SUPPLEMENTARY MATERIAL

Chemical profiling and anti-psoriatic activity of methanolic extract of *Andrographis nallamalayana*

J.L.Ellis

Sunitha Parlapally^{a,b,1}, Neeraja Cherukupalli^{a,1}, Sudarshana Reddy Bumireddy^{c,d1}, Prabhakar Sripadi^{c,d}, Ravindernath Aniseti^b, Charu Chandra Giri^a, Venkateswara Rao Khareedu^a and Dashavantha Reddy Vudem.^{a,*}

^a Centre for Plant Molecular Biology, Osmania University, Hyderabad 500 007, India

^b Department of Pharmacy and Biotechnology, University College of Technology, Osmania University, Hyderabad 500 007, India

^c National Centre for Mass Spectrometry, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007 Telangana, India.

^d Academy of Scientific and Innovative Research, CSIR-IICT

*Corresponding author:

Dashavantha Reddy Vudem, Centre for Plant Molecular Biology, Osmania University, Hyderabad 500 007, India.
Tel: +914027098087; Fax: +914027096170. E-mail address: vdreddycpmb@yahoo.com

¹ Authors contributed equally to the work

Abstract

Andrographis nallamalayana is being widely used as tribal medicine in the treatment of leucoderma and mouth ulcers. Chemical profiling of methanolic extract of the whole plant (PE), using GC-MS and LC-MS, revealed the presence of compounds viz., α -tocopherol, β -sitosterol, tetradecanoic acid, monostearin, flavones/flavanones and their glycosides, chromones etc. Topical application of imiquimod on the dorsal portion of male BALB/C mice resulted in the development of psoriatic symptoms (erythema, scaling, thickening and folding) with a mean disease activity index (DAI) of >7.0. Topical treatment with 100 μ L PE (~6.4%/12.8%) formulations, for 12-days, resulted in the alleviation of disease symptoms. Compared to water-based formulations, emu oil-based formulation, PE400EO was found more effective in reducing the mean DAI (>84%), keratinocyte count (>65%) ($p < 0.01$) and Interleukin-22 (~70%) ($p < 0.05$). We report, for the first time, anti-psoriatic activity of *A. nallamalayana* having great potential in developing a potent phytomedicine against psoriasis.

Keywords: *Andrographis nallamalayana*; Chemical profiling; Anti-psoriatic activity; Mouse model; Interleukin-22

Experimental

Preparations of plant extract (PE)

Plants of *A. nallamalayana* were collected from Nallamala hills of Kurnool district in the month of April 2013 after obtaining the permission from the Department of Forestry, Government of Andhra Pradesh. Identification of the species was done based on the taxonomic literature and authentication given by Deccan Circle, Botanical Survey of India and deposited the specimen of *A. nallamalayana* (Voucher no. CNS01AN2013). Leaves, stems and roots of the plant were shade dried and cut into small pieces. The dried material was ground into fine powder. Extraction was carried out at 65 °C using soxhlet apparatus (Oyedeki et al. 2012; Vijayalakshmi et al. 2013) by taking 10 g of the fine powder in 300 mL of methanol. The extract was filtered using whatman filter paper and vacuum dried to remove methanol.

GC-MS analysis

The plant extract was subjected to derivatization prior to GC-MS analysis (Mamza et al. 2012; Muthulakshmi et al. 2012; Eddy et al. 2011). The sample was silylated using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). About 2 mg of dried methanolic extract of *A. nallamalayana* was taken in 1 mL of methanol and vortex mixed for 5 min. The mixture was centrifuged for 5 min at 10,000 rpm to remove the particles. The supernatant was transferred to a fresh vial and evaporated to dryness using scanvac (Denmark). The residue was dissolved in 200 µL of acetonitrile (ACN) and 200 µL of BSTFA was added. The vial was sealed with parafilm and kept at 60 °C for one hour in water bath. The reaction mixture was concentrated to 100 µL by nitrogen purging. The same procedure was also applied for generating solvent blank with pure methanol. Samples were sealed with parafilm and stored at -20 °C until subjected to GC-MS analysis.

