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EXPERIMENTAL PAPER

Isolation, characterization and evaluation of anti-proliferative properties of andrographolide isolated from *Andrographis paniculata* on cultured HaCaT cells

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Summary

Introduction: Psoriasis is an inflammatory skin disease characterized by hyper-proliferation, abnormal epidermal keratinocytes and inflammatory infiltration. It affects approximately 4% of the population globally. Herbal extracts have better results with less toxic effects than the synthetic drugs in the treatment of psoriasis.

Objective: Present study was aimed to access the anti-psoriatic effect of andrographolide extracted from *Andrographis paniculata* (*A. paniculata*).

Method: We extracted, characterized, and screened the extracted andrographolide for anti-proliferative characteristics using cultured cell model of human HaCaT keratinocyte.

Results: Andrographolide at 31.25 $\mu\text{g/mL}$ (90 μM) demonstrated significant inhibitory effect on human HaCaT keratinocytes proliferation in cell culture. To our best knowledge, we reported the anti-proliferative potency of andrographolide extracted from *A. paniculata* for the first time.

Conclusion: The results suggest that the andrographolide extracted from *A. paniculata* plant may have potential to be used in the management of psoriasis.

Key words: *andrographolide, Andrographis paniculata, anti-proliferative, psoriasis, epidermal keratinocytes*

Słowa kluczowe: *andrografolid, brodziuszka wiechowata, Andrographis paniculata, działanie antyproliferacyjne, łuszczyca, keratynocyty naskórka*

INTRODUCTION

Psoriasis is a chronic autoimmune and over proliferative skin inflammation characterized by propagated cell growth, modified differentiation of keratinocytes and angiogenesis with marked eczema of blood vessels [1]. This skin disorder is distinguished by periodic emergence of recognizably defined skin conditions in the form of red and thick dermal patches with swollen lesions showing symptoms like itchiness, dryness, blisters/rashes, scales/plaques. The initiating activity of disease takes place in epidermis of the skin where hyper-proliferation, maturation and uncommon differentiation of keratinocytic cells occur due to the abnormal operation of immune system [2, 3].

Wound healing is a mechanism in which cellular stimuli activates dermal cells and begins repairing procedure. The skin regeneration mechanism gets interrupted when cellular responses or immune system becomes faulty [4]. This condition is inherited and distinguished by erythematous and marginated scaly plaques. The elbow, palms, fingertips, scalp, under the breast gluteus, umbilicus, genitals, knees, shin, sacrum and soles are the most affected body parts. The skin of a psoriatic patient becomes very loose due to the hyper-proliferation of epidermal cells and finally exfoliation of fish-like skin occurs. It is to believe that gene variation could be a possible reason for the same phenomenon [5].

There is lack of complete or medicinal cure for the disease due to the hyperproliferation of epidermal keratinocyte cells in diseased condition. The therapies associated with the treatment of psoriasis target the regulation of keratinocytes proliferation [6]. The anti-psoriatic drugs restore the homeostatic control and balance of keratinocyte growth of hyper-proliferative skin [7]. Current anti-psoriatic therapeutics includes arsenic derivatives, anthralin, and various corticosteroids. These therapeutics exhibit anti-proliferative activity *via* apoptosis. However, these synthetic drugs may lead to the local irritation (anthralin), heart

attack, headache (acritine), nervous system problems (calcipotriene-associated), etc. [8].

Recent studies reveal that various crude extracts or formulations from individual plants and polyherbs have been used in the treatment of dermal diseases including psoriasis. The efficacy of several herbs and formulations has been reported in treating psoriasis due to their anti-proliferative action [9, 10]. More than 60% of global population utilizes bioactive substances derived from plants due to their less or no side effects [11]. The medicinal herbs such as *Annona squamosa*, *Curcuma longa* and *Alpinia galanga* have shown anti-psoriatic activity [12]. The available evidence on anti-proliferative and apoptotic properties of plant-based bioactive substances in the treatment of psoriasis inspired us to carry out this study.

Andrographis paniculata (Burm. f.) Nees (*A. paniculata*) is a vital herb belonging to the *Acanthaceae* family. The profitable preparations of this plant extract are used in several forms. However, it is essential to standardize the improved efficacy of these preparations. The aerial parts of *A. paniculata* composed of diterpene glycosides, diterpenoids, flavonoid glycosides, lactones and flavonoids. Roots and leaves are being used in different diseases like hyperglycaemia, inflammation, cancer, tuberculosis etc. as a folklore remedy [13]. *A. paniculata* has a wide range of pharmacological potencies, including antihepatitis, antidiarrheal, anti-HIV, antimicrobial, antioxidant, anti-inflammatory, antimalarial, cardiovascular, antihyperglycaemic, cytotoxic, immunostimulatory, hepatoprotective and anticancer properties [14-17]. Since *A. paniculata* is used for the treatment of many diseases, its anticipated benefits require to be evaluated for antiproliferative activity in the management of psoriasis. Therefore, the present communication aimed to isolate and characterize andrographolide from *A. paniculata* for its anti-proliferative activity in the management of psoriasis. To our best knowledge, in this present study, we reported the anti-proliferative potency of andrographolide extracted from *A. paniculata* for the first time.

