Isolation, Characterization of Neoandrographolide from Andrographis macrobotrys Nees and Evaluation of its effect on LPS induced TNF-α Activity

Medha A. Bhat, Hosakatte Niranjana Murthy*

ABSTRACT

Introduction: Andrographis macrobotrys Nees is an important species of genus Andrographis with applications in traditional medicine. Neoandrographolide is one of the constituents in this plant. Current work is undertaken to concentrate on isolation, characterization, and evaluation of tumor necrosis factor-alpha (TNF-α) inhibition activity of neoandrographolide from A. macrobotrys.

Materials and Methods: For the isolation process techniques like column chromatography, thin-layer chromatography (TLC), and preparative TLC were used. Characterization was done by ultra visible (UV)-visible spectroscopy, Fourier transform infrared (FTIR), proton nuclear magnetic resonance (1H NMR), carbon-13 (13C NMR) nuclear magnetic resonance (13C NMR) analysis. 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay was done for the preliminary cytotoxicity test to standardize the sample concentration for the TNF-α inhibition study. The flowcytometric method was used to determine TNFα inhibiting ability in a human monocytes cell line (THP-1).

Results: Neoandrographolide was isolated from methanolic extract of A. macrobotrys which had a melting point of 174-175ºC. FTIR results had shown stretching for –OH, 342758 cm⁻¹, sp²-CH, lactone, and α, β unsaturated ester. NMR data confirmed 26 carbon structures. Cytotoxicity of isolated neoandrographolide was 22.59 μg/ml. Further lipopolysaccharide (LPS) induced TNFα inhibition was highest in the case of isolated neoandrographolide in comparison with the crude extract of A. macrobotrys.

Conclusion: Neoandrographolide can be used as a new source of neoandrographolide with anti-inflammatory abilities by inhibiting the TNF-α release in THP-1 cells.

Key words: Andrographis, Anti-inflammation, Terpenoids, THP-1 cells, TNF-α.

INTRODUCTION

Andrographis is a genus that belongs to the family Acanthaceae with medicinally potential species out of which about 28 species are distributed in India. Among the species of this genus, few species like Andrographis paniculata are extensively examined for their medical applications while other species are being used folk medicines. Andrographis macrobotrys Nees is one such ethnomedicinally significant plant distributed in Karnataka and Kerala. It is also known as Guude Kirathakaddi, Guude Kalamegha (in Kannada) by the vernacular people with traditional medicinal importance to treat a snake bite, fever, muscle pain, antipyretic, and skin diseases.

Phytochemical analysis of A. macrobotrys showed the presence of phytochemicals like glycosides, flavonoids, steroids, triterpenoids, tannins, saponins, and phenolic compounds with antimicrobial effects. Two new flavonoids were isolated and characterized from the plant, while it also possessed an important diterpenoid, neoandrographolide in an appreciable quantity. Neoandrographolide was previously reported and no flowcytometry-based studies of TNF-α inhibition in human monocytes (THP-1) induced by neoandrographolide. Thus this work was taken up aiming to isolate and characterize neoandrographolide from A. macrobotrys and to evaluate the ability to inhibit TNF-α production in THP-1 cells using fluorescein isothiocyanate (FITC) mouse anti-human TNF-α antibodies.

MATERIALS AND METHODS

Andrographis macrobotrys Nees plants were collected from Puttur (12.7648°N 75.1842°E), Karnataka.
Plants were identified using Flora of British India and voucher specimen (DSR-038) was deposited at Herbarium, Department of Botany, Shivaji University, Kolhapur (SUK), India. Plants were maintained at the experimental garden. Healthy leaves were shade dried and made into powder. 60 g of such powder was used for the extraction in 500 ml of methanol (Analytical grade, HiMedia, Mumbai, India) as solvent using soxhlet apparatus at 70°C for 7-8 h. A sticky green colored paste was obtained after evaporating the solvent. In the end, 8 g of Andrographis macrobotrys extract (AME) was obtained after evaporating the solvent with a percentage of yield of 13.3%.

