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Original Article

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. But there are no previous reports of isolation of neoandrographolide from *A. macrobotrys*. 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 (^{1}H NMR), carbon-13 (C13) nuclear magnetic resonance (^{13}C NMR) analysis. 3-(4,5-dimethylthiaxo-2yl) 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 TNF-α 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 174-175°C. FTIR results had shown stretching for –OH, 3427.58 cm^{-1},sp^{3}-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 TNF-α inhibition was highest in the case of isolated neoandrographolide in comparison with the crude extract of *A. macrobotrys*.**Conclusion:***A. macrobotrys* 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.

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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 ethno-pharmacologically significant plant distributed in Karnataka and Kerala.[1] It is also known as Gudde Kirathakaddi, Gudde 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.[2] Two new flavonoids were isolated and characterized from the plant,[3] while it also possessed an important diterpenoid, neoandrographolide in an appreciable quantity.[4] Neoandrographolide was previously reported as isolated from *Andrographis paniculata* with biological activities like anti-inflammatory activity,[5]chemosensitizing activity,[6]and hypolipid effect.[7]

Tumor necrosis factor-α (TNF-α) is the most significant inflammatory cytokine which plays role in immune responses. Overproduction of TNF-α leads to various kinds of health issues like asthma, rheumatoid arthritis, psoriatic arthritis, septic shock, diabetes, and inflammatory bowel diseases. Synthetic drugs are always considered within the boundary of their limitations. Thus scientists are focussing on natural plant products to regulate the production of TNF-α.[8]

It was found that there are no reports of isolation of neoandrographolide from *A. macrobotrys* 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.

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Plants were identified using Flora of British India and voucher specimen (DSD-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 70ºC 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 yieldof 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 80ºC 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 sulfoxide (DMSO)-$d_6$and analyzed for ^{1}H NMR (399.8 MHz) and ^{13}C NMR (100.53 MHz) (Jeol, India).

Cytotoxicity by MTT assay

THP-1cells 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 well-known drug for anti-inflammatory activity was also tested along with isolated andrographolide and AME. The formazan crystals formed are dissolved in 100 µl of DMSO and optical density wasmeasured at 570 nm ($OD_{570-620}$). IC_{50} of Aspirin, isolated neoandrographolide and AME were calculated by the dose-response curve.The nontoxic concentration of the samples was set for further experiment.

In vitro evaluation of TNF- α activity

The flowcytometric method was followed to evaluate the TNF- α activity induced by the isolated neoandrographolide in comparison with AME and aspirin. THP-1 cells were pre-cultured in 6 well plates at a density of 3×10^5 cells/2 ml for 24 h at 37ºC in CO_2 (5%) incubator. 2 µg/ml of lipopolysaccharide was used to stimulate inflammatory response except in one well which was considered as control. Then 1µl BD GolgiStop™, protein transport inhibitor (containing monensin) to all the wells to help in the accumulation of expressed cytokines within the cell. After 4 h of incubation nontoxic concentration (5 µg/ml) of test samples (isolated neoandrographolide, AME, and aspirin) were added and again incubated for 6 h. Such cells are then harvested and washed with phosphate-buffered saline, fixed with 2% paraformaldehyde and incubated for 20 m, and treated with 0.1% of bovine serum albumin (BSA). After 10 m of incubation, 100 µl of FITC mouse anti-human TNF-α was added and incubated at room temperature for 30 m before the flowcytometric analysis. FL 1 detector was used with a 525 nm bandpass filter for green fluorescence.

RESULTS

The *Andrographis macrobotrys* crude extract (AME) was obtained from the dried leaves and was used for the isolation of a major diterpenoid, neoandrographolide. The thin layer chromatogram of AME is shown in Figure 1A after removing the non-polar compounds. A thin layer chromatogram of the isolated compound after column chromatography and preparative TLC along with the standard neoandrographolide is shown in Figure 1B. The characteristic yellow band corresponding to neoandrographolide was observed with an Rf value of 0.88 in pure ethanol.

