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First Report of Isolation of Moupinamide as a Chemical Marker from Natural Source *Polygonatum verticillatum*

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ABSTRACT

Polygonatum verticillatum (*Mahameda*) is an important ingredient of Ashtawarga and many other important rejuvenative *Ayurvedic* formulations. Now-a-days, it comes under the category of endangered plants due to large scale and indiscriminate collection of wild material. To overcome the scarcity, substitutes of *Mahameda* that are different from authentic plant by *Ayurvedic* as well as pharmacologic theory of drug action are commonly used in market and lead to substitution/adulteration. The present study was designed to isolate and identify chemical marker of *Mahameda* for detection of unauthorized substitution. Methanolic extract of rhizomes of *Mahameda* was subjected to preliminary phytochemical screening which shown the presence of flavonoids, carbohydrates, terpenoids, phenolics and alkaloids. It was followed by column chromatography for isolation of marker compound. Initially, the column was eluted with pure *n*-hexane and polarity of solvent was gradually increased. Total 7800 fractions were collected and pooled on the basis of Thin Layer Chromatography (TLC) profiling. A pure white crystalline powder was isolated by column chromatography and characterized as (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl) ethyl] prop-2-enamide (commonly known as moupinamide) with the help of chemical tests, melting point and spectral analysis. The isolated compound, moupinamide, is identified as novel compound reported for first time from natural source and can be used as a chemical marker for regulating adulteration with substandard/spurious substitutes in market formulations.

INTRODUCTION

Polygonatum genus is an erect or decumbent perennial herb belonging to the family Liliaceae with about 57 species around the world. It is widely distributed in the temperate regions of the northern hemisphere and concentrated in the Himalayas [1]. The generic name of *Polygonatum* has been derived from the morphological feature of rhizome that bears resemblance to Yovi (a knee) because it bears many little knee shapes [2]. It is found in India, East Asia (China, Japan, etc.), Pakistan, Korea, Nepal, Afghanistan, Bhutan, Russia, and in moderate climate zones of North America and Europe. In India, *Mahameda* is found in the temperate Himalaya from Kashmir (at an altitude of 2000-3600m asl) to Sikkim (at an altitude of 2600-4000 m asl), Himachal Pradesh and Uttarakhand (1600-3500m asl) [3]. A thick, fleshy, and creeping sympodial rhizome is the characteristic feature of *Polygonatum*. It has a very high therapeutic value and globally used in herbal medicine for thousands of years. All plant parts of *Polygonatum* have some medicinal value but the rhizome is the most important than other parts because of its innate adaptogenic, anti-oxidant, cardio-tonic, demulcent, diuretic, energizer, hypoglycemic, tonic, anti-bacterial and anti-fungal activities [4]. It is also used in the treatment of pulmonary problems for dry cough and tuberculosis [5, 6]. Traditionally, *Mahameda* is effective against emaciation, senility, pain, pyrexia, weakness, burning sensation, phthisis, pulmonary affections, and also has other significant effects like a tonic, galactagogue, emollient, aphrodisiac, insecticidal and leishmanicidal [3, 7]. The rhizome of *Mahameda* is proven for therapeutic activities because it contains steroidal saponins and polysaccharides [8]. Rhizomes of *Mahameda* have been proven for anti-nociceptive, anti-malarial, anti-oxidant, anti-microbial, anti-bacterial, anti-spasmodic, anti-diarrheal, anti-pyretic, anti-convulsant, tracheorelaxant, and anti-inflammatory activities [9-16].

P. verticillatum is commonly known as *Mahameda* in Hindi and Whorled Solomon's seal in English. Rhizomes of *Mahameda* are a fundamental part of Ashtawarga group and numerous *Ayurvedic* formulations like *Chyawanprash, Vachadi Taila, Vajikaran Ghrita, Astavarga Churna, Chitrakadi Taila, Mahakalyan Ghrita, Mahamayura Ghrita, Jivaniya Ghrita, Mahapadma Taila, Brahini Gutika, Indrokta Rasayan*, etc. [3]. Due to loss of habitat, decreased cultivation, extensive usage, increased market demand followed by destructive collection from natural resources/forest, *Mahameda* plant has been pushed in the category of endangered plants [7, 8].