MATERIALS AND METHODS

Plant material

A. paniculata plant was collected from the Herbal Garden, Guru Jambheshwar University of Science and Technology, City and District Hisar, Haryana, India in July 2017. The authentication and certification of plant was done at NISCAIR, New Delhi. The leaves were dried under shade and then powdered using blender. Obtained powder was stored in an airtight vessel till further use.

Materials

Reference standard of andrographolide was purchased from Yucca Enterprises, Mumbai, India. Methanol (HPLC grade) was purchased from Sigma Aldrich, Germany. Water for analysis was purified by Millipore purified system. The TLC plates (aluminium foil pre-coated with silica 60F254) were purchased from Merck, Japan. The solvents and reagents used were of AR grade.

Extraction and isolation of andrographolide from *A. paniculata*

A. paniculata leaves (200 g) were macerated overnight in ethanol and filtered using muslin cloth. The marc was packed into a thimble and placed in the Soxhlet assembly. The extraction was done using 500 ml of ethanol (90%) for a period of 12 h and the temperature was maintained at $75\pm 5^\circ\text{C}$. The extract was collected, and the solvent was removed, using vacuum. Dark green coloured crystalline mass was obtained and washed with toluene until the complete removal of colour from the residue. The remaining crystalline material (yield 1.8%) was recrystallized using hot methanol followed by cooling under refrigeration. The process was repeated until colourless constant plates were obtained [18].

Characterization and standardization of andrographolide

The purity of standard andrographolide and isolated andrographolide was checked by the following methods:

Physicochemical analysis

Andrographolides were characterized for colour, odour, melting point and solubility. Solubility was determined in methanol, acetone, ethanol, and phosphate buffer (pH 6.7). Melting point of the extract was determined using differential scanning calorimetry (DSC).

Spectral analysis

Thermal analysis

Thermal behaviour of the andrographolides was determined using differential scanning calorimetry (DSC) (Pyris 6 DSC, Perkin-Elmer, USA). The instrument was calibrated using indium as reference standard (transition point: 156.60°C). The sample was crimped in aluminium pans and heated from $50\text{--}300^\circ\text{C}$ at a heating rate of $10^\circ\text{C}/\text{min}$ under dry nitrogen at constant supply of 30 ml/min.

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectrum of pure andrographolide was recorded to check the structure of individual molecule and mixture composition using Shimadzu FTIR spectrophotometer by the KBr disc method. Briefly, 1 mg of the andrographolide sample was mixed with dried potassium bromide and compressed using the KBr press at 10 tons for 2 min to form a pallet. The sample pallet was placed in FTIR sample holder and scanned from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} .

X-ray diffraction study

The X-ray diffraction analysis is an efficient technique characterizing the physical state (crystalline or amorphous) of unknown materials. The X-ray diffraction pattern of pure andrographolide was recorded by X-ray powder diffractometer (Bruker AXS, D8 Advance, Massachusetts, USA). The X-ray diffraction pattern was recorded using $\text{Cu K}\alpha$ radiations ($\lambda=1.5405980\text{ \AA}$), a voltage of 40 kV and a current of 30 mA. The sample was analysed over $5\text{--}60\ 2\theta$ range with a scan step size of 0.02 and 0.50 s per step.

Chromatographic analysis

Chromatographic analysis of the andrographolide was carried out by HPLC method. The stock solution was formed by dissolving 1 mg drug sample in 1 ml methanol (HPLC grade). The chromatographic separation was carried out using Luna 5u C18 100A (250 × 4.6 mm) column (phenomenex, USA). The mobile phase was composed of acetonitrile: Milli Q water: methanol (90:8:2% v/v). The solvent mixture was filtered using 0.45 μm membrane filter and degassed. The mixture was pumped with the flow rate of 0.5 ml/min under isocratic condition and analysed with detection wavelength of 223 nm. The sample analysis was carried out in triplicate (IP, 2007).