The GC-EIMS analyses were performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 5973N mass selective detector and a HP-5MS capillary column of length 30 m, 250 µm i.d. and 0.25 µm of film thickness. One µL of the sample (containing 20 µg PE) was injected into the GC-MS under split injection mode with 20:1 split ratio. The column oven was programmed initially from 50 °C with 2 min holdup time to the final temperature of 280 °C with 10 °C/min ramp and the final temperature holdup time was 5 min with a total runtime of 30 min. Helium was used as the carrier gas in constant flow mode at a flow rate of 1.0 mL/min. The inlet and GC/MS interface temperatures were kept at 250 °C and 280 °C, respectively. EI source and quadrupole analyzer were kept at 230 °C and 150 °C, respectively. MS scanning was kept in full scan mode from m/z 29 to 600.

LC-MS Analysis

The sample was subjected for direct ESI-HRMS analyses under positive and negative ion modes. (Song et al. 2013; Barrios et al. 2010) The number of signals and intensities were found to be better in negative ion mode. Hence, the sample was subjected for LC-MS analysis under negative ion mode. Prior to the sample run, a solvent blank run was performed with the methanol/water (65:35, v/v) to know the peaks related to system background. The LC-PDA-MS analysis of methanolic extract of *A. nallamalayana* was performed on Exactive Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) in negative ion mode connected to Thermo Accela 600 ultra high performance liquid chromatography (UHPLC) with quaternary gradient pump. Accela autosampler (Thermo Scientific, USA) was used to inject the samples. Data was acquired using Xcaliber software (Thermos Scientific). Chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 μ m; Agilent, Santa Clara, CA, USA). After separation, samples were allowed to enter Photo Diode Array detector (Accela PDA, 80 Hz) operated at the range of ultraviolet-visible wavelength (200 nm to 600 nm) using deuterium and tungsten lamps. Two component mobile phase consisting of 0.02% formic acid in methanol (A) and 0.02% formic acid in 5 mM ammonium formate buffer (B), with gradient mode of elution was employed for the separation with a flow rate of 0.6 mL/min. The column temperature was set at 40 °C and 5 μ L of sample (containing 100 μ g PE) was injected and subjected to 30 min run. MS source was equipped with heated electrospray ionization probe and the typical operational conditions include, 300 °C probe temperature, capillary temperature 280 °C, capillary voltages of 85 V, spray voltage of 4.5 kV, tube lens voltage of 175 V. Nitrogen was used as both auxiliary and sheath gas at a flow rate of 45 and 15 mL/min, respectively. Mass range of 100 to 1000 m/z with a resolution of 40,000 of full width at half maximum was used.

Animal housing

Five weeks-old BALB/C male mice were procured from Sainath agencies. All animal procedures have been approved by the Animal Ethical Committee (No: 033/C/07/CPCSCA) in accordance with animal experimentation and care guidelines provided by IAEC/CPCSEA, India. The animals were acclimatized to laboratory conditions prior to initiation of experiment. They were given feed in the form of pellets and water *ad libitum*. Forty two mice were randomized according to their body weight and divided into 7 groups (G1-G7), comprising of 6 mice each with a mean body weight variation not exceeding $\pm$ 20 % between the groups. Hair on the dorsum portion (nearly 2 $\times$ 2 cm²) of each mouse was removed by using depilatory cream from Reckitt Benckiser Company, U.S.A.