Isolation of neoandrographolide

AME was loaded on analytical TLC silica plates (MERK, Mumbai, India) prepared in a concentration of 2 mg/ml. Different solvents were used to optimize the solvent system viz. pure chloroform, pure ethyl Acetate, pure ethanol, chloroform: ethanol (9:1), chloroform:1 ethanol (1:9), ethanol: ethyl acetate(6:4), chloroform: ethanol: ethyl acetate (1:6:3). Among these only chloroform had shown the maximum number of bands after visualizing in iodine chamber saturated with iodine crystals.

AME was washed with hexane and chloroform to remove nonpolar and colored compounds. Then it was saturated with silica (60-120 mesh) and subjected to column chromatography where the internal diameter of the column was 4.5 cm, the length of the stationary phase was 20 cm, and the length of the sample binding gel was 1.5 cm. The elute rate was set to 1 ml/min. The solvent system used was chloroform: ethanol: ethyl acetate in a ratio of (1:6:3). The fraction matched with the standard neoandrographolide (Natural Remedies, Bangalore, India) was taken for further purification.

Preparative TLC was performed to get pure compound in a higher quantity. 5 g of silica in 10 ml of distilled water was coated in a glass plate after air dry it was activated by keeping it in a hot air oven at 80°C overnight. A long streak of the fraction was made and let run in pure ethanol as solvent. The single band obtained was dissolved in ethanol and silica gel was allowed to settle at the bottom. The supernatant was dried at room temperature and used for further studies.

Chemical characterization

The melting point was determined using a melting point apparatus where visual observation was done to note down the temperature at which the compound changed its state. Isolated neoandrographolide was dissolved in ethanol at a concentration of 1 mg/ml. It was scanned for maximum absorption in the UV-visible region from 190-1000 nm using a UV-visible spectrophotometer (Jasco V 750, Japan). To determine the functional groups Fouriertransform infrared spectroscopy (Thermo Fisher Scientific, Nicolet 6700, USA) was used where KBr discs with 5% of the sample. IR transmission spectra were obtained after scanning at the wavenumbers ranging from 400 to 4000 cm⁻¹. For further confirmation, isolated neoandrographolide was dissolved in dimethyl sulphoxide (DMSO) and analyzed for 1H NMR (399.8 MHz) and 13C NMR (100.53 MHz) (Jeol, India).

Cytotoxicity by MTT assay

THP-1 cells were cultured in Roswell park memorial institute (RPMI-1640) medium (HiMedia, Mumbai, India) along with 10% of fetal bovine serum (FBS) and subjected to MTT assay. Aspirin which is a vital role in inflammatory actions. Terpenoids are a large group of phytochemicals that are associated with different kinds of biological activities. Diterpenes like trilobolide, α abietic acids, were reported as inhibitors of LPS induced TNF-α activity. Characterization of the isolated compound showed a similar TLC band as that of the commercially available neoandrographolide (Figure 1). Melting point results were 174-175°C and no absorption in the UV-visible region. FTIR results showed stretching at 3427.58, 2926.41, 2848.83, 2965.51, 1650.71 cm⁻¹ and –C–H bending at 1445.01, 1026.41, and 1072.18 cm⁻¹ (Figure 2A), and these results were in concurrence with standard andrographolide (Figure 2B). 1H NMR (399.8 MHz) and 13C NMR (100.53 MHz) results from the isolated compound were compared with that of standard neoandrographolide and data were represented in Figure 3A and Figure 3B respectively. Isolated neoandrographolide was tested for the cytotoxic effect on THP-1 and also for LPS induced TNF-α inhibiting activity. The IC₅₀ of aspirin, isolated neoandrographolide, and AME were 30.58 μg/ml, 57.72 μg/ml, and 22.59 μg/ml respectively. The flowcytometric results indicating the percentage of FITC mouse anti-human TNF-α high and low cells are represented in Figure 4.

