of antitumor activity of stigmasterol, a constituent isolated from Bacopa monnieri Linn aerial parts against Ehrlich Ascites Carcinoma in mice. *Oriental Pharmacy & Experimental Medicine*, 11(1) 41-49. DOI: 10.1007/s13596-011-0001-y.

Hogg, J. A. (1992). Steroids, the steroid community, and Upjohn in perspective: a profile of innovation. *Steroids*, 57(12) 593-616. DOI: 10.1016/0039-128X(92)90013-Y.

IUCN. (2001). IUCN Red List Categories and Criteria, Version 3.1. International Union for Conservation of Nature and Natural Resources, Gland, Switzerland.

Jaiswal, Y. S., Williams, L. L. (2017). A glimpse of Ayurveda--The forgotten history and principles of Indian traditional medicine. *Journal of traditional and complementary medicine*, 7(1) 50-53. DOI: 10.1016/j.jtcme.2016.02.002

Jun-Hua, H. A. N., Yue-Xin, Y. A. N. G., Mei-Yuan, F. E. N. G. (2008). Contents of phytosterols in vegetables and fruits commonly consumed in China. *Biomedical and Environmental Sciences*, 21(6) 449-453. DOI: 10.1016/S0895-3988(09)60001-5.

Kametani, T., Furuyama, H. (1987). Synthesis of vitamin D3 and related compounds. *Medicinal research reviews*, 7(2) 147-171. DOI: 10.1002/med.2610070202.

Kaur Virk, J., Bansal, P., Kumar, S., Singh, R., Kumar Rawal, R. (2016). Isolation and Quantification of Chemical Marker of Polygonatum verticillatum: First Report. *Current Traditional Medicine*, 2(3) 216-224.

Kokate CK, Khandelwal KR, Pawar AP, Gokhale SB (2000) Practical Pharmacognosy, Nirali Prakashan, Pune, 45-46.

Kuniyal, C. P., Bisht, V. K., Negi, J. S., Bhatt, V. P., Bisht, D. S., Butola, J. S., et al. (2015). Progress and prospect in the integrated development of medicinal and aromatic plants (MAPs) sector in Uttarakhnad, Western Himalaya. *Environment, development and sustainability*, 17(5) 1141-1162. DOI: 10.1007/s10668-014-9595-9.

Navarro, A., De Las Heras, B., Villar, A. (2001). Anti-inflammatory and immunomodulating properties of a sterol fraction from Sideritis foetens Clem. *Biological and Pharmaceutical Bulletin*, 24(5) 470-473. DOI: 10.1248/bpb.24.470.

Panda, S., Jafri, M., Kar, A., Meheta, B. K. (2009). Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from Butea monosperma. *Fitoterapia*, 80(2) 123-126. DOI: 10.1016/j.fitote.2008.12.002.

Raj, S., Karthikeyan, S., Gothandam, K. M. (2011). Ayurveda-A glance. *Research in Plant Biology*, 1(1) 1-14. ISSN: 2231-5101

Sagar, P. K. (2014). Adulteration and substitution in endangered, ASU herbal medicinal plants of India, their legal status, scientific screening of active phytochemical constituents. *International Journal of Pharmaceutical Sciences and Research*, 5(9) 4023-4039.

Saha, D., Ved, D., Ravikumar, K., Haridasan, K., Dhyani, A. (2015). Lilium polyphyllum. The IUCN Red List of Threatened Species, Available at: www.iucnredlist.org. [Accessed on 25/November/2019].

Shukla, A. K., Dhanya, R. (2017). Standardization and Evaluation of Ayurvedic Medicines-Needs. *Journal of Ayurveda and Integrated Medical Sciences*, 2(2) 190-195. ISSN 2456-3110.

Sididiqi, S., Siddiqui, B. S., Adil, Q., Begum, S. (1990). Constituents of Mirabilis Jalapa. *Fitoterapia*, 61(5) 471-474. ISSN: 0367-326X

Sundararaman, P., Djerrassi, C. (1977). A convenient synthesis of progesterone from stigmasterol. *The Journal of organic chemistry*, 42(22) 3633-3634. DOI: 10.1021/jo00442a044.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale pharmaceutica sciencia*, 1(1) 98-106.

Virk, J. K., Bansal, P., Gupta, V., Kumar, S., Singh, R. (2015). Lack of pharmacological basis of substitution of an endangered plant group Ashtawarga-A significant ingredient of polyherbal formulations. *Am J Phytoned Clin Ther.*, 2 690-712.