INTRODUCTION

Microstylis muscifera (Lindley) Kuntze is a rare, terrestrial perennial, endangered medicinal orchid of Himalayan region and belongs to family Orchidaceae. Microstylis muscifera (Lindl.) Kuntze is commonly known as Jeevaka or Jeevaka in Hindi. Jeevaka is distributed from tropical to alpine areas of the world having more than 35,000 species with 800 genera in which 166 genera and 1141 species are presented in India. Microstylis genus is well known for several therapeutic species that are used as a vital component of Ayurvedic and pharmaceutical products having aphinsiac (M. acuminata D. Don), diaphoretic (M. versicolor Sant. {Rishabhā} and Kapadia), and rejuvenating properties [M. acuminata, M. muscifera (Lindl.) O. Ktze.] [1-8] Numerous review articles related to antioxidant property, antioxidant property, anti-inflammatory activity, antipyretic activity, febrifuge and spermopiotic activity of Microstylis muscifera (Lindl.) Kuntze have been published but no report for evaluation of pharmacological activity of isolated compounds exists. The therapeutic importance of orchids is due to presence of phytocompounds such as alkaloids, glycosides, steroids, terpenoids, flavonoids, tannins, phenolics, saponins, carbohydrates, proteins, amino acids etc. [9] Due to resurgence of use of Ayurvedic formulations followed by high demand and low supply of this plant manufacturers are using cheap substitutes. However literature reveals that more than 60% Ayurvedic parameters as well as pharmacological actions of Ashtawarga plants do not match with their substitutes leading to reduced efficacy of the drugs along with loss of faith for use of herbal drugs. [10-11] 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 identification of authentic plant. So this study was carried out to find marker compound of plant that could help in authentication and regulate adulteration. Present communication presents first report of isolation and characterization of marker compound, 5-O-cafeoylquinic acid from methanolic extract of plant using various analytical techniques and can be used as marker for identification of the plant in market formulations. Isolated compounds from the present study can be used as a chemical marker for identification of this plant as the cost of synthetic compound is very high and industry people cannot add it from outside just to prove the presence of Jeevaka.

Research Article

ISOLATION OF 5-O-CAFFEOYLQUINIC ACID FROM NATURAL SOURCE MICROSTYLIS MUSCIFERA (LINDL.) KUNTZE: A POTENTIAL CHEMICAL MARKER FOR IDENTIFICATION

Raturi R1, Maithani M1, Gupta V1, Singhal RG2, Rawal RK3, Kumar S4, Singh R5 and Bansal P1*

1MRU, UCER, Baba Farid University of Health Sciences, Faridkot, Punjab, India
2Shobhit University, Meerut, Uttar Pradesh, India
3Department of Chemistry, Maharishi Markandeshwar, Mullana, Haryana, India
4Central Ayurveda Research Institute for Respiratory Disorders, Patiala, Punjab, India
5AVIPS, Shobhit University, Saharanpur, Uttar Pradesh, India

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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 number of rejuvenating Ayurvedic formulations. Ever rising demands, lack of natural sources and quantity insufficient to meet the requirements of 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 identification of authentic plant. So this study was carried out to find marker compound of plant that could help in authentication and regulate adulteration. Present communication presents first report of isolation and characterization of marker compound, 5-O-cafeoylquinic acid from methanolic extract of plant using various analytical techniques and can be used as marker for identification of the plant in market formulations. Isolated compounds from the present study can be used as a chemical marker for identification of this plant as the cost of synthetic compound is very high and industry people cannot add it from outside just to prove the presence of Jeevaka.

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formulation. The situation is being exploited by manufacturers because regulatory authorities lack the quality control tools (marker compound) needed for identification of authentic plant. Hence the present study was designed to isolate chemical marker of Jeevaka using column and TLC techniques.

MATERIAL AND METHODS

Plant material

*Microstylis muscifera* (Lindl.) Kuntze bulbs were procured from Himachal Pradesh and identified by the cultivator vide letter number No. HRG/Testimonial-NMPB/02/2015-2016 and further authenticated by Central Instrumentation Facility, National Botanical Research Institute (Lucknow) with Ref No: NBRI/CIF/669/2018. Bulbs were washed and dried at room temperature (<40°C). A voucher specimen has been stored in air tight container in the Department of Herbal Drug Technology at University Center of Excellence in Research, Baba Farid University of Health Sciences, Faridkot for future use.

Chemicals

All the solvents and reagents used in the study were of AR/GR grade. Precoated aluminum-backed TLC plates manufactured by E. Merck (Germany) with 0.2 mm layer of silica gel 60 F254 (20 cm × 10 cm) were purchased from local authorized dealer and used in isolation studies.

Preparation of Extract

Bulbs of *Microstylis muscifera* (Lindl.) Kuntze were coarsely powdered and defatted with petroleum ether followed by extraction with methanol through continuous hot extraction process. It was filtered, evaporated to obtain a semi solid mass and extracts were stored in desiccator for further use.\(^{[12-15]}\)

Phytochemical screening

Preliminary phytochemical screening was performed for the detection of phyto-constituents’ like alkaloids, glycosides, fatty acids, steroids, terpenoids, flavonoids, tannins, phenolics, saponins, carbohydrates, proteins, lipids, amino acids etc.\(^{[16-17]}\)

