Secondary metabolite profiling and characterization of diterpenes and flavones from the methanolic extract of *Andrographis paniculata* using HPLC-LC-MS/MS

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**Abstract**

**Background:** *Andrographis paniculata* is a well-known medicinal plant that contains various classes of bioactive secondary metabolites. It is widely used by the traditional medicinal healers for treatment of malaria and other diseases. There is an urgent need for screening of potent novel compounds from the methanol extract of *A. paniculata*. Earlier, we obtained appreciable in vitro anti-malarial activity (IC\(_{50}\)-10.75 \(\mu\)g/ml) in the same plant. In current study, we developed novel analytical methods for rapid identification and characterization of diterpenes and flavones using chromatographic and spectroscopic techniques and identified major compounds that might possess anti-malarial activities.

**Results:** Based on the chromatographic and mass spectrometric features, we have identified a total of 74 compounds (25 compounds from positive ion mode; 49 compounds from negative ion mode). The mass spectrum data predicted andrographolide (15%) presence in the highest amount in both positive and negative ion modes. Based on the percentage purity, Andrographolide and skullcapflavone I was selected as representative class of diterpenes and flavones for fragmentation studies.

**Conclusions:** The result led to identification of Neoandrographolide, andrographolactone, 14-dehydroxy-11,12-didehydroandrographolide, skullcapflavone I, and 5-Hydroxy-2',7,8-tri methoxy flavone from the methanolic extract of *A. paniculata* that is used in traditional medicine by tribal healers of Amarkantak region for treating malaria. These could be lead compounds for the development of novel anti-malarial drugs.

**Keywords:** Flavones, Diterpenes, HPLC, Liquid chromatography, Skullcapflavone I, Andrographolide

**Background**

From historic times, plants are used to treat various disorders and diseases of mankind [1, 2]. Amarkantak, Madhya Pradesh (India), is a biodiversity-rich region that has around 1498 plant species, including several medicinal plants. As per World Health Organization (WHO) estimate, herbal medicines are used by around three-fourth of the world population for meeting primary health care requirements [3, 4]. We have earlier documented 19 medicinal plants (including *Andrographis paniculata*) being used by traditional medicinal healers of Amarkantak region and prepared their Geographical Information System (GIS) maps [5, 6]. Based on frequency of usage, *A. paniculata* was selected for carrying out in vitro anti-malarial assays, where we obtained promising results in the methanolic (IC\(_{50}\)-10.75 \(\mu\)g/ml)
whole plant extract against drug-sensitive 3D7 and drug resistance K1 strains (10.64 μg/ml) of *Plasmodium falciparum* [7]. *A. paniculata* belonging to family Acanthaceae, which is a tropical traditional medicinal plant used by native medicinal healers of Philippines, India, Malaysia, Taiwan, Hong Kong, China, Indonesia, and many South Asian countries [2, 8, 9].

Medicinal plants are an essential source of new natural products, which have huge potential as enzyme inhibitors, anti-oxidants, herbicides, and anti-parasitic molecules [10, 11]. Plant extracts typically contain mixture of various types of bioactive constituents that are known to work in a synergetic manner in most of the herbal medicines [11]. Identification of bioactive phytoconstituents in the plant extract is a tedious process [12]. Pharmacologically active phytochemicals have been the basis of many current drugs and their structures have laid foundations for the synthesis of several derivatives with improved activities. The phytochemicals obtained from the plants are a source of tremendous chemical diversity, necessitating the development of specific and sensitive chemical profiling methods for plant extracts [12]. For the accurate separation, identification, and characterization of bioactive phytoconstituents from plant extracts, various types of chromatographic techniques in combination with different types of detection methods capable for efficient screening of respective extracts are being used [12, 13]. Separation, identification, and systematic profiling of active bioactive constituents followed by consistent evaluation of the identified compounds can be accomplished by the use of high-performance liquid chromatography (HPLC) that has been vastly used for chromatographic fingerprinting of the phytoconstituents. The liquid chromatography mass spectrometry utilizes Electro-spray ionization as ion source that is the most successful interface and a powerful approach for the identification of unknown phytochemicals [11].