Nuclear magnetic resonance (NMR) spectroscopy

¹H and ¹³C analysis at 600 MHz were carried out in ¹H NMR. The number of peaks represented the number of protons present in the compound. ¹³C NMR represented the number of C-atoms present in the compound. Sample was prepared by dissolving 1 mg of andrographolide in 0.7 ml deuterated methanol and the sample was run for ¹H and ¹³C NMR to obtain the spectrums.

Anti-proliferative activity

Anti-proliferative activity of andrographolide was carried out at Radiant Research Lab, Bangalore, India (RR/190224/CB/AP/07-19). The *in vitro* cytotoxicity study was carried out for the test substance on human keratinocyte cells to find toxic concentration of sample substance which was further used to determine the level of proliferation rate in the presence of test substance.

Preparation of test solution

The sample of herbal extract (10 mg) was taken separately and dissolved in Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) supplemented with 2% foetal bovine serum (FBS) in inactivated form to obtain a stock solution of 1 mg/ml concentration. Filtration method was used for the sterilization of solution using 0.22 μm syringe filter. Two-fold serial dilutions were prepared to carry out cytotoxic studies.

Cell line and culture medium

DMEM-HG media was utilized for the culture of HaCaT (Human Keratinocytes) cell line (National Centre for Cell Science, Pune, India), using 10% inactivated FBS, streptomycin (100 μg/ml), penicillin (100 IU/ml) and amphotericin B (5 μg/ml) in 5% CO₂ humidified atmosphere at 37°C. The cells were dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution containing 0.02% EDTA, 0.05% glucose, 0.2% trypsin in PBS. The culture stock was grown in 25 cm² culture flasks. All the experiments were conducted using 96 well microtitre plates.

Cytotoxicity studies

For the cytotoxicity studies, single layer of cell culture was trypsinized and 100,000 cells/mL cell count was adjusted using DMEM-HG containing 10% FBS. Diluted cell suspension (0.1 mL) was added each well. A partial monolayer was formed after 24 h and the supernatant was decanted off. The monolayer was washed with medium (DMEM with 10% FBS). Different test dilutions (100 μL each) of test substances were added to the microtitre plates containing partial monolayer. The plates were incubated for 72 h at 37°C in the atmosphere containing CO₂ (5%). The microscopic examination of samples was carried out at every 24 h interval. After 72 h, the test solution was discarded from the wells and to each well 50 μL of MTT in PBS was added. The plates were shaken gently and incubated for 3 h at 37°C in the atmosphere containing CO₂ (5%). The supernatant was decanted, and propanol (100 μL) was added. To solubilize formazan, the plates were shaken gently, and absorbance was measured at 540 nm using a microplate reader. The growth inhibition percentage was calculated and CTC₅₀ value (concentration of sample drug required to cause inhibition of cell growth by 50%) for each cell line was estimated from the dose response curves.

Anti-proliferation activity of isolated andrographolide in HaCaT cell line

HaCaT cells were entrenched into a 96-well plate with a density of 5 × 10³ cells/well and supplemented with DMEM for 24 h to grow the cells. The cells were washed with PBS and treated with non-toxic concentration of each test substance and standard control. The addition of the cell control solutions

was carried out and the plates were incubated for 72 h at 37°C in the atmosphere containing CO₂ (5%). Upon incubation, the cell supernatants were removed off and the cell viability was estimated by MTT assay method.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

Extraction and isolation of andrographolide

The yield of andrographolide was found to be 1.6% w/w. The yield was significantly higher than the value reported by Kumoro and Hasan (0.46% w/w) [19]. However, it was slightly lower than the value reported by Rajani *et al.* (1.9% w/w) [18].

Physicochemical analysis

Isolated andrographolide was pale white crystalline order-less powder. The melting point of isolated andrographolide was found to be 236±2°C (reference melting point is 232–242°C) [18]. The isolated andrographolide was found to be sparingly soluble in water, sparingly soluble in phosphate buffer, having pH 6.8 and freely soluble in methanol [20].

Thermal analysis

The DSC analysis is a technique useful in investigation of thermal behaviour of materials. It provided both qualitative and quantitative information about physicochemical state of andrographolide. The DSC thermogram of isolated andrographolide gave a single endothermic peak at 236°C representing the melting point of andrographolide (fig. 1). This indicated the purity of isolated andrographolide [18].

Fourier Transform Infra-Red (FTIR)

The FTIR spectral analysis was carried out to investigate the presence of functional groups and the identification of the compound. FTIR spectrum of isolated andrographolide showed characteristic peaks at 2979.18cm⁻¹(asymmetric-CH), 2848.98cm⁻¹(symmetric - CH), 2929.03 cm⁻¹(=CH₂), 3398.72 cm⁻¹(-OH), 1457.28 cm⁻¹(C=C), 1365.66 cm⁻¹(O-C=O) (fig. 2). The characteristic peaks confirmed purity of isolated andrographolide [21].