DISCUSSION

Tumor necrosis factor-α is a type of transmembrane protein released from a wide spectrum of immune cells such as mast cells, T cells, neutrophils, natural killer cells when there is induced stress. These play a vital role in inflammatory actions. Terpenoids are a large group of phytochemicals that are associated with different kinds of biological activities. Diterpenes like trilobolide, α abietic acids, were reported as inhibitors of LPS induced TNF-α activity. Characterization of the isolated compound showed a similar TLC band as that of the commercially available neoandrographolide (Figure 1). Melting point results were 174-175°C which was also incomparable with the previous reports of neoandrographolide isolated from A. paniculata. FTIR results –OH stretching at 3427.58 cm⁻¹ and sp²-C=H stretching at 2926.41, 2848.83, 2965.51, lactone stretch was at 1750, α, β unsaturated ester stretching was seen at 1354.08 cm⁻¹ that were in correspondence with the previous reports. The fingerprinting region was almost the same as the commercial neoandrographolide confirming its structure (Figure 2A and 2B). 1H-NMR (400 MHz, 399.8 MHz) and 13C NMR (100.53 MHz) results from the isolated compound were compared with that of standard andrographolide (Figure 2B).
Figure 1: Thin layer chromatography. A. TLC sheet where 1 corresponds to AME after removal of non-polar compounds, 2 corresponds to standard neoandrographolide. B. TLC sheet where 1 is an isolated compound, 2 is standard neoandrographolide.

Figure 2: FTIR transmittance spectrum at 400-4000/cm. A. Isolated compound. B. Standard neoandrographolide.
Bhat, et al.: Isolation, Characterization of Neoandrographolide from *Andrographis macrobotrys* Nees and Evaluation of its effect on LPS induced TNF-α Activity

DMSO-d6 δ 7.46-7.40 (1H), 4.87-4.72 (6H), 4.59-4.53 (1H), 4.39-4.31 (1H), 4.03-3.95 (1H), 3.88-3.80 (1H), 3.67-3.55 (1H), 3.44-3.34 (1H), 3.10-2.95 (4H), 2.93-2.85 (1H), 2.33-2.17 (1H), 2.03-1.55 (8H), 1.54-1.12 (4H), 1.02-0.73 (4H), 0.63-0.53 (3H) (Figure 3A). 13 26 carbons could be observed supporting the molecular formula C_{26}H_{40}O_{8} (Figure 3B).

Flowcytometric analysis of TNF-α inhibiting activity of isolated neoandrographolide along with standard anti-inflammatory drug aspirin and crude methanolic extract revealed that TNF-α low cells were high (96.6%) in case of untreated cells (Figure 4A) (not treated with LPS and test samples). When the THP-1 cells were treated with LPS, there was a higher active TNF-α production where TNF-α high cells were 30.8% (Figure 3B), while it was 3.39% when the cells were not treated with LPS (Figure 3A). Among the test, samples analyzed aspirin showed the best inhibiting activity, while neoandrographolide (TNF-α high- 96.7% and TNF-α low- 3.25%) was showing better inhibiting activity than the crude. In the previous report of Liu et al., neoandrographolide was also showing TNF-α inhibition when added with the LPS in RAW 264.7 macrophages.

Figure 3: NMR spectral analysis. A. ^1^H NMR spectra of isolated compound. B. ^13^C NMR spectra of isolated compound.
CONCLUSION

The present study suggests *A. macrobotrys* as source neoandrographolide isolation with considerable yield. A simple methodology for the isolation makes this plant a better option over *A. paniculata*. Neoandrographolide has proved to be an anti-inflammatory agent by the preliminary studies of LPS induced TNF-α inhibition making way to explore more for its anti-inflammatory abilities.

