SUPPLEMENTARY MATERIAL

Composition and in vitro cytotoxic activities of essential oil of *Hedychium spicatum* from different geographical region of Western Himalaya by principal component analysis

Authors and Affiliations

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Abstract

The rhizome of *Hedychium spicatum* has been widely used in traditional medicines. Present study deals with the evaluation the cytotoxic potential of rhizome essential oil of 4 different regions of western Himalaya (India) along with, comparative correlation analysis to characterize the bioactive cytotoxic component. The essential oils coded as MHS-1, MHS-2, MHS-3 and MHS-4, characterized using GC-FID and GCMS. The main volatile compounds identified were 1, 8-Cineol, Eudesmol, Cubenol, Spathulenol and α -Cadinol. The in vitro cytotoxic activities were assessed against human cancer cell lines such as Lung (A549), Colon (DLD-1, SW 620), Breast (MCF-7, MDA-MB-231), Head and Neck (FaDu) and Cervical (HeLa). MHS-4 is significantly active in comparison to other samples against all the cancer cell lines. Sample MHS-4 have major proportion of monoterpene alcohol mainly 1, 8-cineol. Principal Component Analysis (PCA) has been done for the experimental results and all four samples clustered according to their percentage inhibition at different doses.

Keywords: *H. spicatum*; rhizome essential oil; cytotoxic activity; PCA

Experimental Part

1. Plant Material

H. spicatum rhizomes of four accessions were collected from Western Himalayan regions of India in April, 2013. Samples were processed and identified following standard collection procedures (Jain et al. 1977). Voucher specimens have been prepared for all the samples (Table 2), and deposited in the herbarium of CSIR-National Botanical Research Institute, Lucknow, India. The species was identified and authenticated by Mr. Baleshwar Meena, CSIR-National Botanical Research Institute, Lucknow (India).

2. Isolation of essential oils

The essential oil from fresh *H. spicatum* rhizomes (100g/sample) was obtained by hydro-distillation for 6 h using Clevenger-type apparatus (Clevenger et al. 1928). The extraction of essential oil was carried out in triplicates. The percentage oil content was calculated on the basis of fresh weight of plant materials. The oils were dried over anhydrous Na₂SO₄ and stored in sealed vials under refrigeration prior to analysis.

3. GC-MS Analysis

Chemical constituents of *Hedychium spicatum* rhizome essential oils were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) on a Thermo Fisher TRACE GC ULTRA using a TR 50MS column (30m x 0.25mm ID x 0.25 µm, film thickness). Constant flow at 1 ml/min of carrier gas (Helium) was used for whole analysis. The injector temperature of the instrument was 220⁰C; oven temperature was programmable starting from 50⁰C, with hold time 2.0 min rising at 2⁰C/min to 120⁰C and at 3⁰C/min. to 250⁰C with hold time of 15 minutes. Sample was injected in split mode (1:50) with injection volume 1 µl. The relative proportion of the sample constituents were obtained (% area) by FID peak–area normalization, without the use of response factor. Retention indices (RI) of the sample components and authentic compounds were determined on the basis of homologous n- alkane hydrocarbons. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed with a Thermo Fisher TRACE GC ULTRA coupled with DSQ II

Mass Spectrometer instrument using a TR 50MS column (30m x 0.25mm ID x 0.25 μ m, film thickness). Constant flow at 1 ml/min of carrier gas (Helium) was used for whole analysis. The injector temperature of the instrument was 220⁰C; oven temperature was programmable starting from 50⁰C, with hold time 5.0 min to 250⁰C with ramp of 4⁰C/min, with hold time of 5 minutes. Sample was injected in split mode (1:50) with injection volume 1 μ l. The ion source temperature was set at 220⁰C; transfer line temperature was 300⁰C. The ionization of the sample components was performed in EI mode at an ionization voltage of -70eV. Mass range was used from m/z 50 to 650 amu.

3.1 Identification of constituents

The retention indices were obtained by co-injecting the oil components with C₈ – C₂₀ linear hydrocarbon mixtures (Adams et al 2004). The volatile components were analyzed by GC/MS and identification was done by comparing retention indices and mass spectra with those in the literature, as well as computerized matching of the acquired mass spectra with those stored in the NIST/ Wiley mass spectral library and other published mass spectra.

4. In-vitro Cytotoxic Activity

4.1 Cell culture and sample preparation

The human cancer cell lines, i.e. Lung (A549), Colon (DLD-1), Breast (MCF-7), Head and Neck (FaDu), Cervical (HeLa) were maintained in RPMI-1640 medium, whereas other cell lines like Breast (MDA-MB-231) and Colon (SW 620) in DMEM medium. The test samples/ molecules were weighed in micro-centrifuge tubes and stock solutions of 100 mg/ml were prepared by dissolving the samples in DMSO. Stock solutions were stored at -20⁰C. A working solution of 200 μ g/ml was prepared by diluting the stock solution in culture medium (RPMI-1640 with 5 % FBS) prior to the assay.

