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INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

ISSN 2349-7203



Human Journals

Research Article

March 2020 Vol.:17, Issue:4

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Isolation of Sitostanol Caffeate from *Microstylis muscifera*: A New Chemical Marker for the Identification of Adulteration and Substitution



IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals



ISSN 2349-7203

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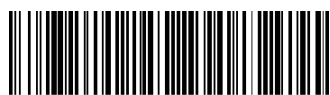
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Submission: 25 February 2020
Accepted: 2 March 2020
Published: 30 March 2020

Keywords: *Microstylis muscifera*, sitostanol caffeate, Marker, Isolation, Identification

ABSTRACT

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads. *Microstylis muscifera* plant has been recommended for its use in several rejuvenating Ayurvedic formulations. Ever rising demands, lack of natural sources and quantity insufficient to meet the requirements of the market for the raw material has lead the manufacturers towards the use of official substitutes recommended by Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) that has further encouraged them for adulteration of formulations by other substandard/spurious raw drugs. More than 60% Ayurvedic parameters, as well as pharmacological actions of Ashtawarga plants, do not match with their substitutes hence consumers are forced to pay for the material which has never been used for high cost claimed formulations. The situation is being exploited by manufacturers because regulatory authorities lack the tools (marker compound) needed for the identification of the authentic plant. So this study was carried out to find a marker compound of plant that could help in authentication and regulate adulteration. Present communication presents the first report of isolation and characterization of a marker compound, sitostanol caffeate (SC) from methanolic extract of the plant using various analytical techniques and can be used as a marker for identification of the plant in the market formulations. Isolated compounds from the present study can be used as a chemical marker for the identification of this plant as the cost of the synthetic compound is very high and industry people cannot add it from outside just to prove the presence of *Microstylis muscifera*.



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INTRODUCTION

Microstylis muscifera (Lindley) Kuntze is a rare, terrestrial perennial, endangered medicinal orchid of the Himalayan region and belongs to family Orchidaceae. *Microstylis muscifera* (Lindl.) Kuntze is commonly known as Jeevak or Jeevaka in Hindi. *Microstylis* widely grows from alpine to tropical areas of the world having more than 35,000 species with 800 genera. In India, 166 genera and 1141 species of this plant are distributed mainly in high altitude areas.^[1-4] The Genus *Microstylis* is well recognized for many therapeutic species that are used as essential constituents of *Ayurvedic* and pharmaceutical products having rejuvenating [*M. acuminata*, *M. muscifera* (Lindl.) O. Ktze.], diaphoretic (*M. versicolor* Sant.) and aphrodisiac properties (*M. acuminata* D. Don).^[4-7] The presence of a variety of high quantity/rich amounts of phytochemicals such as glycosides, amino acids, flavonoids, alkaloids, terpenoids, saponins, tannins, proteins, steroids, phenolics, carbohydrates, etc makes orchids highly significant for therapeutic purposes.^[8] There is a fresh spurt for the use of traditional formulations worldwide. High demand and a low supply of Ashtawarga plants lure the manufacturers to use cheap substitutes in drug manufacturing. Literature shows that the majority (60%) of Ayurvedic parameters, as well as pharmacological actions of Ashtawarga plants, do not comply with attributes of their substitutes leading to reduced efficacy of the drugs that finally lead to loss of faith for use of Ayurveda based formulations.^[9-10] Consumers are compelled and befooled to pay for the material which has never been used for high cost claimed formulation. The situation is being exploited by manufacturers because regulatory authorities lack the quality control tools (marker compound) needed for the identification of the authentic plant. Therefore the present study aimed to isolate chemical marker from Jeevaka by different chromatographic as well as spectral techniques.

MATERIALS AND METHODS

Plant material

The pseudobulbs of *Microstylis muscifera* (Lindl.) Kuntze was procured from Himachal Pradesh and was authenticated from Central Instrumentation Facility (CIF), National Botanical Research Institute (NBRI), Uttar Pradesh with Ref No: NBRI/CIF/669/2018. Plant materials were washed and dried at room temperature (<40°C).