Preparation of test drug formulations

Stock solutions of various test drug formulations taking 200 mg PE or 400 mg PE/kg body weight of the animal were prepared freshly (Jiao et al. 2013). Considering the average body weight of each mouse as 32 g, formulations of PE200/PE200EO (determining the dose 200 mg/kg body weight) were constituted by dissolving 38.4 mg of dried methanolic extract in 180 μ L of DMSO and made up the volume to 600 μ L

by adding 420 μ L of water/emu oil. Daily each animal was administered with 100 μ L of formulation containing 6.4 mg of test drug. Similarly PE400/PE400EO formulations (determining the dose 400 mg/kg body weight) were prepared by doubling the content of plant extract and 100 μ L of formulation consisting of 12.8 mg of PE was administered daily to each animal.

Induction of psoriasis

Group1 (G1) served as control and topically administered with 50 mg of vaseline on the shaved dorsal surface of the skin of each mouse once daily for 11 days. Psoriasis was induced in the groups 2-7 (G 2-G7) by topical application of 50 mg of IMQ cream (Glenmark pharmaceuticals) once daily for 11 days. Mice were evaluated daily, 4 hours prior- and post-application of IMQ. To score the severity of inflammation of the dorsum, clinical Psoriasis Area and Severity Index (PASI) scoring system was used (Sun et al. 2013). Signs of psoriasis, erythema, scaling, thickening and folding of the skin were scored independently on a scale from 0 to 3 (0- without any disease symptoms, 1- mild, 2- Severe and 3- Very severe symptoms of disease). Disease Activity index (DAI) was calculated using cumulative scores of four disease parameters (Erythema + Scaling + Thickening + Folding) on a scale 0 to 12 and served as a measure of severity of the induced-disease condition.

Treatment of psoriasis

All the animals were re-randomized based on mean DAI and subjected to treatment daily with the topical application of test drug formulations for 12 days. Application of IMQ (50 mg) was continued throughout the treatment phase, to all the mice from G2-G7 groups, to ensure that the recovery, if any, in the mice is due to the test drug formulations. In addition to IMQ, animals of groups 2, 3, 4, 5 and 6 were administered with 100 μ L of PE200, PE400, PE200EO, PE400EO and commercial drug BMT, respectively. Group 7 without any treatment served as untreated disease-induced mice. Mice from control group (G1), without disease, were administered with vaseline (50 mg) and 100 μ L 30 % DMSO.

Body weight, food and water intake of each animal were recorded daily. Erythema, thickness, scaling and folding was scored daily 4 hours pre- and post-application of the test drug formulation and IMQ. Reduction in the signs of psoriasis was measured by PASI scoring, histopathological observations of skin sections and quantification of plasma Interleukin-22 (IL-22).

Collection of samples

At the end of experiment, blood samples were collected by retro orbital into the vials containing EDTA. Plasma was separated from blood by centrifugation and was stored at -70 °C. Skin tissues of all the treated mice were excised, washed in Phosphate Buffered Saline (PBS) (9.64 mM phosphate buffer + 0.8 % NaCl pH: 7.4) and fixed in 10 % neutral buffered formalin.

Histological studies

After one week of fixation, wax embedded blocks of excised skin tissue was prepared in routine process. The sections (3 μ) were made using microtome (Leica RM 2245) on to glass slides. After complete removal of wax, the sections were subjected to Hemotoxylin and Eosin (H&E) staining (Riedel et al. 2015) and observed under microscope. Number of keratinocytes was counted under 40X magnification of H&E stained skin sections of normal, disease-induced and disease-induced mice treated with test drug formulations.

Enzyme linked immunosorbant assay (ELISA)

Plasma samples were used for the quantification of IL-22 in all the groups by ELISA using the kit ELISA MAX Deluxe Sets (Cat. No. 436304) from Biolegend, Inc., U.S.A and performed the assay according to the manufacturers protocol using recombinant IL-22 of mouse as standard. The experiment was performed in duplicates and the means and standard deviations were calculated.

Statistical analysis

To evaluate recovery of mice from induced psoriasis, statistical significance was calculated for DAI, keratinocyte proliferation and IL-22 levels between the disease-induced group vs respective treatments. Data are presented as mean $\pm$ standard error mean (SEM). Statistical significance of data was performed by unpaired-*t* test using graphpad software (www.graphpad.com) (Priyanka et al. 2012).