The melting point determined was found to be 174-175ºC 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). ^{1}H NMR (399.8 MHz) and ^{13}C 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 IC_{50} 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 triptolide,[9] abietic acids,[10] 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 neoandographolide (Figure 1). Melting point results were 174-175ºC which was also incomparable with the previous reports of neoandrographolide isolated from *A. paniculata*.[11,12] FTIR results –OH stretching at 3427.58 cm^{-1} and sp^3-CH stretching at 2926.41, 2848.83, 2965.51, lactone stretch was at1750, α, β unsaturated ester stretching was seenat 1354.08 cm^{-1} that were in correspondence with the previous reports.[12,13] The fingerprinting region was almost the same as the commercial neoandrographolide confirming its structure (Figure 2A and 2B). ^{1}H-NMR (400 MHz,

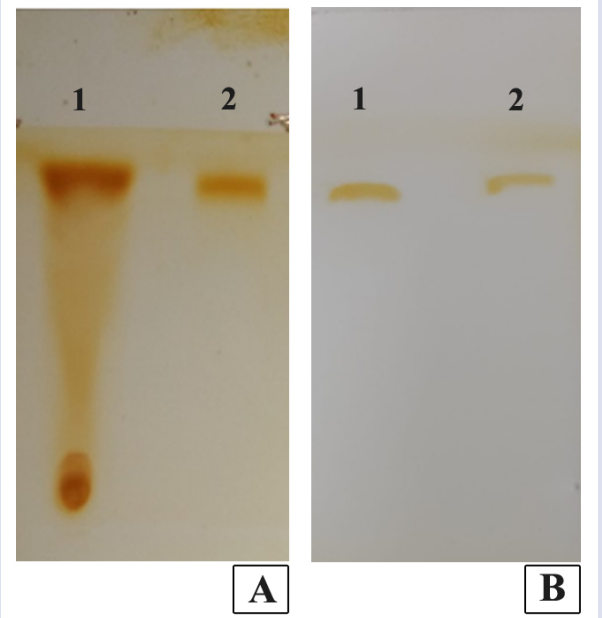

Figure 1: Thin layer chromatography. A. TLC sheet where 1 corresponds to AME after removal of non-polar compounds, 2 corresponds to standard neoandrographolide. B. TLC sheet where 1 is an isolated compound, 2 is standard neoandrographolide.

