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Chemical composition of *Microstylis wallichii* Lindl. from Western Himalaya

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Microstylis wallichii **Lindl. is a Rasayana and belongs to the "*Astverga".* It is an important medicine traditionally known since Vedic period but study on its phytoconstituents is very limited. Metal content and volatile constituents *in M. wallichii* (family Orchidaceae) collected from Uttarakhand were analyzed by Atomic Absorption Spectrophotometer and GC and GC-MS respectively. Chemical analysis revealed that wild *M. wallichii* contained 6.48 ppm Cu, 43.00 ppm Zn, 35.00 ppm Mn, 331.00 ppm Fe, 21600.00 ppm K, 9000.00 ppm Ca, 2800.00 ppm Mg, 198.00 ppm Al, 26.70 ppm Ba, 55.60 ppm B, 0.30 ppm Mo, 156.00 ppm Cl. Fatty acids analysis revealed the presence of the following constituents-linoleic acid (18:2ω6) 61.20% (w/w), α-linolenic acid 18.10% (w/w), oleic acid 12.00 % (w/w), palmitic acid (16:0) 6.00% (w/w), stearic acid (18:0) 2.10% (w/w), γ-linolenic acid (18:3ω6) 2.20% (w/w), eicosanoic acid (20:0) 0.81 % (w/w), eicosenoic acid (20:1) 0.42% (w/w) and eicosadienoic acid (20:2) 0.04% (w/w). Cultivated *M. wallichii* contained 7.18 ppm Cu, 49.50 ppm Zn, 37.00 ppm Mn, 352.45 ppm Fe, 23000.00 ppm K, 13000.00 ppm Ca, 5300.00 ppm Mg, 217.50 ppm Al, 37.50 ppm Ba, 59.70 ppm B, 0.27 ppm Mo, 148.00 ppm Cl. Analysis of fatty acids reported following constitutents of cultivated *M. wallichii* - linoleic acid (18:2ω6) 65.23% (w/w), α-linolenic acid 15.50% (w/w), oleic acid 14.87 % (w/w), palmitic acid (16:0) 5.90% (w/w), stearic acid (18:0) 2.50% (w/w), γ-linolenic acid (18:3ω6) 1.87% (w/w), eicosanoic acid (20:0) 0.69 % (w/w), eicosenoic acid (20:1) 0.52% (w/w) and eicosadienoic acid (20:2) 0.07% (w/w). Other chemical constituents which were isolated from wild and cultivated *M. wallichii* were vitamins α–tocopherol and γ-tocopherol 12.00-9.80 ppm and 695.00-786.7 ppm respectively while terpenoids 18.00-20.50%. It has an acid value of 1.20-1.39 and saponification value of 103.00-110.50.**

Key words: *Microstylis wallichii*; metal content; volatile constituents; GC-MS

INTRODUCTION

Orchids occupy a wide range of habitats and exhibit highly specialized morphological, structural and physiological characteristics (Dressler, 1990). *Microstylis wallichii* Lindl. has been reported by another vernacular name *Malaxis acuminata* D. Don. It belongs to family Orchidaceae, also known as Jeevak. It is a terrestrial herb,