To overcome the absence of the original plant and to meet the market pressure, today, substitutes of *Mahameda* and other Ashtawarga plants are commonly used in pharmaceutical preparations. The official and commercial substitutes of *Mahameda* have been suggested by the Department of AYUSH and by ancient texts however its substitutes have different phytochemical, pharmacological and *Ayurvedic* profile of drug action [7, 17]. As the substitutes/adulterants of these plants are available at very low rates, the manufacturers opt for using substandard raw material for medicine production. This substitution and adulteration further discourage the cultivators of these rare plants and other endangered medicinal plants [7, 18,19]. As an overall scenario, the manufacturers fearlessly adulterate the herbal formulations leading to a setback to faith in *Ayurvedic* formulations as well as challenge and malign the authenticity of the *Ayurvedic* system [7, 18]. This is happening because of the lack of chemical markers of *Mahameda* with the regulatory authorities to detect and define the substitution/adulteration by sub-standard raw drugs/substitutes etc. Hence, it is very important to find out the chemical markers of these rare plants that could help in the detection of substitutes. The present study was conducted to identify and isolate a chemical marker for *Mahameda*.

MATERIALS AND METHODS:

Plant material

Rhizomes of *Mahameda* were procured from an approved cultivator from Himachal Pradesh and authentication was done by the cultivator vide letter No. HRG/Testimonial-NMPB/02/2015-2016. Authentication of the plant was also done by Central Instrumentation Facility, National Botanical Research Institute (Lucknow) with Reference No: NBRI/CIF/524/2016. Crude plant material was dried under shade (<40°C). The dried material was coarsely powdered and stored in a desiccator for future use.

Chemicals

Analytical grade chemicals and reagents were used during the experimental work and these were purchased from different companies like Qualikems, Finar, and Merck, etc. Silica gel 60 F_{254} (Merck) plates were used for Thin Layer Chromatography (TLC) and silica gel (60-120mesh size / 0.120-0.250mm particle size) was used for column chromatography.

Extraction

The coarse powder of rhizomes of *Mahameda* was defatted with petroleum ether and the methanolic extract was prepared by the continuous hot extraction process. It was filtered and the filtrate was concentrated by distillation to obtain a semi-solid residue. The percentage yield was calculated and the extract was kept in a desiccator for further use [20-23].

Phytochemical screening

The extract of *Mahameda* was subjected to preliminary phytochemical screening for the detection of various phytoconstituents viz. alkaloids, glycosides, steroids, terpenoids, phenolic compounds, saponins, carbohydrates, flavonoids, tannins, proteins and amino acids [24, 25].

Isolation of marker compound

The extract was subjected to column chromatography for the isolation of the marker compound. It comprises of following steps:

Preparation of extract slurry

Rhizome extract (8.5g) was dissolved in the least amount of methanol and a sufficient quantity of silica gel (60-120mesh size) was added through trituration for uniform mixing. After proper mixing, the slurry was dried on a water bath to get a free-flowing powder [20-23].

Packing of column

The wet packing method was used for column packing. About 680g of silica gel was suspended in *n*-hexane and poured into the glass column. The column was allowed to stand overnight for uniform bed packing. After saturation of bed, drug slurry was loaded into the column, and elution was done with solvents of increasing polarity [20-23].

Elution of column

Initially, the column was eluted with *n*-hexane and the solvent polarity was increased gradually by the addition of chloroform in the ratio i.e. 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9 up till pure chloroform. It was followed by a change in polarity by the addition of ethyl

acetate to chloroform until the use of pure ethyl acetate for elution. Afterward, the polarity was again changed by the use of methanol until pure methanol was used for elution. A total of 7800 fractions (each of 150ml) were collected with a flow rate of 4ml/minute as per the prescribed flow rate and TLC of collected fractions was done by using solvents of different polarities. The solvent system for TLC was standardized by the hit and trial method. Fractions with similar TLC profiles were pooled to give major fractions and a total of 50 pooled sub-fractions were collected. The white-colored compound was obtained from fractions numbered 4570 to 4750 and it was purified by recrystallization with methanol. The isolated compound was kept in the refrigerator to get the crystalline compound [20-23]. Identification of isolated compound was confirmed by chemical tests, melting point, spectral analysis (IR and Mass spectra), and further by comparison with literature.

Characterization of isolated compound

Chemical test

The extract was treated with a few milliliters (ml) of dilute HCl, filtered, and subjected to the tests. In Dragendorff's reagent test, to 2-3ml of filtrate, 0.5ml of Dragendorff's reagent was added. The formation of orange-brown precipitates reveals the positive test for alkaloids. In the case of Mayer's reagent test, to 2-3ml filtrate, 0.5ml of Mayer's reagent was added. The formation of cream-colored precipitates reveals the positive test for alkaloids [21].

Melting point

The melting point of the crystallized compound was determined by using the melting point apparatus.