X-ray diffraction study

Pure andrographolide possessed a series of characteristic peaks at 2 Theta of 12.86, 15.42, 16.31, 18.22, 19.05, 19.81, 22.08, 23.07, 26.08 and 27.07 because of its crystallinity (fig. 3).

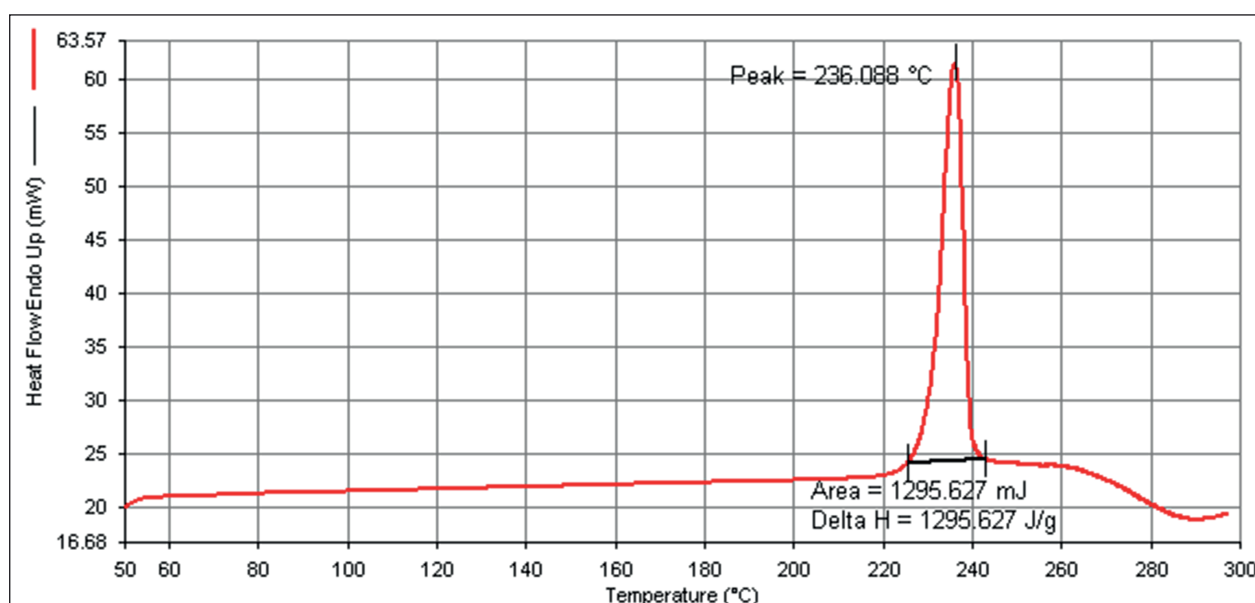


Figure 1.