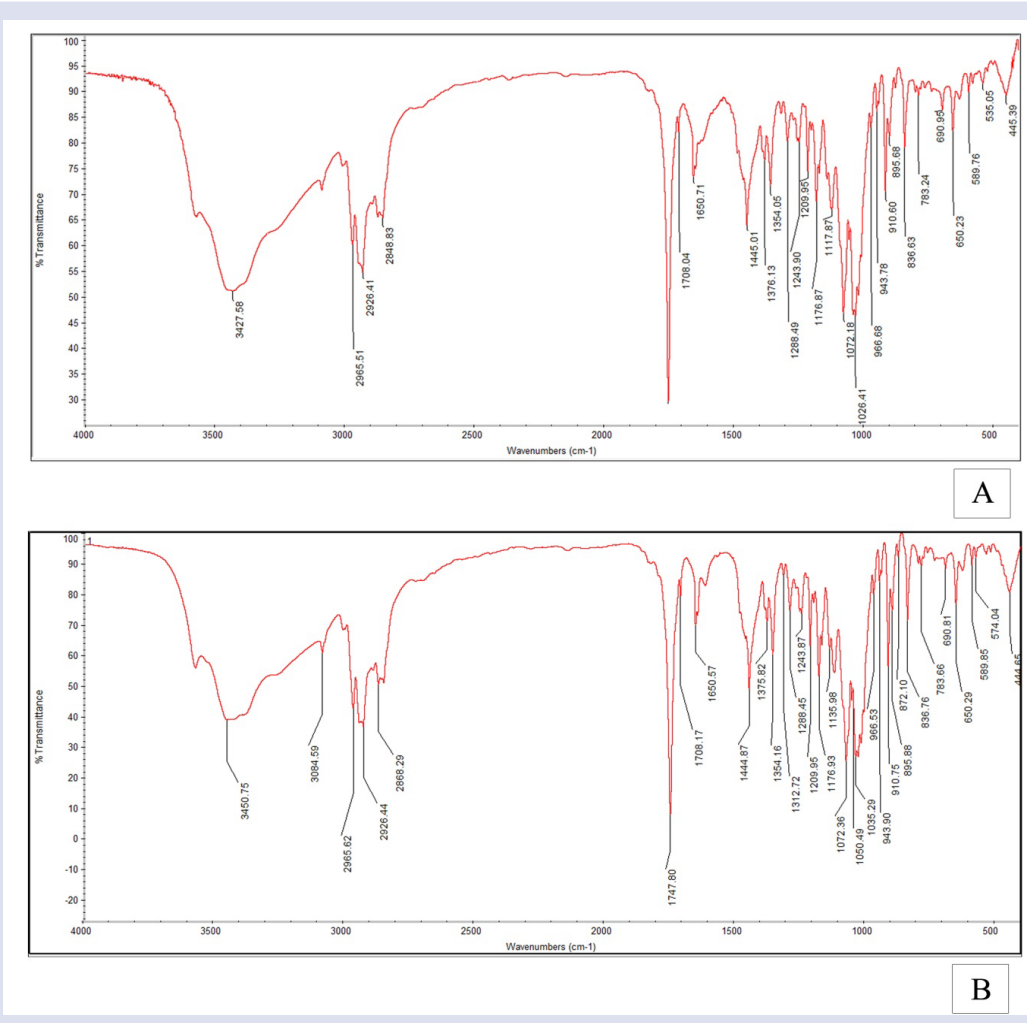

Figure 2: FTIR transmittance spectrum at 400-4000/cm. A. Isolated compound. B. Standard neoandrographolide