Spectral analysis

Different spectroscopic techniques such as Infra-Red (IR) and Mass spectral analysis were used to identify the structure of the isolated compound [24, 26]. An IR spectrum was recorded on FTIR Perkin Elmer and the mass spectrum was recorded on Mass Spectrometer Model Q-ToF Micro Waters equipped with Electrospray Ionization (ESI) at Panjab University, Chandigarh.

TLC

The aim of TLC was to develop a method capable to separate the marker from the extract. About 40 mobile phases were used to achieve the separation of an isolated compound from the extract. Out of trials, mobile phase, *n*-hexane: ethyl acetate (4:6v/v) was shown the best separation of a marker from extract [27,28].

RESULTS AND DISCUSSION:

RESULTS

Physical evaluation

The dark brown colored semi-solid mass of extract was obtained and the percentage yield was found to be 14% (w/w).

Phytochemical screening

Phytochemical screening of extract was shown the presence of carbohydrates, terpenoids, flavonoids, phenolics, and alkaloids.

Identification of an isolated compound

Physicochemical characterization

The isolated compound was found as a white crystalline powder with the melting point in the range of 143-145°C (Lit. 144.5 - 145°C).

Chemical test

The isolated compound was shown the positive test for alkaloids.

IR spectrum

The IR spectrum was shown the peaks at 3198.68cm^{-1} (phenolic O-H stretching), 2955.64-2924.62cm^{-1} (aromatic C-H stretching), 2854.66cm^{-1} (aliphatic C-H stretching), 1735.65cm^{-1} (carbonyl C=O stretching of –CONH group), 1617.68cm^{-1}, 1457.67cm^{-1} (aromatic C=C, olefinic C=C Stretching), 1401.64 (OCH_3 bending), 1169.67cm^{-1} (phenolic C-O stretching), 832.71cm^{-1} (C-N stretching); confirm the skeleton of (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide (Figure 1).

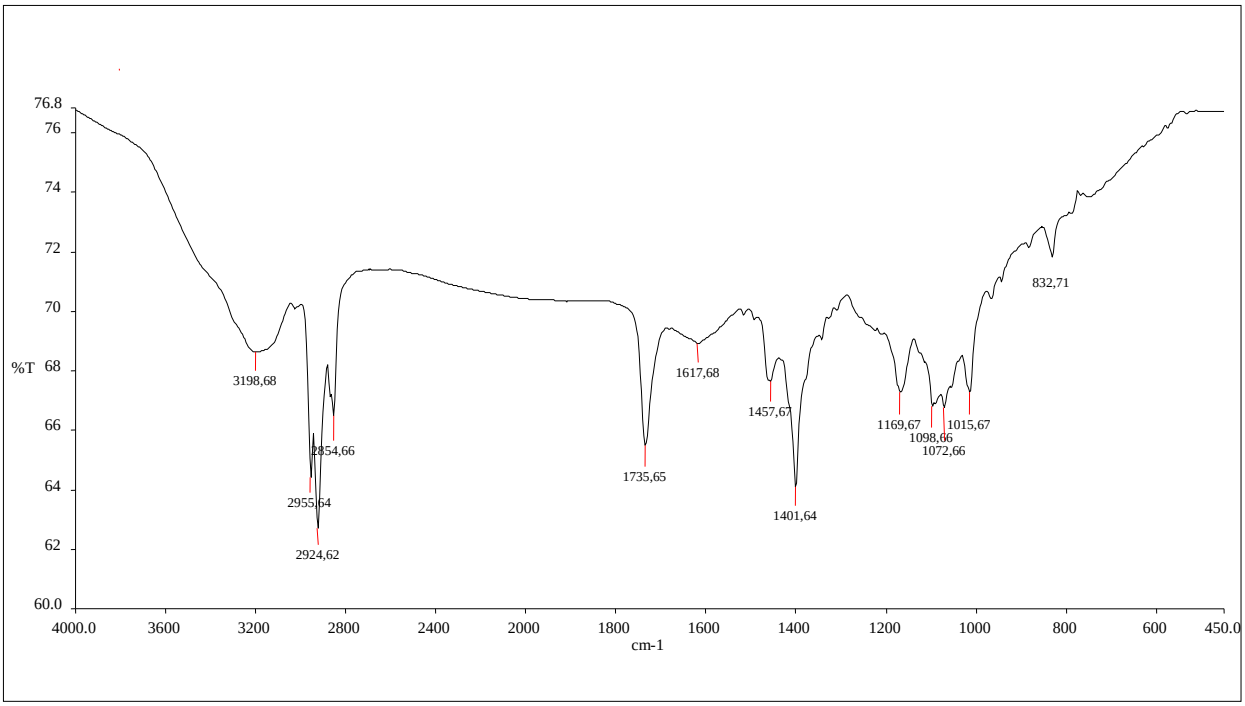

Figure No. 1: IR spectrum of isolated compound.