Differential scanning calorimetry thermogram of isolated andrographolide

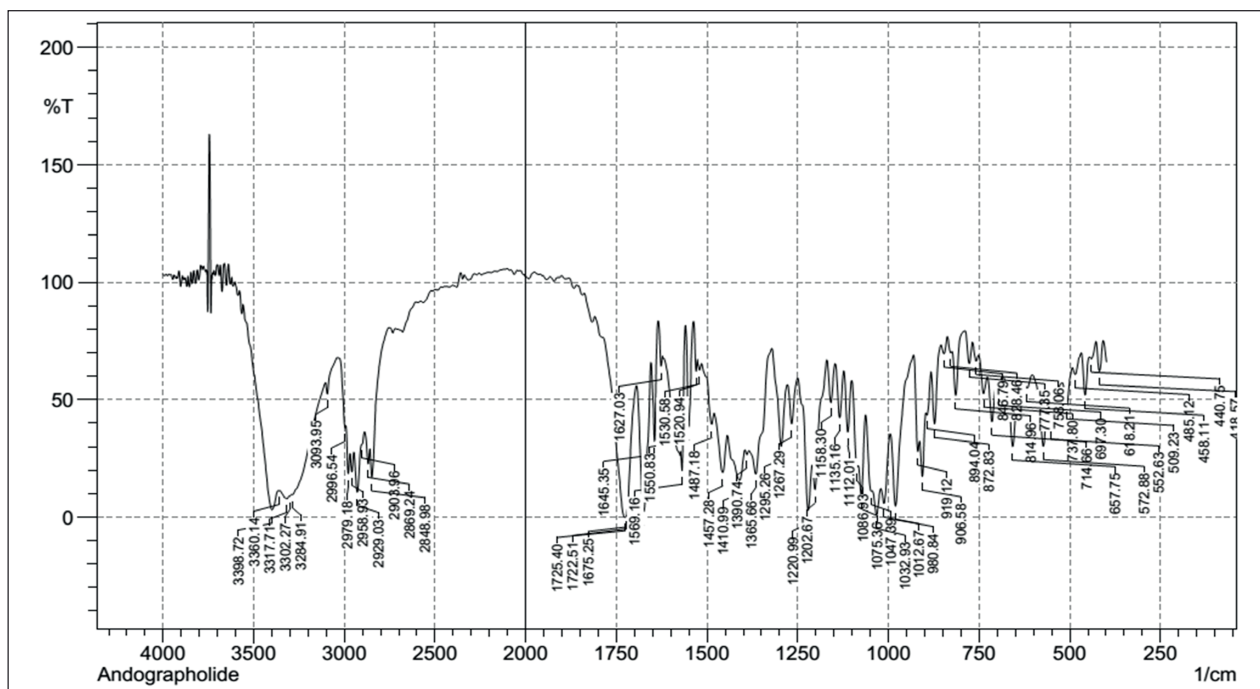


Figure 2.

Fourier transform infra-red of isolated andrographolide

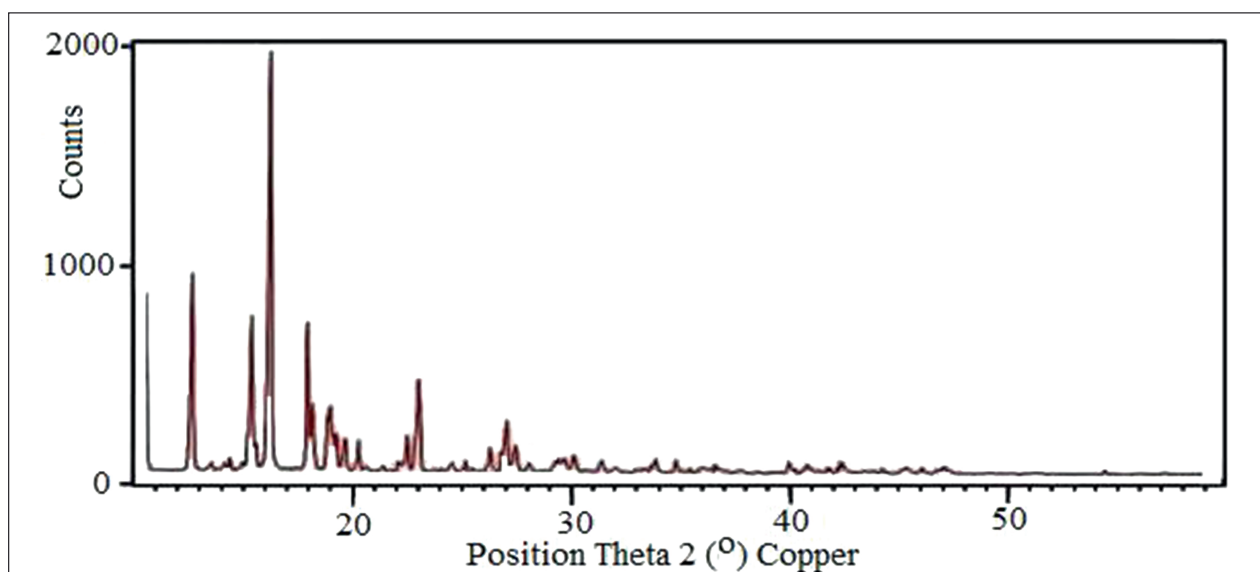


Figure 3.

X-ray diffraction pattern of andrographolide.

Chromatographic analysis

Chromatographic analysis was done to determine the purity of isolated andrographolide based on its retention time. The retention time of pure andrographolide was found to be 5.124 min. (fig. 4). The obtained retention time was similar to the previously reported value [22].

Nuclear magnetic resonance (NMR) spectroscopy

The ^1H NMR and ^{13}C NMR spectrum of isolated andrographolide are presented in figures 5 and 6, respectively. All the functional groups along with their δ value matched with the literature values [20], which confirmed the structure of andrographolide.

Cytotoxicity study

Cytotoxicity is one of the most important indicators for biological evaluation of chemicals and drugs. In present study, the *in vitro* cytotoxicity studies were carried out on HaCaT cells using MTT assay

exposing the cells to various concentrations of andrographolide extracted from 1000 to 7.8 $\mu\text{g/ml}$ [23, 24]. The moderate toxicity (CTC_{50}) of extracted andrographolide was observed at a concentration of $608.41 \pm 3.6 \mu\text{g/ml}$. The result of cytotoxic studies of test substance against HaCaT cells is presented in figure 7.