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10-25cm high; bulbous at base covered by old leafy scales. Leaves 3(4), are ovate- lanceolate, membranous and measuring 10-15 X 5-6.5cm showing acute apex and undulate margins. Flower pale-green tinged purple, shortly stalked, 1-1.2cm across, on many flowered, 8-10cm long spikes; bracts linear, minute. Sepals oblong; lateral broad and short with recurved margins. Petals are linear, longer than sepals. Lip shield like, broadly ovate, somewhat convex, tip notched, auricles at base straight or overlapping. It shows flowering and fruiting from August-October (Gaur, 1999).*M. wallichii* is a plant of temperate to alpine region. The alpine climatic conditions are often characterized by high ultraviolet radiation, low atmospheric pressure and oxygen concentration, with the minimum air temperature dropping near freezing point every night. Alpine plants, however still manage to grow under these conditions and form a large group of medicinal plants in the alpine region. They appear as different growth forms and synthesize secondary metabolite to tolerate stress conditions. Secondary metabolites are in the form of alkaloids, amino acids, trisaccharides, amines and purines and they are stored in underground part. Availability of these secondary metabolites has gained maximum attention and curiosity among modern pharmaceutical companies for these plants. The increasing use of herbal medicines all over the word has further increased demand of these plants (Nautiyal and Nautiyal, 2004).*M. wallichii* is a Rasayana that belongs to the "*Astverga".* It constitutes a group of eight drugs named; *Jivak, Rishbhak, Mahameda, Meda, Kakoli, Khirkakoli, Ridhi* and *Bridhi* (Chunekar, 1982; Singh, 2006). These drugs are important constituent of a number of *Ayurvedic* preparations (Sharma *et al*., 2009). Rhizomes are used in skin diseases (Kushtha), piles (Arsha), diseases of children (Balrog), burning sensation (Daha) and Fistula-in-ano (Bhagandra) (Anonymous, 2008). Locally the bulbs are used in bronchitis as well as given as a tonic (Gaur, 1999). Paste of pseudobulb can be applied externally in case of insect bites and mixture with other plants is used in the treatment of rheumatism (Chakarvarty, 1976). Different formulations of the plant species are also well documented (Anonymous, 2006). The literature survey of the plant revealed that very little research work dealing with its chemistry has been carried out so far. In this study, we aimed to provide the chemical composition of *M wallichii* for the acclaimed medicinal use and to determine the quality of the drug prepared using this herb.

MATERIALS AND METHODS

Plant

Pseudobulbs of *M. wallichii* were collected from wild locations of Almora, Kumaun, Western Himalaya and experimentally cultivated at SMP Garden, Regional Research Institute of Himalayan Flora, Tarikhet, Ranikhet. After completion of agro trials for three subsequent years another sample of peudobulbs was collected from the cultivated and treated garden and pseudobulbs sample showing best yield were again chemically analyzed to see the effect of *ex-situ* cultivation on chemical constituents of this herb (Figure 1). The plant material was identified and authenticated taxonomically by Drug Standardization Research Unit, C.R.I. (ay), Lucknow. A voucher specimen of the collected sample was deposited in the institutional herbarium for future reference.

(a) (b)

Figure 1. (a) Whole plant and (b) Rhizomes of *M. wallichii* Lindl.

Preparation of ethanolic extract

The shade dried plant materials was crushed, powdered and exhaustively extracted by overnight maceration with 10 volumes of 50% ethanol. The extracts were filtered, pooled and concentrated on rotary evaporator (Buchi, USA) and dried in lyophilizer (Labconco, USA) under reduced pressure to obtain 10% of solid residue.

Plant grinding

All the samples were examined prior to grinding to determine if they are dry enough to be ground, are properly labelled, and free from anything unusual, such as fungus growth on them etc. Proper drying of each sample was ensured by vacuum drying without heating. Samples were ground with a grinder. The ground plant sample was then sieved through a series of mesh sizes utilizing a Tyler Industrial Product, model RX24, portable shaker.

Extract preparation

Fine powder (100 g) was mixed thoroughly with 500 ml of 50% ethanol and kept at room temperature overnight. On the second and on the 3rd day, 500 ml of fresh 50% ethanol was added to this mixture, and after mixing thoroughly, kept at room temperature overnight. Similarly, on the fourth day, the mixture was filtered and processed for rotavaporation to concentrate the filtrate. Then the concentrated filtrate was freeze dried (lyophilized) to get a powder. The samples were then processed as per the specific protocols for different analysis.

Metal analysis

Metal analysis was done using the ground powder of each sample (not with the extract). One gram of each of the ground sample in triplicate was digested by gentle heating, initially with 10 ml concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$ [4: 1 v/v] in Kjeldahl flask until no brown fumes appeared (Vanloon and Lichwa, 1973). The final volume was made up to 10 ml in a volumetric flask with 1% HNO_3. Analysis of the metal was carried out on an Atomic Absorption Spectrophotometer. The measurements were made using an external standard calibration graph. Each analysis was done in triplicate including blanks.