4.2 Cytotoxicity assay (SRB Assay)

The cells were dispensed in a 96-well tissue culture plate. Each well received 100 μ L of the cell suspension containing 10,000-30,000 cells (depending upon the nature of cell line). The cells were then incubated at 37⁰C in 5% CO₂ concentration for 24 hrs before addition of the test

samples/standard drugs. The test sample (100 µl) was added to the cell monolayer to give a final concentration of 100µg/sample, and duplicate wells were included. In each assay plate, both positive and vehicle controls were included. In positive control well, adriamycin (Doxorubicin) was added at 10 µM concentration, where as in vehicle control wells, DMSO was added. In all assay wells the final concentration of DMSO was 0.1%. The plates were then incubated at 37°C in 5% CO₂ concentration for 48 hrs. After incubation, cells attached to substratum of the plate were fixed by adding cold 50% trichloroacetic acid (TCA, 50 µl/well) on top of the medium and incubated at 4 °C for 1 h. After that the plate was gently washed 5 times with slow running tap water via plastic tubing to remove TCA, culture medium and dead cells. After washing, the plates were allowed to dry in air (plates can be stored for long periods at room temperature after fixing and drying step). To dry plates, 50 µl/well of SRB solution was added and left at room temperature for 30 min. At the end of the staining period, unbound SRB was removed by quickly rinsing plates 4-5 times with 1 % (v/v) acetic acid. Plates were allowed to air-dry at room temperature (Stained and dried plates can be stored indefinitely at room temperature). Thereafter, 150 µl of 10 mM Tris base solution was added to each well and plate was shaken for 15 min on a gyratory shaker to solubilize the protein-bound dye. Absorbance was measured at 510 nm in a microplate spectrophotometer.

4.3 Data analysis

Percentage of cell growth inhibition in presence of the test sample was calculated as follows:

$$\% \text{ of cells killed} = 100 - \left[\frac{(\text{mean OD}_{\text{test}})}{(\text{mean OD}_{\text{control}})} \times 100 \right]$$

Principal Component Analysis was performed using SAS 9.1 software.

4.4 Selection criteria

During initial screening, the sample that showed equal to or more than 80% growth inhibition of cancer cells at 100µg concentration were considered as ‘Hits’ and further screened at 2-fold serial dilutions against cancer cell lines to calculate their half maximal inhibitory concentration (IC₅₀) value and IC₅₀ values were derived using Graph Prism software.

Table S1. Essential oil composition (%) of *H. spicatum* rhizome collected from four different geographical regions

Compound name and class	RI	MHS-1	MHS-2	MHS-3	MHS-4
α -Pinene	930	0.02	ND	0.01	0.53
β -Pinene	955	0.19	0.13	0.51	0.67
Limonene	985	0.25	0.08	0.28	0.60
1,8-Cineol	1020	7.64	5.00	17.36	25.78
4-Thujenol	1050	0.01	ND	ND	ND
β -Linalool	1080	0.22	0.04	0.27	4.05
4-Terpineol	1150	0.17	0.30	0.25	0.51
α -Terpineol	1165	0.49	0.35	0.64	0.87
α -Cubebene	1170	0.01	0.01	ND	ND
Copaene	1280	0.08	0.59	0.04	0.03
β -Cadinene	1380	1.74	6.77	2.07	1.93
β -Farnesene	1450	0.40	0.06	0.06	0.08
τ -Murolene	1460	ND	0.98	ND	0.18
α -Muurolene	1460	0.41	0.78	0.45	1.19
Germacrene-D	1469	0.38	ND	0.28	ND
β -Caryophylline	1498	0.97	1.46	0.88	0.57
τ -Elemene	1502	0.57	ND	ND	ND
Cubenol	1509	1.65	8.82	2.11	1.84
Trans Nerolidol	1554	ND	0.93	ND	ND
Germacrene-D-4-ol	1555	0.47	3.51	0.91	0.94
Hedycaryol	1564	17.69	1.10	22.38	4.19
-(-)-Spathulenol	1572	7.25	5.41	13.83	1.67
Caryophylline Oxide	1574	0.12	0.23	0.04	0.05
Aromadendrene	1590	0.18	0.12	0.02	ND
τ -Eudesmol	1642	12.35	2.68	ND	12.01
τ -Murollol	1644	ND	ND	6.63	6.63

β -Eudesmol	1645	26.57	15.77	0.02	ND
α -Eudesmol	1648	1.29	0.65	0.24	0.96
α -Cadinol	1650	5.89	29.19	19.16	26.05
Monoterpene Hydrocarbon		0.46	0.21	0.8	1.80
Monoterpene Alcohol		8.53	5.69	18.52	31.21
Sesquiterpene Hydrocarbon		4.74	10.77	3.8	3.98
Oxygenated Sesquiterpene		0.12	0.23	0.04	0.05
Sesquiterpene Alcohol		73.16	68.06	65.28	54.29
Total Identified		87.01	84.96	88.44	91.33

Table S2. Plant material Details used for study

Sample	Place	Lattitude	Longitude	Altitude (in meters)	Specimen Number
MHS-1	UK; Uttarkashi, Pirithi	31 ⁰ 04.401 ⁰	078 ⁰ 15.04 ⁰	2155	253416
MHS-2	UK; Nainital, Pangot	29 ⁰ 25.821 ⁰ N/S	079 ⁰ 25.331 ⁰ E/W	2060	253433
MHS-3	UK; Nainital, Pangot	29 ⁰ 25.741 ⁰ N/S	079 ⁰ 25.484 ⁰ E/W	2053	253435
MHS-4	UK; Almora, Binsar	29 ⁰ 41.701 ⁰ N/S	079 ⁰ 45.062 ⁰ E/W	2264	253480

Figure S1. Percentage Inhibition against drug concentration of all 4 essential oil samples against colon cancer cell line

Figure S2. Percentage Inhibition against drug concentration of all 4 essential oil samples against breast cancer cell line.

Figure S3. Percentage Inhibition against drug concentration of all 4 essential oil samples against Head and Neck cancer cell line

Figure S4. Percentage Inhibition against drug concentration of all 4 essential oil samples against lung cancer cell line.

Figure S5. Percentage Inhibition against drug concentration of all 4 essential oil samples against cervical cancer cell line.

Figure S6. Percentage Inhibition of Positive control (Doxorubicin) at 10 μ M concentration against all cancer cell line.

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