Mass spectrum

The mass spectrum of the compound was shown a molecular ion peak at m/z 313 (M^+, **I**). It was also shown fragmentation peaks at 281 (**II**), 207 (**III**), and 149 (**IV**+1), being in agreement with the proposed structure of (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)ethyl] prop-2-enamide (Figure 2-3).

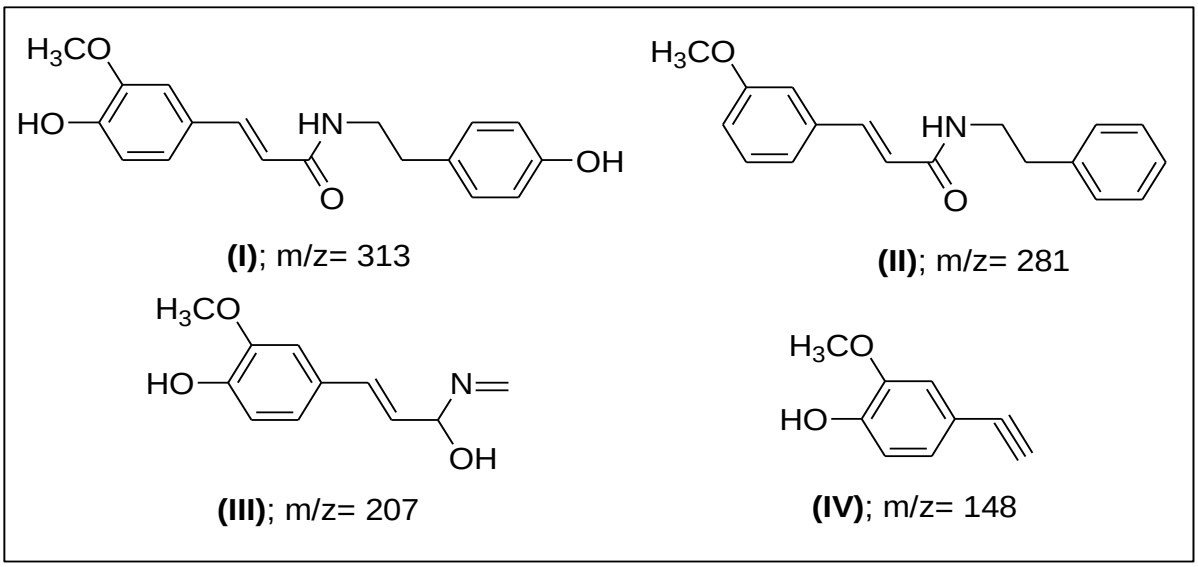

Figure No. 2: m/z value of isolated compound.