Chemicals

In the present study, all the reagents and solvents of either AR or GR grade were used. For isolation of markers, Precoated aluminum-backed TLC plates with a 0.2 mm layer of silica gel 60 F₂₅₄ (20 cm × 10 cm) manufactured by E. Merck (Germany) were purchased from a local authorized dealer.

Preparation of Extract

Pseudobulbs of *Microstylis muscifera* (Lindl.) Kuntze was powdered coarsely and defatted with nonpolar solvent petroleum ether. The continuous hot extraction process was used for extraction using methanol as a solvent. To obtain a semi-solid mass, the extract was filtered, evaporated and finally stored in a desiccator for further use. [11-14]

Phytochemical screening

For the detection of phytoconstituents like alkaloids, steroids, carbohydrates, flavonoids, glycosides, terpenoids, proteins, tannins, amino acids, phenolics, fatty acids, saponins, lipids, etc preliminary phytochemical screening was carried out. [15-16].

Isolation of chemical marker compound

Column chromatography of extract was done for the isolation of a single compound. The slurry was prepared by adding 12.2g of extract in methanol followed by the addition of an adequate quantity of silica gel (60-120 mesh size / 0.120–0.250mm particle size). The slurry was mixed uniformly using trituration and dried on a water bath to get a free-flowing powder. About 790 g silica gel with n-hexane was used to charge the glass column (1000mm × 50mm) for the preparation of the silica bed. After saturation of silica bed, the slurry was charged into the column and allowed to stand overnight for uniform bed packing. Packed column was eluted with n-hexane followed by solvent with increased polarity prepared by using different ratios of toluene, isopropyl alcohol, chloroform, ethyl acetate, and methanol. About 150mL of the fraction was collected at an optimum flow rate of 4mL/min and the TLC profile of similar fractions was collected to give major fractions. Convenient hit and trial method using different polarity solvents was chosen for TLC analysis. Three compounds with R_f 0.32, 0.43, and 0.62 were separated by TLC in solvent system toluene using a mobile phase of Chloroform: Ethyl acetate: Formic acid (3:7:0.1 v/v). The single compound was

isolated by cutting and pooling of the TLC plate of a compound having R_f 0.62. The compound was obtained in fractions numbered 510 to 625 and purified by recrystallization with methanol. The fraction was kept in the refrigerator to get the crystallized compound as per the earlier standard method. [17-20]

Characterization of the isolated compound

Characterization of the isolated compound was done by melting point, chemical test, spectral analysis and compared with literature.

TLC of the isolated compound alone and with extract

The rationale behind TLC is to create a method that is suitable for the separation of the marker from the extract. The best separation of the isolated compound in the extract was achieved in the mobile phase consisting of Chloroform: Ethyl acetate: Formic acid (3:7:0.1 v/v) after trying more than 27 mobile phases and R_f was found to be 0.62 for the isolated compound.

RESULTS

Physical evaluation of extract

The physical appearance of the isolated compound as a white crystalline powder.

Phytochemical screening of the extract

Methanolic extracts of the plant were subjected to preliminary phytochemical analysis that confirmed the presence of flavonoids (with lead acetate test) and amino acids (with Ninhydrin test).

Identification of an isolated compound

Physicochemical description

The isolated compound was having a melting range between 180-185°C.

Spectral Analysis

Infrared (IR) spectra of Isolated Compound

Very strong overlapping bands in the IR spectrum, which appear in the region of 3438.4 cm^{-1} , were assigned to the different O–H vibrations. Both the C–H stretching modes of aromatic and aliphatic moiety were found at 2857.3 , 2927.2 and 2957.5 cm^{-1} (Fig. 1). The strong intensities band at 1734.1 (IR) was assigned to the C=O stretching modes of the caffeate ester with Stigmastanol (sitostanol) phytosterol. Also, the bands of the medium and strong intensities at 1458.7 cm^{-1} were assigned to the C=C stretching modes of the acyclic chain and benzene moiety which confirm the skeleton of sitostanol caffeate (SC).