Figure S1 Structures of the compounds identified in the GC-MS analysis of methanolic extract of *Andrographis nallamalayana*.

Figure S2 LC-ESIMS total ion chromatograms of methanolic extract of *A. nallamalayana* (upper), solvent blank (below).

Figure S3 Extracted ion chromatograms (EICs) and their negative ion ESI mass spectra obtained in LC-MS analysis of the sample.

Figure S4 Reduction in the signs of psoriasis after administration of test drug formulations of *A. nallamalayana* for 12 days and their respective H&E stained skin sections of mice: (A) Control mouse showing normal skin and keratinocytes with thin epidermal and subcutaneous tissue (B) Disease-induced mouse displaying the signs of erythema, scaling, thickening, folding and abnormal keratinocyte proliferation with thickened epidermal and subcutaneous tissues (C) PE200 treated mouse showing reduced signs of psoriasis and mild reduction in the keratinocyte proliferation (D) PE400 treated mice exhibiting improved recovery from the signs of psoriasis and reduction in keratinocyte proliferation but the thickness of epidermal and subcutaneous tissue still persists (E) PE200EO treated mice showing increased reduction in the signs of psoriasis, thickness of epidermal and subcutaneous tissue along with reduced keratinocyte proliferation (F) PE400EO treated mice exhibiting maximum alleviation in the signs of psoriasis and reduction in the thickness of epidermal and subcutaneous tissue with minimum keratinocyte proliferation similar to control (G) Positive control commercial drug BMT treated mice showing reduction in the signs of erythema, scaling, thickness of epidermal, subcutaneous tissue and keratinocyte proliferation but extreme folding of the skin was observed as a side effect.

Table S1. Accurate mass values and plausible structural information for the LC-MS peaks (Details are provided in the Figure S3).

Accurate mass (m/z) of $[M-H]^-$	RT (min)	Peak Area	Category	Plausible structures
353.0874	2.32	113107696	Chromone	Unknown
461.1124	2.54	93569621	Flavone-glycoside/ flavanone-glycoside
445.1198	2.78	91027180	Flavone-glycoside
491.1178	2.93	438193345	Flavone-glycoside
313.0737	3.36	112745365	Flavones
283.0580	3.59	753579133	Flavones/ Flavanones
295.2236	3.85	35652270	Fatty acids	Unknown
485.2750	4.33	510938013	6-O-Acetyl glycosydic flavones	Unknown
457.0965	2.34	111983075	catechin	Unknown
447.1315	2.66	31793190	Flavanone-Glycoside

Table S2. Reduction in the DAI, keratinocyte proliferation and IL-22 levels in the disease-induced mice treated with methanolic extract of *A. nallamalayana*

	Control	PE200	PE400	PE200EO	PE400EO	BMT	Disease induced
Day1	0	7.66±0.33	7.5±0.20	7.0±0.62	7.3±0.30	7.3±0.30	7.5±0.38
Day2	0	7.33±0.45	7.5±0.22	7.0±0.36	6.5±0.42	6.6±0.42	7.6±0.40
Day3	0	7.33±0.33	7.3±0.21	6.6±0.33	6.3±0.33	6.1±0.30	7.4±0.42
Day4	0	7.0±0.36	7.1±0.16	6.5±0.56	6.1±0.30	6.2±0.53	7.4±0.61
Day5	0	5.5±0.53	5.5±0.22	5.6±0.36	5.1±0.42	6.0±0.57	7.3±0.18
Day6	0	5.4±0.22*	5.1±0.47*	5.3±0.47*	5.1±0.61*	5.2±0.34*	7.3±0.44
Day7	0	5.4±0.46*	5.1±0.30*	5.3±0.30*	5.0±0.33*	4.6±0.22*	7.4±0.18
Day8	0	5.0±0.28*	5.3±0.33*	5.0±0.36*	4.6±0.49*	3.4±0.46*	7.5±0.60
Day9	0	3.4±0.22*	3.5±0.42*	3.5±0.50*	3.8±0.30*	2.6±0.22*	7.5±0.46
Day10	0	3.1±0.30*	2.5±0.42*	2.4±0.22*	2.0±0.36*	1.8±0.34*	7.8±0.34
Day11	0	2.0±0.44*	1.8±0.30*	1.5±0.22*	1.5±0.22*	1.2±0.53*	7.8±0.32
Day12	0	1.8±0.36*	1.6±0.33*	1.5±0.22*	1.2±0.21*	1.2±0.53*	7.8±0.33