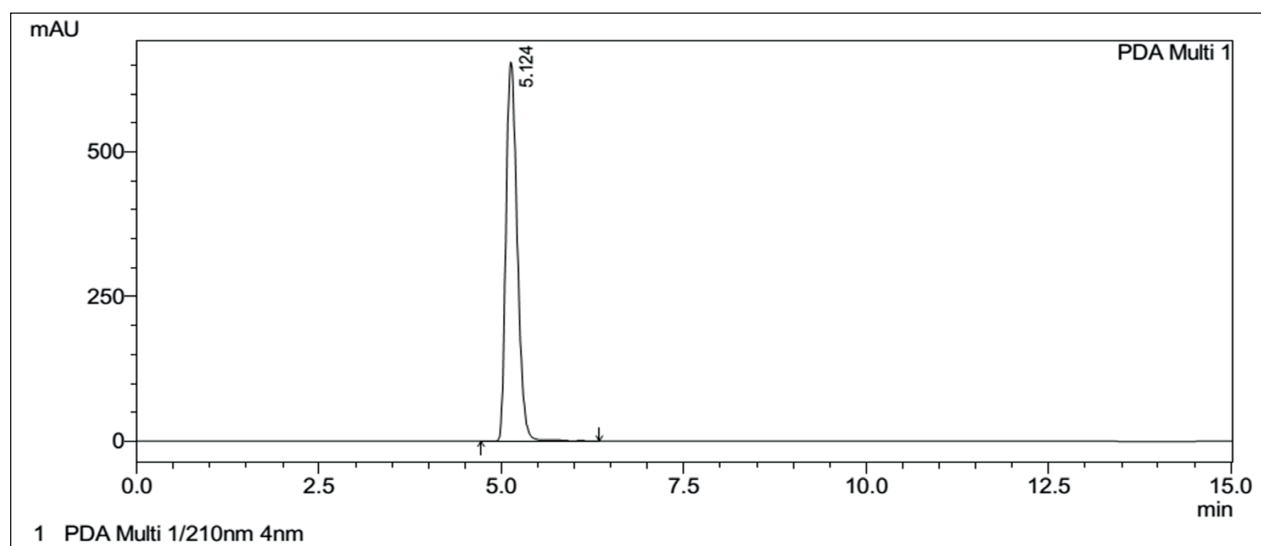


Figure 4.

HPLC chromatogram of andrographolide.