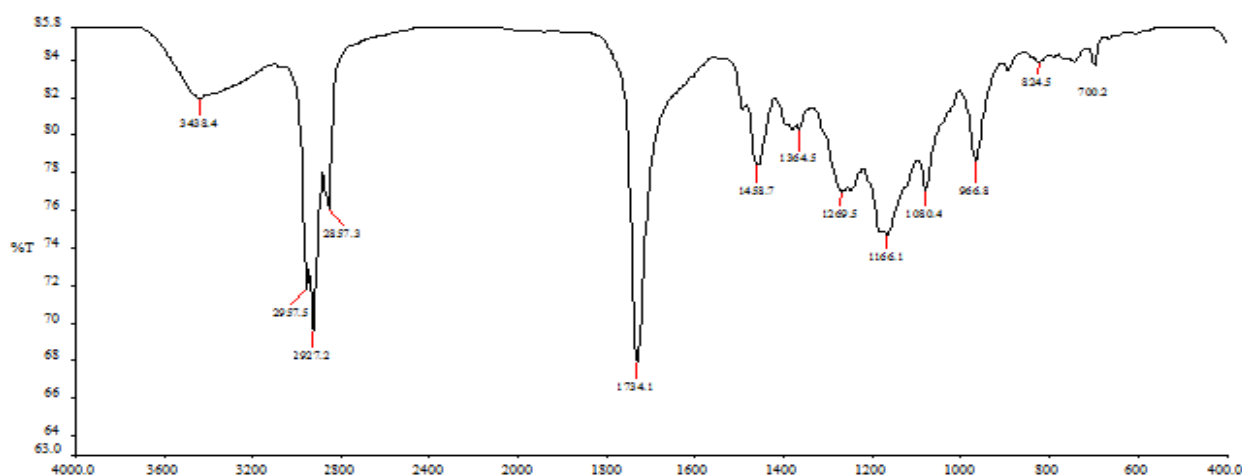


Figure No. 1: IR spectra of isolated compound

NMR

$^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 0.69-0.94 (m, 10H), 1.07-1.44 (m, 12H), 1.60 (m, 4H), 1.67 (t, 1H, $J=6$ & 6.5), 2.00-2.05 (m, 4H), 2.17-2.21 (m, 7H), 2.29-2.35 (m, 4H), 3.53-3.75 (m, 4H), 4.04-4.05 (m, 1H), 4.13-4.16 (m, 1H), 4.18-4.25 (m, 2H), 4.27-4.30 (m, 1H), 5.25 (bs, 1H, OH), 5.34 (bs, 1H, OH), 7.16 (dd, 1H, $J=8.5$ & 2.5), 7.35 (s, 1H), 7.52-7.53 (dd, 2H, -CH=CH-), 7.69-7.71 (m, 1H). $^1\text{H-NMR}$ spectrum shows phenolic –OH at δ 5.25 & 5.35, the characteristic signal of two proton of olefinic proton of -CH=CH- in the form of doublet at 7.52 and 7.54, respectively (Fig. 2) which confirm the structure of SC.