We identified compounds present in the methanol extract of *A. paniculata* using basic analytical techniques such as thin layer chromatography, UV-Visible spectroscopy, Fourier transform infrared spectrophotometer (FTIR), and HPLC. The major constituents of potent anti-malarial active methanol extract of *A. paniculata* were characterized by liquid chromatography mass spectrometry (LC-MS) based on the retention time and MS spectrum. We have also reported the distribution of diterpenes and flavones based on the obtained spectrum. Chromatography and spectroscopy techniques may be quite useful for the identification and determination of active constituents in medicinal plants used in the traditional medicinal system.

**Methods**

### Chemicals and reagents

Chloroform (analytical grade) and methanol (LC grade), acetonitrile (HPLC and LC grade), and all other analytical grade reagents were procured from Central Drug House. Ultra-pure water (Millipore, Milford, MA, USA) was used in all preparations.

**Plant authentication and extraction process**

*A. paniculata* was collected at the elder stages and generally at midday in the month of July to September, 2019 from Amarkantak, Madhya Pradesh, India. Dr. Ravindra Shukla, Department of Botany, identified the plant specimen. The voucher specimen (AM/DOB/004) was deposited for the future references. After washing with water the plant was dried in shade for 20 days at room temperature (RT), powdered and stored till further use. Plant powder (200 g) was subjected to extraction using methanol, chloroform, *n*-hexane, and double-distilled water. After four cycles of extraction, extracts were concentrated using a rotary evaporator. The semi-solid extracts were stored at 4 °C until the samples were submitted for the anti-malarial activity. The methanol extract of plant was found potentially active against drug sensitive 3D7 and drug resistance K1 strains.

**Identification and characterization of anti-malarial active compounds**

### Thin layer chromatography

Thin layer chromatography (TLC) was performed at RT using the mixture of chloroform: methanol (90:10) as mobile phase. Visualization of the spots was done by the introduction of the TLC plates (Merck-silica gel 60 F254) inside a UV inspection cabinet (254 nm) for few minutes. The Rf values were calculated for possible identification of the compounds.

### UV-visible spectroscopy

Methanol extract of *A. paniculata* was diluted in methanol (0.10 mg/ml) and the spectrum was recorded in a UV-Vis-Spectrophotometer (Shimadzu, UV-1800) between 200 and 800 nm range. Methanol was used as a blank.

### Fourier Transform infrared spectrophotometer

IR spectra of the methanol extract was recorded by a FTIR analyzer (Thermo scientific iD7 ATR). Semi-solid methanol extract was diluted in chloroform and scanned between 400 and 4000 cm⁻¹ range.

### High-performance liquid chromatography

Methanol extract of *A. paniculata* was used for chromatographic separation using HPLC (Water-HPLC Model no. waters 2489 UV/Visible). In brief, methanol extract (100 mg) was taken in a round bottom flask and diluted with 50 ml of methanol. The resultant solution was sonicated for 5 min and filtered through a 0.45 μm membrane filter. The filtrate was injected into a C18 HPLC...
column (250 mm × 4.6 × 5 μm) and detected using detector (2489, Waters). Isocratic elution was carried out using the acetonitrile:water (60:40 v/v) mobile phase at a flow rate of 1 ml/min at 1600 psi pressure. Then, 10 μl of sample was injected and run time was 30 min. The integration and calibration was done using the Empower 3 software.

**Liquid chromatography mass-spectrometry**
The liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis was performed on Waters ACQUITY QSM-TQD #QBB1152. The system was attached to a diode-array detector HPLC system equipped with quaternary pump system, auto-sampler and column compartment.