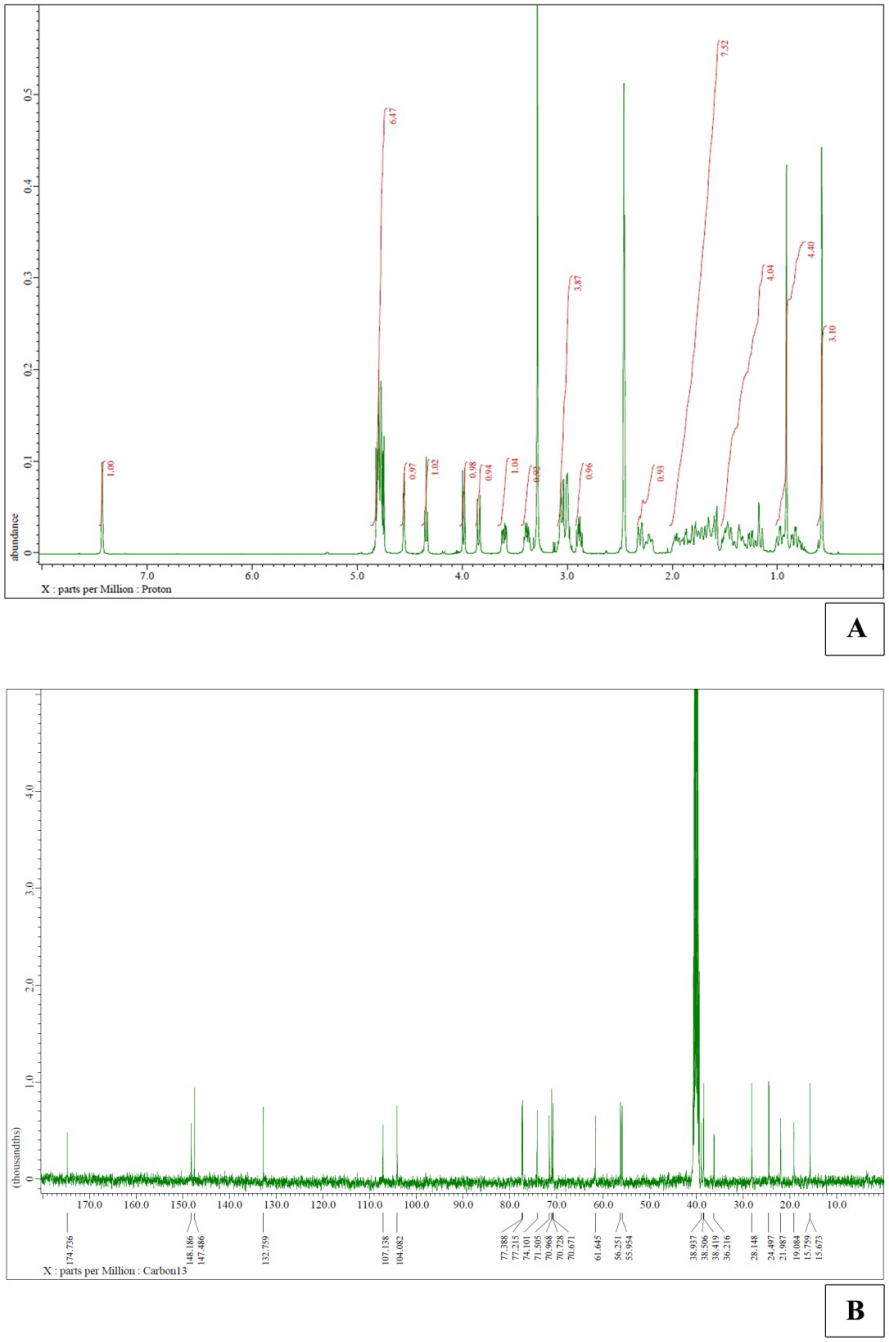

Figure 3: NMR spectral analysis. A. ^{1}H NMR spectra of isolated compound. B. ^{13}C NMR spectra of isolated compound.

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.[5] neoandrographolide was also showing TNF-α inhibition when added with the LPS in RAW 264.7 macrophages.