Analysis of alkaloids, fatty acids and other volatiles

GC-MS combined with headspace solid-phase micro extraction (HS- SPME) method was used to analyze the profiling of free volatile compounds in given plant extracts. Prior to the analysis, conditions for sample preparation (SPME fiber type, extraction time, extraction temperature and dilution solvent) and GC-MS conditions were standardized to get optimized methodologies and results. Following the standardization, the extracts were suspended thoroughly with water and 8 ml of sample was placed in a 20 ml headspace vial with addition of NaCl; a polydimethylsiloxane SPME fiber was used for extraction at 40 ^{0}C for 30 minutes with continuous stirring. Identification of components was obtained by comparison of their retention time with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of n- hydrocarbons. Further validation and authentication of the product was done using Library of databases available with GC-MS. The calculations and quantification were done using software provided with the equipment.

Statistical analysis

Comparison of data was done by using the Student's t-test and is presented as mean ± standard deviation. Comparison of data was made in triplicate for quantification. Values of $p < 0.05$ were considered significant.

RESULTS

Phytochemical analysis results obtained for wild and cultivated samples of *Microstylis wallichii* are tabulated in Tables 1 to 3. The analysis showed high variety of nutrients/metals, fatty acids and other chemical constituents are present in wild crafted as well as in cultivated conditions and can be used as drugs in different compositions of medicines.

Chemical analysis results revealed that wild sample of *Microstylis wallichii* contains metals/nutrients- 6.48 ppm Cu, 43.00 ppm Zn, 35.00 ppm Mn, 331.00 ppm Fe, 21600.00 ppm K, 9000.00 ppm Ca, 2800.00 ppm Mg, 198.00 ppm Al, 26.70 ppm Ba, 55.60 ppm B, 0.30 ppm Mo, 156.00 ppm Cl and fatty acids- Linoleic acid (18:2ω6) 61.20% w/w, α -Linolenic acid (18:3ω3) 18.10% w/w, Oleic acid (18:1ω9) 12.00 % w/w, Palmitic acid (16:0) 6.00% w/w, Stearic acid (18:0) 2.10% w/w, γ-Linolenic acid (18:3ω6) 2.20% w/w, Eicosanoic acid (20:0) 0.81% w/w, Eicosenoic acid (20:1) 0.42 % w/w and Eicosadienoic acid (20:2) 0.04% w/w. Other chemical constituents which were isolated from *Microstylis wallichii* are vitamins α –Tocopherol and γ-Tocopherol 12.00 ppm and 695.00 ppm respectively, terpenoid 18.00%. It has an acid value of 1.20, saponification value of 103.00.

The cultivated sample of *Microstylis wallichii* contained metals/nutrients- 7.18 ppm Cu, 49.50 ppm Zn, 37.00 ppm

Table 1. Analysis of metal/ nutrients.

Metal/nutrients	Wild sample (ppm) ± SD	Cultivated sample (ppm) ± SD
Cu	6.48*±0.52	7.18±0.32
Zn	43.00*±0.52	49.50±0.70
Mn	35.00±1.32	37.00±2.18
Fe	331.00*±0.82	352.45±4.80
K	21600.00*±47.70	23000.00±52.12
Ca	9000.00*±26.63	13000.00±42.72
Mg	2800.00*±45.51	5300.00±59.63
Al	198.00*±3.61	217.50±7.21
Ba	26.70*±1.30	37.50±1.00
B	55.60*±0.52	59.70±1.28
Mo	0.30±0.06	0.27±0.06
Cl	156.00±3.00	148.00±4.00

*significance at p≤ 0.05

Table 2. Analysis of fatty acids

Fatty acid	Wild sample Percent (w/w) ± SD	Cultivated sample Percent (w/w) ± SD
Linoleic acid (18:2ω6)	61.20*±1.20	65.23±0.47
α -Linolenic acid (18:3ω3)	18.10*±0.26	15.50±0.50
Oleic acid (18:1ω9)	12.00*±1.00	14.87±0.28
Palmitic acid (16:0)	6.00±0.10	5.90±0.36
Stearic acid (18:0)	2.10±0.17	2.50±0.36
γ-Linolenic acid (18:3ω6)	2.20±0.17	1.87±0.11
Eicosanoic acid (20:0)	0.81*±0.02	0.69±0.05
Eicosenoic acid (20:1)	0.42±0.08	0.52±0.08
Eicosadienoic acid (20:2)	0.04±0.00	0.07±0.03