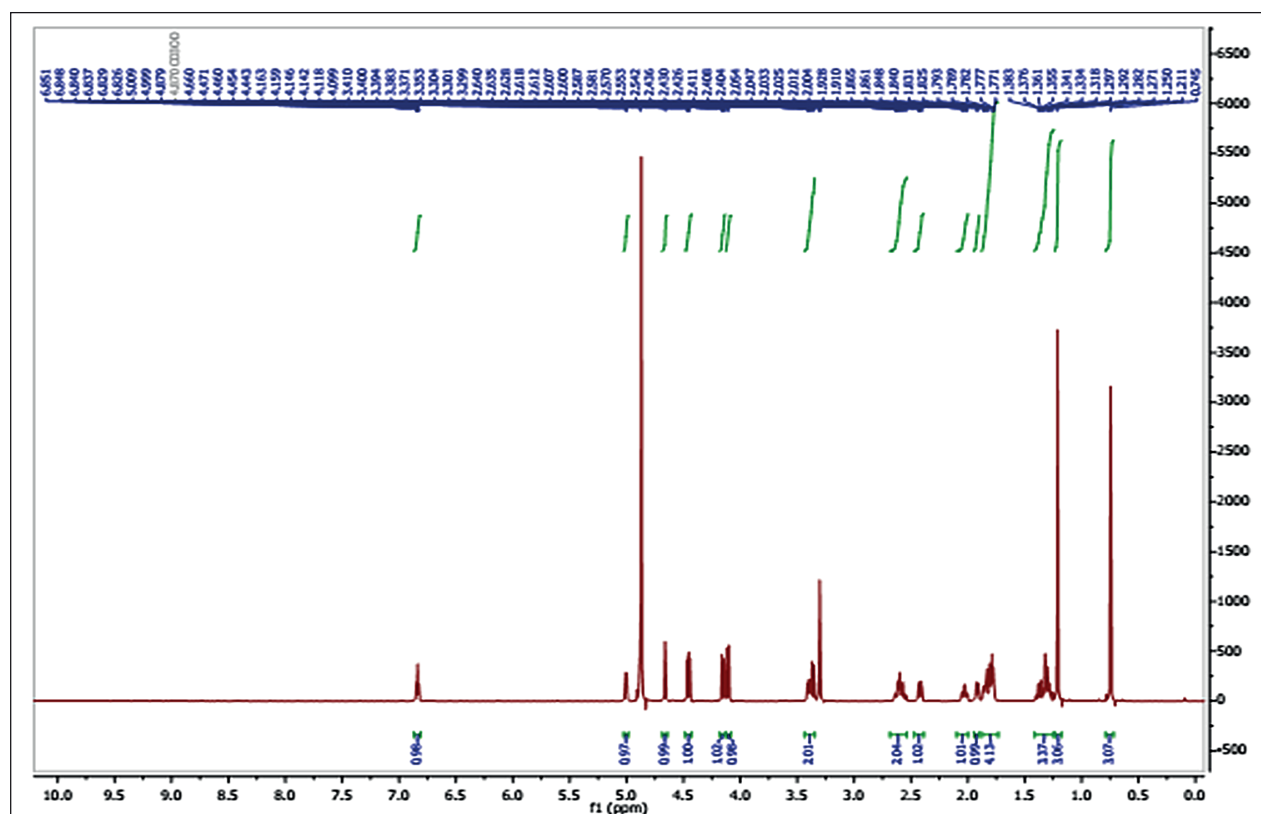


Figure 5.

^1H nuclear magnetic resonance spectrum of andrographolide

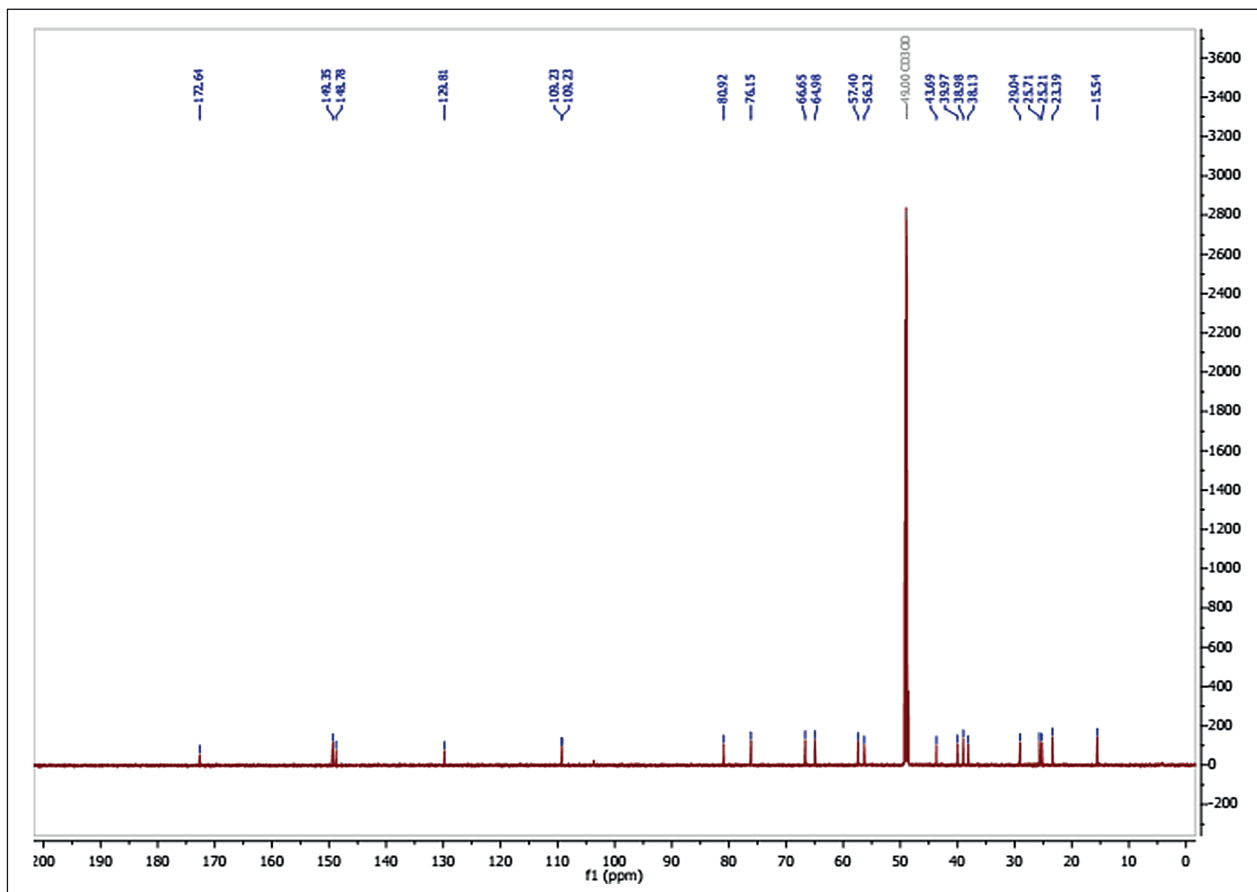


Figure 6.