REFERENCES
1. Bhat KG. Flora of Udupi. 2003;470:913.
2. Alagesabopathi C. Phytochemical analysis and antimicrobial evaluation of *Andrographis macrobotrys* Nees- An endangered medicinal plant of India. Int J Sci Res. 2014;3(10):1617-23.
3. Reddy BA, Reddy MV, Gunasekar D, Murthy MM, Caux C, Bodo B. *Two new flavonoids from Andrographis macrobotrys*. Indian J Chem. 2005;44:1966-9.
4. Dalawai D, Aware C, Jadhav JP, Murthy HN. *RP-HPLC analysis of diterpene lactones in leaves and stems of different species of Andrographis*. Nat Prod Res. 2019;1:4.
5. Liu J, Wang ZT, Ji LL. In vivo and in vitro anti-inflammatory activities of neoandrographolide. Ann J Chin Med. 2007;35(2):317-28.
6. Pfisterer PH, Rollinger JM, Schyschka L, Rudy A, Vollmar AM, et al. Neoandrographolide from *Andrographis paniculata* as a potential natural chemosensitizer. Planta Med. 2010;76:1698-700.
7. Yang T, Shi H, Wang Z, Wang C. Hypolipidemic effects of andrographolide and neoandrographolide in mice and rats. Phytother Res. 2013;27:618-23.
8. Iqbal M, Verpooorte R, Korthout HAAJ, Mustafa NR. Phytochemicals as a potential source for TNF-α inhibitors. Phytochem Rev. 2012;12:86-93.
9. Zhou HF, Niu DB, Xue B, Li FQ, Liu XY, He OH, et al. Triptolide inhibits TNF-α, IL-1β, and NO production in primary microglial cultures. Neuroreport. 2003;14(7):1091-5.
10. Takahashi N, Kawada T, Goto T, Kim CS, Taimatsu A, Egawa K, et al. Abietic acid activates peroxisome proliferator-activated receptor-γ (PPARγ) in RAW264.7 macrophages and 3T3-L1 adipocytes to regulate gene expression involved in inflammation and lipid metabolism. FEBS Lett. 2003;550:190-4.
11. Sharma V, Qayum A, Kaul S, Singh A, Kapoor KK, Mukharjee D, et al. Carbohydrate modifications of Neoandrographolide for improved reactive oxygen species-mediated apoptosis through mitochondrial pathway in colon cancer. ACS Omega. 2019;4:20435-42.
12. Matsuda T, Kuroyanagi M, Sugiyama A, Umehara K, Ueno A, Nishi K. Cell differentiation-inducing diterpenes from *Andrographis paniculata* Nees. Chem Pharm Bull. 1994;42(6):1216-25.
13. Gupta S, Choudhry MA, Yadava JNS, Srivastava V, Tandon JS. Antidiarrhoeal activity of diterpenes of *Andrographis paniculata* (Kal-Megh) against *Escherichia coli* enterotoxin in invivo models. Int J Crude Drug Res. 1990;27(4):273-83.
GRAPHICAL ABSTRACT

ABOUT AUTHORS

Medha A. Bhat is research scholar at Department of Botany, Karnatak University, Dharwad, India. She has completed Master of Science in Botany from same university.

Hosakatte Niranjana Murthy professor in Department of Botany, Karnatak University, Dharwad, India, has obtained Ph.D. degree from the same university. He has tremendous passion for research and academics. Since 1986, Apart from his teaching experience of 34 years, he possesses extensive research experience in the area of plant biotechnology. Prof. Murthy has post-doctoral and collaborative research experience in many foreign research institutes. He has successfully completed more than 15 research projects funded by various agencies and guided several Ph.D. students. Prof. Murthy has published more than 200 research articles in international peer reviewed journals with high impact factor.

Cite this article: Bhat MA, Murthy HN. Isolation, Characterization of Neoandrographolide from Andrographis macrobotrys Nees and Evaluation of its effect on LPS induced TNF-α Activity. Pharmacog J. 2021;13(3): 669-74.