Isolation of chemical marker compound

Column chromatography of extract was done for isolation of single compound. Slurry of extract was prepared by dissolving 11.0g of extract in methanol followed by addition of adequate quantity of silica gel (60-120 mesh size / 0.120-0.250mm particle size). It was uniformly mixed by trituration and dried on water bath to get a free flowing powder. Further, Silica bed was prepared by pouring 810 g silica gel suspended in n-hexane into the glass column (1000mm × 50mm). After saturation of silica bed, slurry was charged into the column and allowed to stand overnight for uniform bed packing. Initially column was eluted with n-hexane followed by increase in polarity of solvent by addition of toluene, isopropl alcohol, chloroform, ethyl acetate and methanol in different ratio. Each fraction of 150mL was collected with optimum flow rate of 4mL/min. Fractions with similar TLC profile were pooled to give major fraction. TLC was performed by using different polarity solvents with hit and trial method. Elution with the solvent system toluene yielded a pool of three compounds with \(R_f\) 0.44, 0.51, and 0.71 on TLC plates by use of mobile phase composition of Chloroform: Ethyl acetate: Formic acid (3:7:0.1 v/v). Single compound was isolated by cutting and pooling of TLC plate of compound having \(R_f\) 0.71. The compound was obtained in fractions numbered 749 to 900 and purified by recrystallization with methanol. 5-O-caffeoylquinic acid (CQA) fraction was kept in refrigerator to get the crystallized compound as per earlier standard method.\(^{[18-21]}\)

Characterization of isolated compound

Isolated compound was characterized by chemical test, melting point, spectral analysis and compared with literature.

TLC of 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 extract. Out of 15 tried mobile phases, Chloroform: Ethyl acetate: Formic acid (3:7:0.1 v/v) has shown best separation of isolated compound in extract. \(R_f\) of isolated compound has been found to be 0.71.

RESULTS

Physical evaluation of extract

The isolated compound was white crystalline powder.

Phytochemical screening of extract

Preliminary phytochemical analysis of the methanolic extracts of plant has shown presence of flavonoids (with lead acetate test) and amino acids (with Ninhydrin test).

Identification of isolated compound

Physicochemical description

Isolated compound was found as white crystalline powder having melting point in the range of 205-209°C.

Spectral Analysis

IR of Isolated Compound

Very immense and strong overlapping bands in the IR spectrum, which appear in the region of 3800–2900 cm\(^{-1}\), were assigned to the different O–H vibrations. Both the C–H stretching modes of aliphatic and aromatic moiety were found at 2854.3 and 2924.3 cm\(^{-1}\). The bands of the medium and strong intensities at 1730.4 (IR) and 1695.3 cm\(^{-1}\) (IR) were assigned to the C=O stretching modes of the acyclic and quinic moiety. In addition, the bands of the medium and strong intensities at 1636.3, and 1461.2 cm\(^{-1}\) were assigned to the C=C stretching modes of the acyclic chain and benzene moiety which confirm the skeleton of CQA (Figure 1).

Mass Spectra

The molecular ion peaks were found at m/z 354.38 (M \(^{+}\)) and 355.24 (M+1) \(^{+}\) in mass spectra of the isolated compound. The
mass spectra also showed parent ion peaks at 353.36 (M-H)− which was being in agreement with the proposed structure of CQA (Figure 2).

**Structure and Molecular Formula of Isolated Compound**

The molecular formula of isolated molecule is CQA (Figure 3) that is confirmed by IR and mass spectral data available in literature. Its IUPAC name is (1S,3R,4R,5R)-3-{[(E)-3-[(3,4-dihydroxyphenyl)acryloyl]oxy]-1,4,5-trihydroxycyclohexane carboxylic acid.

**TLC of isolated compound alone and with extract**

TLC of isolated compound with extract and isolated compound alone was performed. *Rf* of isolated compound has been found to be 0.71. TLC of isolated compound.

**DISCUSSION**

CQA also known as Chlorogenic acid is one of the most available acids among phenolic acid compounds which can be naturally found in fruits/vegetables (apples, pears, carrots, tomatoes, and sweet potatoes) and in coffee as well as tea.[22-24] CQA is an important and biologically active dietary polyphenol, playing several important therapeutic roles such as natural antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, anti-hypertension, free radicals scavenger and a central nervous system (CNS) stimulator.[25-26] In addition, it has been found that CQA could modulate lipid metabolism and glucose in both genetically and healthy metabolic related disorders. It is speculated that CQA can perform crucial roles in lipid and glucose metabolism regulation and thus help to treat many disorders such as hepatic steatosis, cardiovascular disease, diabetes and obesity as well. Furthermore, this phenolic acid (CQA) causes hepatoprotective effects by protecting animals from chemical or lipopolysaccharide-induced injuries.[27-30] The hypocholesterolemic influence of CQA can result from the altered metabolism of nutrients, including amino acids, glucose and fatty acids. So probably that is the basis of including this plant in very important anti-aging and health promoting Ayurvedic formulations. Hence substitution of Jeevaka plant with substandard drugs and substitutes may play with desired therapeutic effect of plant. Hence the chemical marker isolated in the study can be used for differentiating use of substitutes in high cost formulations claiming to use this rare medicinal plant. It is important to mention here that the market price of CQA is $80/g (approximately) and it will be difficult for commercial manufacturers to replace Jeevaka plant with CQA just to claim the presence of Jeevaka.[30-36] However, if equivalent amount of Jeevaka is added then it will be a cheaper for the industry, in addition to its original status as a drug.

**CONCLUSION**

In the present study authors isolated CQA from bulbs of *Microstylis muscifera* (Lindl.) Kunze using column chromatography and TLC that was confirmed by chemical test, melting point, IR and Mass spectroscopy. As per our knowledge, this is first report on chromatographic method of isolation of CQA from natural source *Microstylis muscifera* (Lindl.) Kunze (Common name: Jeevaka). This compound can be used as a chemical marker for identification of this plant as the cost of synthetif compound is very high and industry people cannot add it from outside just to prove the presence of Jeevaka.

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**Conflict of interest**

The authors confirm that this article content has no conflict of interest.

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