**Chromatographic conditions**
An Aqua C18, 150 × 2.1, 2.6 μm column was utilized for gradient elution, using solvent A (95% H2O: ACN), solvent B (ACN), solvent C (Methanol), solvent D (5 mM NH4 in H2O:ACN 95:5), constant flow rate (0.250 ml/min), and flow ramp rate: 0.45 min. Column temperature was 30 °C and run time was 40 min. One microliter sample was injected and the highest pressure limit was 15,000 psi.

**Mass spectrometric condition**
Mass spectroscopy electro spray ionization (MS-ESI) spectrum was obtained in both, positive and negative, ion modes. The capillary voltage was set to 3.50 (kV) in both the modes, while collision gas flow was 0.23 ml/min in positive and 0.10 ml/min in negative ion mode. Then, 120 °C source temperature and ion energy I (0.50) and II (0.30) were used for positive and negative ion modes. The mass range was between 100 and 1000 m/z. Before sample analysis, external instrument calibration was performed to attain correctness for bunch threshold of 0.1459. Mass spectrometer operated in positive ESI mode and spectra were recorded in the mass range from m/z 50 to 350 for daughters of 315, m/z 50 to 375 for daughters of 351, and for their dimer m/z 50 to 725 used for MS/MS. The collision energy was set at 25 eV for both the masses.

**Data processing**
The Water’s software (Version: 1.50.1481) was used for processing the accurate mass data of molecular ions. Data was extracted in terms of ion chromatograms, elution order, behavior of fragmentation, elemental compositions of molecular ions, accurate mass measurement, and comparison with reliable data in chemical database.

**Statistical analysis**
All the data analysis including secondary metabolite distribution, purity percentage, and compound distribution was done using Microsoft Excel software.

**Results**

**TLC and UV-visible spectroscopy**
The chromatographic analysis of the *A. paniculata* methanol extract displayed two spots in the visible region (spot 1: Rf 0.66), (spot 2: Rf 0.82) and five spots in the UV region (spot 5: Rf 0.82), (spot 4: Rf 0.74), (spot 3: Rf 0.7), (spot 2: Rf 0.68), (spot 1: Rf 0.66) (Fig. 1A, B). The *A. paniculata* methanol extract was scanned at wavelength from 200 to 800 nm to obtain the qualitative UV-Visible spectrum that displayed four bands at 241, 416, 538, and 667 nm (Fig. 2) having absorption values of 3.838, 2.955, 0.323, and 1.173, respectively.

![Fig. 1 Thin Layer Chromatograph of methanolic extracts of *Andrographis paniculata* whole plant: A Visualization in visible regions. B Visualization in UV-regions (254 nm)](image)
FTIR analysis

IR absorbance bands of the methanol extract of *A. paniculata* is displayed in Fig. 3. On the basis of spectrum peaks in the region of IR radiation, functional groups were identified. In the spectrum, major peaks were obtained at 3360.19, 2928.50, 2851.47, 2848.9200, 2359.69, 2342.19, 1741.13, 1673.79, 1645.04, 1449.80, 1377.80, 1348.69, 1205.52, 1079.97, 1026.87, 894.75, 668.02, and 648.90 cm\(^{-1}\).

HPLC analysis

HPLC was performed for identifying the principal constituents of the methanol extract of *A. paniculata*. Two peaks observed in the chromatogram were identified as andrographolide (RT-5.832, 68.26%), and neoandrographolide (RT-12.933, 24.77%), while two peaks could not be correlated and named unknown (RT-1.739, 4.13%) and unknown (RT-6.822, 2.84%) (Fig. 4).

Liquid chromatography mass-spectrometry identification of compounds

In the present study, LC MS/MS analysis was performed using both the positive and negative ion modes for the better identification and characterization of molecules present in *A. paniculata* whole plant methanol extract. LC–MS/MS analysis identified 74 (41 diastereomers) secondary metabolites that included 24 (16 diastereomers) in positive ion mode and 49 (25 diastereomers) in negative ion mode. We were able to identify majority of diterpenes and flavones in the *A. paniculata*. The employed method presents the researchers to uniquely identify diterpenes and flavones from the plant extracts and can be utilized for quality control purposes. Among the obtained molecules, 83% belonged to diterpenes while remaining 17% were flavonoids. This distribution represents the chemotypical behavior of *A. paniculata* from biodiversity rich Amarkantak region (Fig. 5).