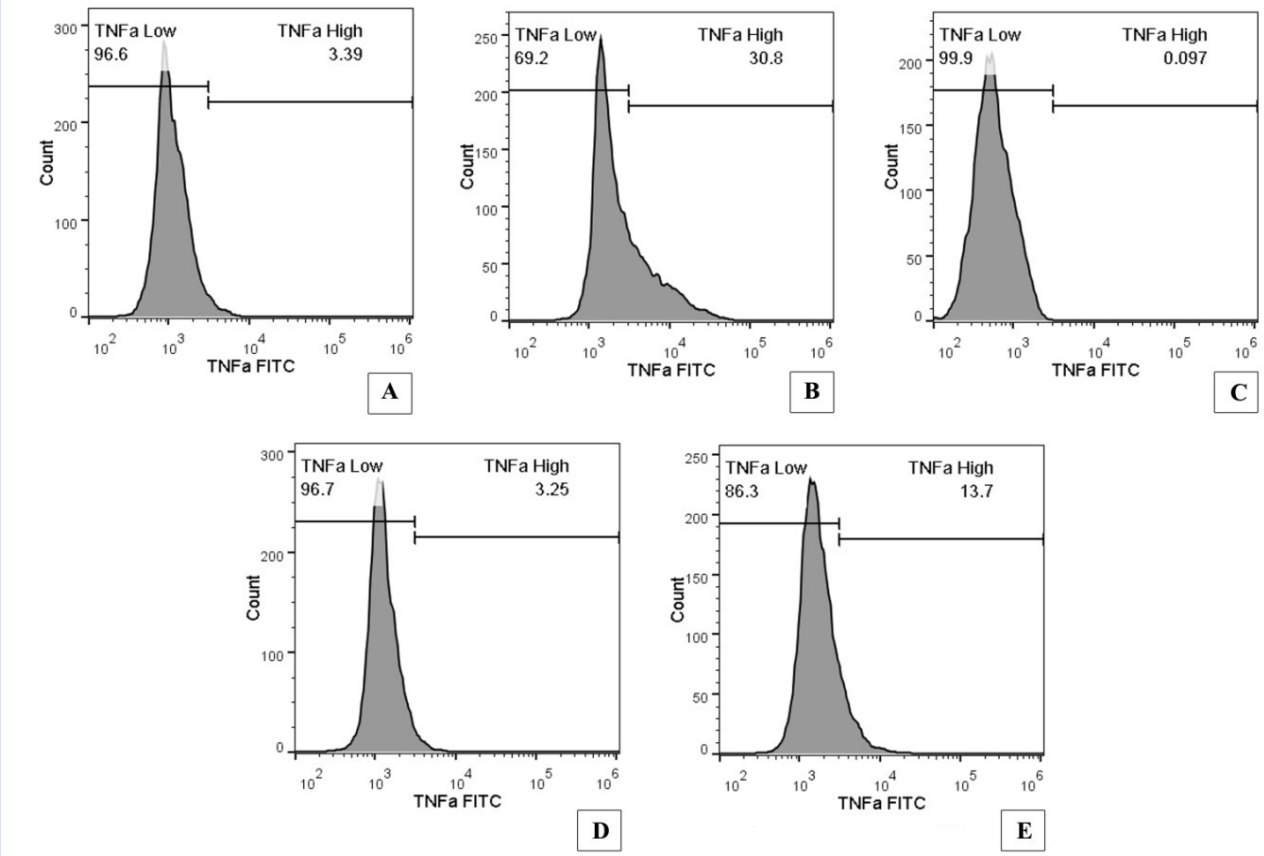

Figure 4: Flow cytometric analysis of TNF-α inhibition activity. A. Untreated THP-1 cells. B. LPS treated THP-1 cells. C. Aspirin treated LPS induced cells. D. Neoandrographolide treated LPS induced cells. E. AME treated LPS induced cells.

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.

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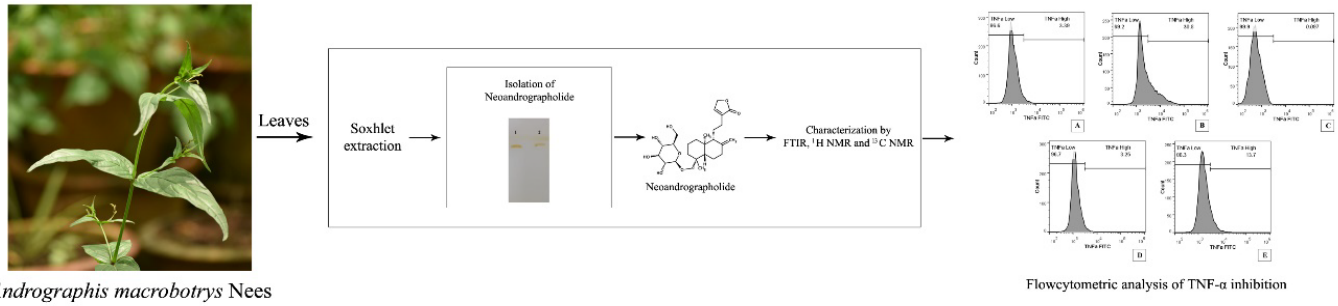
GRAPHICAL ABSTRACT



Andrographis macrobotrys Nees

Flowcytometric analysis of TNF-α inhibition

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