*significance at p≤ 0.05

Table 3. Other chemical constituents

Chemical constituents	Wild sample	Cultivated sample
α –Tocopherol (ppm)	12.00*±0.95	9.8±0.98
γ-Tocopherol (ppm)	695.00*±8.66	786.7±3.11
Acid value	1.20±0.02	1.39±0.07
Saponification value	103.00*±1.73	110.5±0.87
Terpenoid (%)	18.00*±0.44	20.50±0.43

*significance at p≤ 0.05

Mn, 352.45 ppm Fe, 23000.00 ppm K,1300.00 ppm Ca, 5300.00 ppm Mg, 217.50 ppm Al, 37.50 ppm Ba, 59.70 ppm B, 0.27 ppm Mo, 148.00 ppm Cl, and fatty acids-Linoleic acid (18:2ω6) 65.23% w/w, α-Linolenic acid (18:3ω3) 15.50% w/w, Oleic acid (18:1ω9) 14.87% w/w, Palmitic acid (16:0) 5.90% w/w, Stearic acid (18:0) 2.50% w/w, γ-Linolenic acid (18:3ω6) 1.87% w/w, Eicosanoic acid (20:0) 0.69 % w/w, Eicosenoic acid (20:1) 0.52% w/w and Eicosadienoic acid (20:2) 0.07% w/w. Other chemical constituents which were isolated from *Microstylis wallichii* are vitamins α–Tocopherol and γ-Tocopherol 9.80 ppm and 786.7 ppm respectively, terpenoid 20.50%. It has an acid value of 1.39 and saponification value of 110.5.

DISCUSSION

The qualitative analysis of both the samples *viz*. wild and cultivated showed that all the chemical contents which were found in the wild samples were also present in cultivated samples. The quantitative comparison of the wild and cultivated samples revealed that in the cultivated samples, the quantity of most of the metals/nutrients was increased significantly while quantity of some metals/ nutrients was slightly lower but not significant. This increment in the quantity may be due to the presence of nutrients in the fertilizer with which the cultivation beds were supplemented. Quantity of some of the fatty acids and most of the other chemical constituents were also significantly influenced by the type of sample. There appeared to be considerable variation in the quantitative composition of the chemical contents.

One sterol namely β-sitosterol and an alcohol identified as cetyl alcohol, two sugars namely glucose and rhamnose and five basic compounds one of them being cholin were reported from the *M. wallichii* (Bhatnagar *et al*., 1970). Limonene, eugenon, citronellal, 1, 8 cineole, piperitone and p-cymene were reported to occur in *Microstylis wallichii* by thin layer chromatographic separation (Gupta *et al*., 1978). The quality of the wild crafted drug *Microstylis wallichii* Lindl varied with the place of collection (Sharma et al., 2009). *M. wallichii* is important for its medicinal uses in traditional system of medicine since Vedic period but study on the phytoconstituents is very less. *Malaxis* orchids are believed to contain large number of alkaloids, glycosides, flavonoids (Sharma *et al*., 2011). Chemical compounds reported from *Microstylis wallichii* are citronellal, eugenol, limonene, 1-8 cineole, p-cymene, O-methylbatatasin and cetyl alcohol (Bhatnagar, 2001; Sharma, 2003; Caius, 1986).

Extensive literature survey of this valuable plant revealed that no extensive research work dealing with the isolation and characterization of chemical contents of *Microstylis wallichii* has been carried out so far by the scientific community and this valuable medicinal plant of Himalayan region needs to be chemically explored well. Therefore, more useful work for purification, isolation and characterization on bio-active compounds of this plant is required.

CONCLUSION

The present study shows that in the wild and cultivated samples of *Microstylis wallichii*, all the chemical contents qualitatively remained same. However, there is considerable variation in the quantitative composition of the chemical contents of both the samples.

So, it may be concluded that the mass scale cultivation of this herb in the climatic conditions of Ranikhet may help to fulfill the increasing raw drug demand of pharmaceutical companies but still more work in its chemistry is required for its proper use in pharmaceutical formulations.

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