Untreated Vs treated	Keratinocyte count (Mean ± SE)	IL-22 pg/ml (Mean ± SE)
Control	56.25±2.06	13.37±1.4
PE200	138.5±1.94**	41.49±15.2
PE400	112±4.2**	35.44±9.9*
PE200EO	97.25±2.17**	40.40±8.3*
PE400EO	70.5±2.9*	30.94±5.0*
BMT	75±2.12**	53.745±11.5
Disease induced	208±4.81	100.40±19.25

* Significant *p*-value less than 0.05 ** Significant *p*-value less than 0.01

References

- Barrios J, Jose F, Cordero CP, Heredia, Luci A, Morales A, Aristizabal F, Osorio C. 2010. Chemical analysis and screening as anticancer agent of anthocyanin-rich extract from Uva caimarona (*Pourouma cecropiifolia* Mart.) Fruit. J Agric Food Chem. 58:2100–2110.
- Eddy NO, Awe FE, Siaka AA, Magaji L, Ebenso EE. 2011. Chemical information from GC-MS studies of ethanol extract of *Andrographis paniculata* and their corrosion inhibition potentials on mild steel in HCl Solution. Int J Electrochem Sci. 6:4316-4328.
- Jiao Y, Chen PH, Xiong AZ, Wang ZT, Tsim KW, Chou GX, Xu H. 2015. Evaluation of hemostatic and anti-inflammatory activities of extracts from different *Lagochilus* species in experimental animals: comparison of different extractives and sources. Phytother Res. 29:22-29.
- Mamza UT, Sodipo OA, Khan IZ. 2012. Gas chromatography-mass spectrometry (GC-MS) analysis of bioactive components of *Phyllanthus amarus* leaves. Int Res J Plant Sci. 3:208-215.

- Muthulakshmi A, Margret JR, Mohan VR. 2012. GC-MS Analysis of bioactive components of *Feonia elephantum correa* (Rutaceae). J App Pharm Sci. 2:69-74.
- Oyedeki FO, Ojo OSB. 2012. Quantitative evaluation of the antipsoriatic activity of sausage tree (*Kigelia africana*). Afr J Pure Appl Chem. 6:214-218.
- Riedel R, Marrassini C, Anesini C, Gorzalczany S. 2015. Anti-inflammatory and antinociceptive activity of *Urera aurantiaca*. Phytother Res. 29:59-66.
- Song YX, Liu SP, Jin Z, Quin JF, Jiang ZY. 2013. Qualitative and quantitative analysis of *Andrographis paniculata* by rapid resolution liquid chromatography/time-of-flight Mass Spectrometry. Molecules. 18:12192-12207.
- Sun J, Zhao Y, Hu J. 2013. Curcumin inhibits imiquimod-induced psoriasis-like inflammation by inhibiting IL-1beta and IL-6 production in mice. PLoS ONE. 8:e67078.
- Vijayalakshmi A, Ravichandiran V, Velraj M, Nirmala S, Anusha M, Jayakumari S, Masilamani K. 2013. Anti-psoriatic activity of *Smilax china* Linn. rhizome. Ind J Pharma Edu Res. 47:82-89.