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Isolation and Characterization of Stigmasterol from *Fritillaria roylei*

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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 R_f 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*), Rishabbhaka (*Malaxis acuminatea*), 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 (*Chlorophytum 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_{254} (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_f 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_f 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).

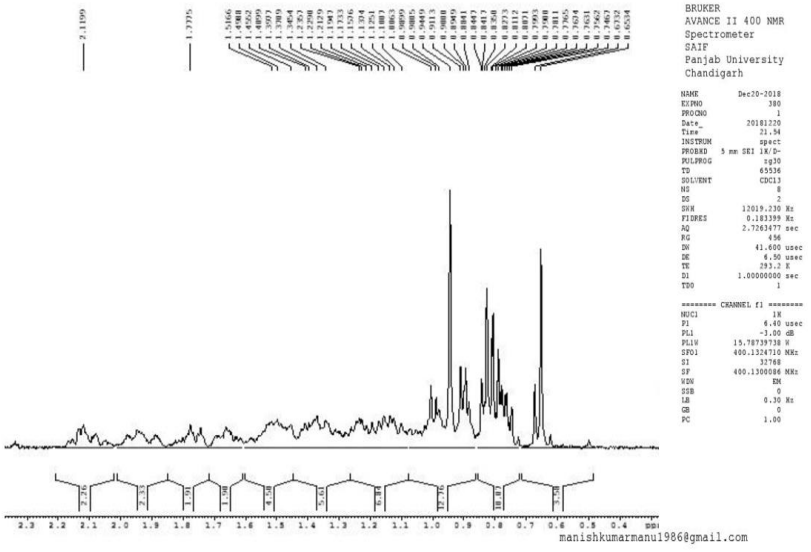

Figure 1. NMR spectra of isolated compound Stigmasterol from *Fritillaria roylei* (Kshirakakoli).

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 $CHCl_3$-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 -CH_3 were observed at 2937 $cm^{-1}$and at 1465 cm^{-1} and vibrations of =CH_2 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 ^{1}H-NMR spectrum of isolated compound showed two olefenic 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, CH_3)] 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 $C_{29}H_{48}O$ that is confirmed by IR, mass spectra and NMR data. Its IUPAC name is (3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl,2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-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 (Sundararaman 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-hypercholestrolemic, 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.

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