Identification of diterpenes and flavones

Seventeen diterpenes were detected in positive ion mode in the *A. paniculata* methanol extract. Diterpenes were analyzed in positive and negative ion mode, thus the method was optimized in both ionization modes. Base peak chromatogram of methanol extract in positive and negative ionization modes are shown in Fig. 6A, B and Table 1. Forty-two diterpenes were detected in negative ion mode in methanol extract of *A. paniculata* whole plant. Some essential diterpenes classified as monomers and polymers of diterpenes lactones are shown in Fig. 7. We could identify diterpenes and flavonoids using both
the ionization modes. Seven flavones detected in each of positive and negative ion mode in methanol extract of *A. paniculata* whole plant (Fig. 6A, B and Table 1).

**Purity percentage of identified compounds**

Based on the mass spectrum, a diterpene andrographolide (15%) was presented in the highest amount in methanol extracts followed by andrographolactone (12%), neoandrographolide (9%), 14-Deoxy-11,12-didehydroandrographolide (7%), and andrographic acid (7%), while flavones such as 5-Hydroxy-2′,7,8-trimethoxyflavone (8%) and skullcapavone I (8%) were presented in positive mode. In negative ion mode, we also found andrographolide (diterpenes) (15%) was presented in high amount followed by neoandrographolide (5%) and andrographic acid (5%), while flavones such as skullcapavone I (4%), skullcapavone-2′-O-b-D-glucopyranoside (4%), tetramethoxyflavone (2%), and 5-Hydroxy-7,8,2′,5′-tetra-Methoxyflavone 5-O-glucoside (2%) were presented in minor quantities. Following compounds were also present in the form of diastereomers such as tetramethoxyflavone, ent-labda-8(17), 13Z-diene-15, 16, 19-triol 19-O-glucoside, 14-Deoxy-11,12-didehydroandrographolide, andrographolide, serpyllin, neoandrographolide, deoxyandrographiside, 6-acetyleneandrographolide, bistandrographolide A, andrographic acid, 7-Hydroxy-14-deoxyandrographolide, 3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17), 11-dien-13-oic acid, andrographolactone, 14-Deoxyandrographolide, and andrograpanin (Fig. 8A, B).

**Characterization (MS/MS) of diterpenes and flavones**

Based on the purity percentage, we have selected one diterpene and one flavone compound as representative compounds for the fragmentation pattern of respective class of compounds. The fragmentation pathways and diagnostic product ions of andrographolide (C_{20}H_{30}O_{5}), and their dimer 2(C_{20}H_{30}O_{5}) and skullcapavone I (C_{17}H_{14}O_{6}) were studied by MS/MS spectra of the (M + H)^+ ions, as shown in Figs. 9, 10, and 11, respectively.

Andrographolide and its dimer were detected as its [M + H]^+ and [2M + H]^+ ion at m/z 351 and 702 (Figs. 9 and 10). The MS/MS spectrum displayed resulting ion at m/z 351 with a loss of C_{20}H_{30}O_{5}. The product ion (m/z 351) underwent hydrolysis and lost water molecule to yield ions at m/z 333 and 315, respectively. The product ions (m/z 315) again underwent hydrolysis resulting in the formation of ion with m/z 297. The ion with m/z 333 lost HCHO to yield the ion at m/z 303 that further formed the product ion (m/z 303) with a loss of CO in a reverse Diels-Alder (RDA) reaction [14, 15] to yield ion at m/z 275. The product ion at m/z 275 with a loss of
H₂O (hydrolysis) formed product ion at m/z 257. Loss of C₃H₄ and HCHO, from m/z 257 and 205 respectively, led to the formation of ions at m/z 205 and 175. Loss of C₂H₂ from m/z 175 created ion at m/z 147. This was followed by consecutive loss of CH₃ (demethylation) and C₂H from ion (m/z 147) and (m/z 133) to yield the ions (m/z 133; m/z 109); the ions at (m/z 109; m/z 95); and finally ions at (m/z 95 and m/z 81).