^{13}C nuclear magnetic resonance spectrum of andrographolide

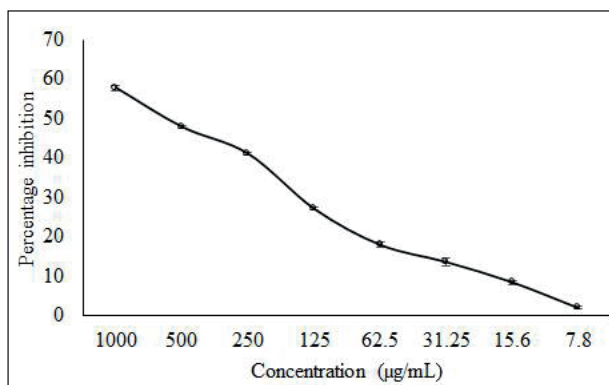


Figure 7.

Results of cytotoxic studies of test substance against Human Keratinocytes cell line

Antiproliferative study

Epidermal homeostasis restores normal epidermal tissue structure of skin by maintaining balance between proliferation and differentiation of keratinocytes of the basal layer of epidermis and the apoptotic process of keratinocytic cell death in superficial

layer of epidermis. Proliferative imbalance of keratinocytes and defect in apoptotic process led to the pathogenesis of psoriasis. Agents that inhibit the keratinocyte proliferation or differentiation and induce keratinocyte apoptosis are potential agents in the treatment of psoriasis [25-28].

In the present study, the andrographolide extract from *A. paniculate* was evaluated to address the problem of psoriasis by reducing abnormal hyperproliferation of keratinocytes. Antiproliferative potential of andrographolide was determined in keratinocytes

Table 1.

Anti-proliferative activity of test substance against HaCaT cell line

Sample	Concentration [μM]	Percentage anti-proliferation*
Andrographolide extract	90	33.04 \pm 1.4
	50	17.38 \pm 1.7
Methodredate standard	40	42.81 \pm 0.9

*Data expressed as the mean \pm standard deviation (n=3).

Table 2.

Results of one-way ANOVA on the anti-proliferative activity of test substance against HaCaT cell line

Source of variation	Sum of square	Degree of freedom	Mean squares	Calculated F	Tabulated F
Between andrographolide extract (90 μ M) and methotrexate standard (40 μ M)					
Between Groups	54.60166667	1	54.60166667	2.569845	7.71
Within Groups	84.98826667	4	21.24706667		
Total	139.5899333	5			
Between andrographolide extract (50 μ M) and methotrexate standard (40 μ M)					
Between Groups	960.388	1	960.388	1237.109	7.71
Within Groups	3.105267	4	0.776317		
Total	963.4933	5			

(HaCaT cells) by MTT assay after 48 h of treatment. The results of antiproliferative study suggested that the test substance had moderate cytotoxicity. The test product had a moderate proliferation activity (tab. 1). Based on the results of CTC₅₀ value obtained in the cytotoxicity study, two non-toxic concentrations, i.e. 31.25 μ g/ml (90 μ M) and 15.60 μ g/ml (50 μ M) were selected for the antiproliferation studies. The test sample at 31.25 μ g/ml (90 μ M) demonstrated significant inhibitory effect of HaCaT cells proliferation in comparison to the untreated (control) cells in a dose dependent manner [23, 24]. On application of one-way ANOVA, no significant difference was observed in the antiproliferative activity among the andrographolide extract (90 μ M) and methotrexate standard (40 μ M) at 95% confidence interval ($p < 0.05$), as the tabulated F value was higher than calculated value (tab. 2). This substantiates the antiproliferative role of 90 μ M andrographolide extract.

CONCLUSIONS

Andrographolide was isolated from *A. paniculata* and characterized using spectral analysis, like FTIR, DSC, XRD and HPLC. The results showed that the isolated andrographolide was pure. The findings of this study revealed that the isolated andrographolide had antiproliferative activity, as confirmed by MTT assay. Thus, from the results of preliminary laboratory results it is said that the *A. paniculata* plant may have potential to be used in the treatment of psoriasis. However, this needs to be proved in a suitable *in vivo* model.

Conflict of interest: Authors declare no conflict of interest.

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