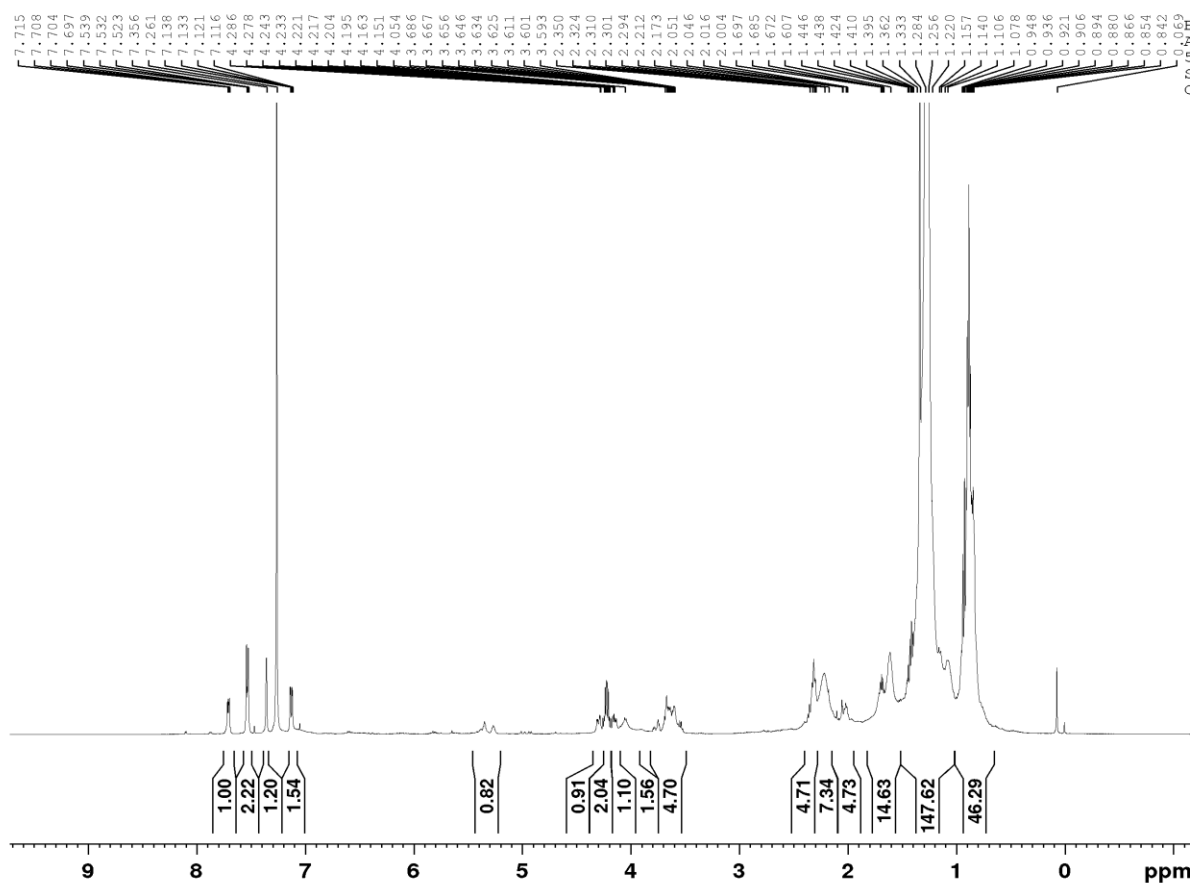


Figure No. 2: NMR spectra of the isolated compound

Mass Spectra

The molecular ion peaks were found at m/z 578.43 (M^+) and 601.43 ($M+Na^+$) in the mass spectra of the isolated compound. The mass spectra also showed a parent ion peak at 601.43 ($M+Na^+$) which was being in agreement with the proposed structure of SC (Fig. 3).