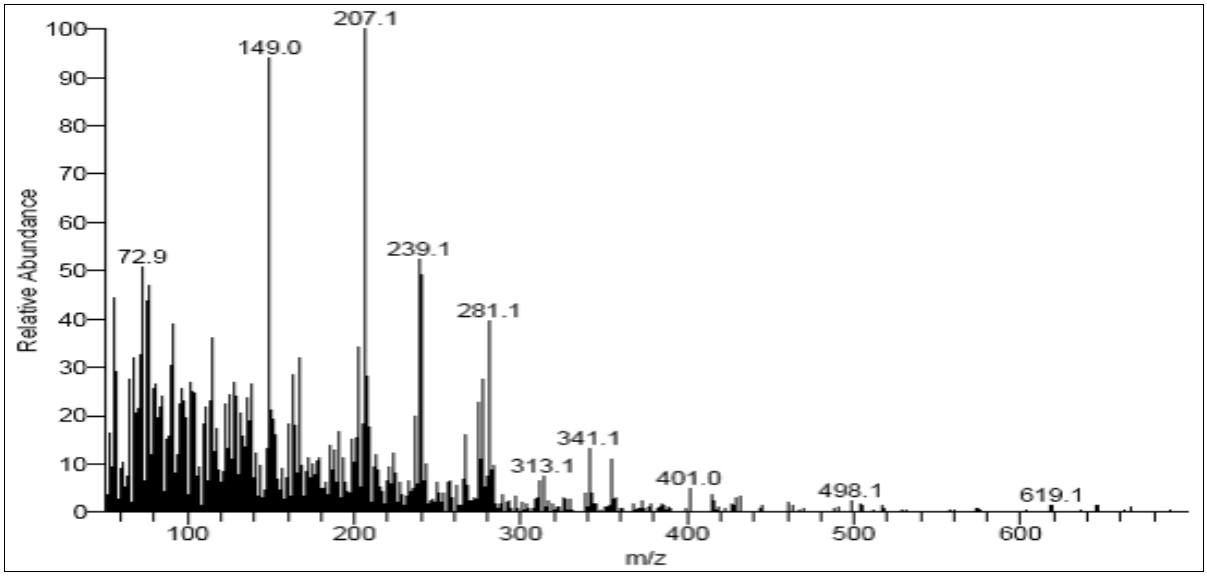

Figure No. 3: Mass spectrum of isolated compound.

Structure

IUPAC name of isolated molecule is (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)ethyl] prop-2-enamide or *N-trans*-feruloyltyramine or moupinamide (Figure 4) that was confirmed by IR and Mass spectral data available in the literature.

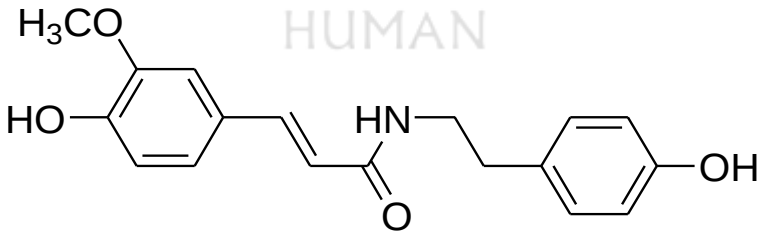

Figure No. 4: Structure of isolated compound.

TLC

TLC chromatogram of extract and isolated compound is shown in Figure 5. R_f of the isolated compound was found 0.56.

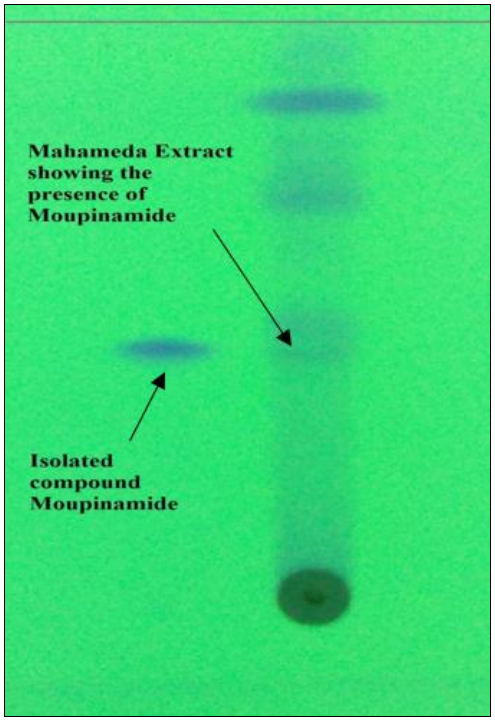

Figure No. 5: TLC chromatogram of the extract and isolated compound.

DISCUSSION:

Moupinamide is an alkaloid and belongs to the category of Methoxyphenols and derivatives [29]. Since *P. verticillatum* is a rare plant and comes under the category of endangered plants, it was pertinent to isolate and identify chemical markers that could be used by regulatory authorities for regulating the unauthorized use of substitutes/adulterants [20-23]. The market price of *moupinamide* is Rs. 32,896 per milligram [30]. If industries add even 10µg of *moupinamide then its cost will be Rs. 328.96 which is even more than the market price of* Chyawanprash, a well-known rejuvenating *Ayurvedic* formulation. So, it is very difficult for industrial organizations to replace *Mahameda* plant with the addition of synthetic *moupinamide* just to claim the presence of *Mahameda*.

CONCLUSION:

In the present study, the investigators isolated *moupinamide* from rhizomes of *P. verticillatum*. It was confirmed by chemical test, melting point, IR and Mass spectroscopy. To the best of our knowledge, this is the first report on the method of isolation of *moupinamide* from *Mahameda*. It is further concluded that it can also be used as a supplement marker for the identification of *Mahameda* because the cost of an isolated

compound is very high and industries cannot add it from outside sources just to claim the presence of *Mahameda*.

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Conflict of interest statement

The authors declared no conflict of interest.

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Graphical abstract