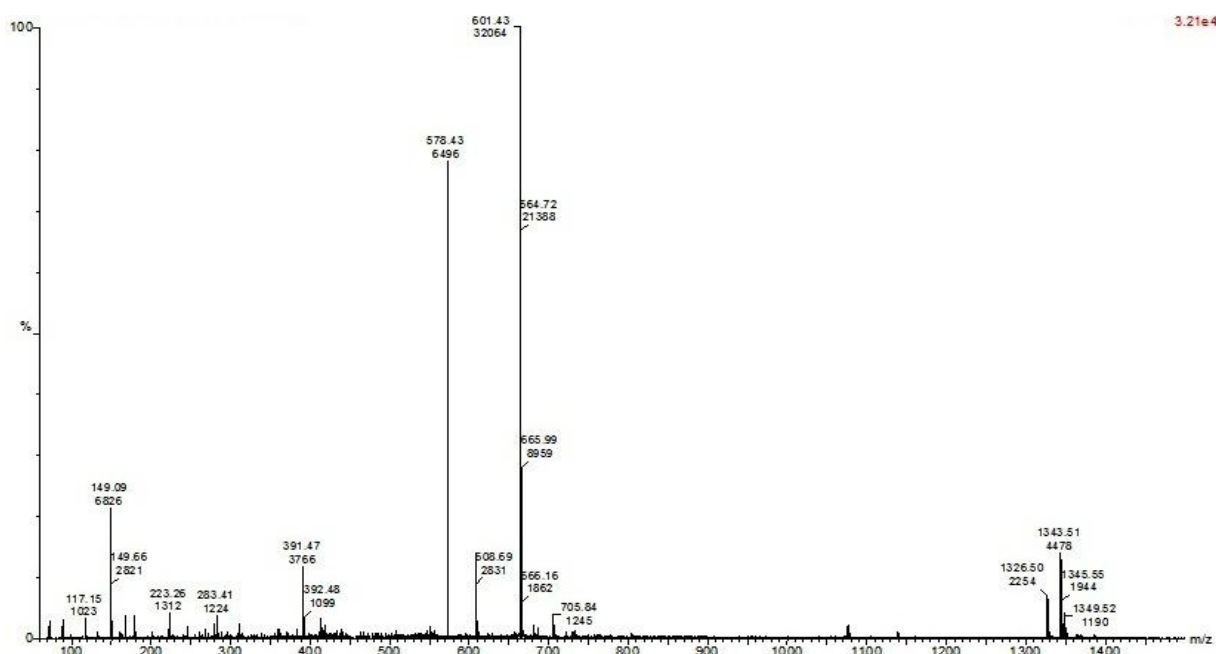


Figure No. 3: Mass spectra of isolated compound SC

Structure and Molecular Formula of Isolated Compound

The molecular formula of isolated molecule SC; (Fig. 4) is $C_{38}H_{58}O_4$ that is confirmed by IR, mass spectra and NMR data. Its IUPAC name is (E)-(5S,8R,9R,10S,13R,14R,17R)-17-((2R,5R)-5-ethyl-6-methylheptan-2-yl)-8,10,13-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-3-yl 3-(3,4-dihydroxyphenyl) acrylate.

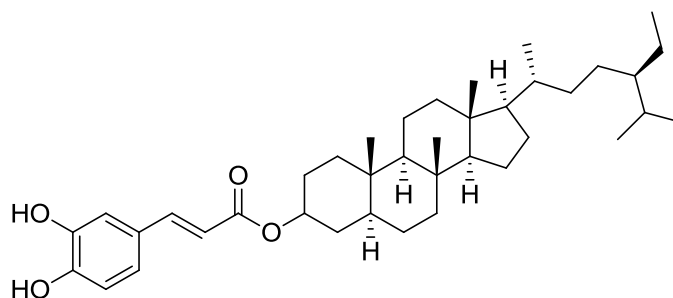


Figure No. 4: Structure of isolated compound SC

TLC of the isolated compound alone and with extract

TLC of the isolated compound with extract and isolated compound alone was performed. The R_f of the isolated compound is 0.62.

DISCUSSION

SC is made from vegetable oils or the oil from pine tree wood pulp and is then combined with canola oil. SC is used for the prevention of heart disease and the treatment of high cholesterol. SC is an ingredient in Benecol margarine and some salad dressings. The U.S. Food and Drug Administration (FDA) allows manufacturers of products that contain SC or related plant chemicals (stanol esters) to claim that the product lowers the risk of getting coronary heart disease (CHD). SC and other plant stanol esters along with a diet low in saturated fat and cholesterol might reduce the risk of CHD by lowering blood cholesterol levels. Although there is plenty of evidence that SC does lower cholesterol levels, so far there is no proof that long-term use lowers the risk of developing CHD. [21-24] Hence the chemical marker isolated in the study can be used for identifying the use of substitutes in high-cost formulations claiming to use this rare medicinal plant. It is pertinent to mention here that the market price of SC is \$3000/g (approximately) and it will be difficult for commercial manufacturers to replace Jeevaka plant with SC just to claim the presence of Jeevaka. [25-29]. However, if an equivalent amount of Jeevaka is added then it will be cheaper for the industry, in addition to its original status as a drug.

CONCLUSION

In the present study authors isolated SC from pseudobulbs of *Microstylis muscifera* (Lindl.) Kuntze using column chromatography and TLC. As per our knowledge, this is the first report on the chromatographic method of isolation of SC from natural source *Microstylis muscifera* (Lindl.) Kuntze. This compound can be used as a chemical marker by regulatory authorities for identification of this plant as the cost of the synthetic compound is very high and industry people cannot add it from outside just to prove the presence of Jeevaka.

Competing interests

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

This work has been supported by the National Medicinal Plants Board, Ministry of AYUSH, Govt. of India, New Delhi. The authors gratefully acknowledge authorities of Baba Farid

University of Health Sciences, Faridkot for necessary permission and providing all research facilities.

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