Skullcapflavone I was detected at m/z 315 and its fragmentation pattern is displayed in Fig. 11. The product ion (m/z 315) lost C₇H₄O₂ and resulted into formation of ion (m/z 195). The resulting ion (m/z 195) further lost CH₂ (demethylation) to form resulting ion (m/z 181). This was followed by loss of 2H₂O (hydrolysis) to produce ions at m/z 145 that further lost CH₂ and C₂H₂ to form ions at m/z 131 and m/z 105. The ion (m/z 105) with a loss of CO formed the ion (m/z 77) undergoing reverse Diels-Alder (RDA) reaction [14, 15].

Discussion

Chromatography and spectroscopic techniques play a major role in phytochemicals profiling and are routinely utilized for qualitative and quantitative analysis of pharmaceutically and biologically active materials. The Rf value can be used to indicate the chemical nature (polar or non-polar) of the compounds present in the sample. Based on the comparison of the observed Rf values with literature data, compounds in the extract were identified as diterpenes (4-deoxy-11, 12-didehydroandrographolide (0.84), andrographolide (0.72), or flavones and glycosides (0.72) [16, 17]. Results confirmed the presence of phenolics, flavonoids, and their derivatives indicating the medicinal importance of A. paniculata. UV spectra of andrographolide are usually observed between 230 and 235 nm range; we also observed a sharp peak within this range confirming the presence of andrographolide in the methanol extract [16, 17]. Similarly, spectra for the...
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|------------------|-------------------------------|----------------------------|-------------------|
| Positive mode (ES+) | | | | | | | |
| 1 | 1.13 | 5 | 358.346 | 5-Hydroxy-7,8,2',5'tetramethoxyflavone (C_{19}H_{18}O_{7}) | Flavone | 381.1 (M+Na) | |
| 2 | 3.83 | 7 | 332.44 | 14-Deoxy-11,12-didehydroandrographolide (C_{20}H_{28}O_{4}) | Diterpenoid | 355.1 (M+Na) | |
| 3 | 4.28 | 8 | 328.32 | 5-Hydroxy-2',7,8-trimethoxyflavone (C_{19}H_{16}O_{6}) | Flavone | 329.1 (M+1) | |
| 4 | 8.32 | 12 | 296.41 | Andrographolactone (C_{20}H_{24}O_{2}) | Diterpenoid | 297.2 (M+1) | |
| 5 | 8.32 | 8 | 314.29 | Skullcapavone I (C_{17}H_{14}O_{6}) | Flavone | 315.2 (M+1) | |
| 6 | 8.82 | 7 | 364.43 | Andrographic acid (C_{20}H_{28}O_{6}) | Diterpenoid | 447.1 (M+1+2ACN) | |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|------------------------|--------------------------|-------------------|-------------------------------|---------------------------|-------------------|
| 7      | 9.49     | 15                     | 350.455                  | Andrographolide (C$_{20}$H$_{30}$O$_{5}$) | Diterpenoid                  | 351.2 (M+1)                | ![Structure 1](image1.png) |
| 8      | 10.71    | 1                      | 350.455                  | Andrographolide (C$_{20}$H$_{30}$O$_{5}$) (diastereomers) | Diterpenoid                  | 701.5 (2M+1)               | ![Structure 2](image2.png) |
| 9      | 10.71    | 2                      | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C$_{20}$H$_{28}$O$_{4}$) (diastereomers) | Diterpenoid                  | 333.2 (M+1)                | ![Structure 3](image3.png) |
| 10     | 11.16    | 4                      | 496.597                  | Andropanoside (C$_{26}$H$_{40}$O$_{9}$) | Diterpenoid                  | 993.6 (2M+1)               | ![Structure 4](image4.png) |
| 11     | 11.79    | 1                      | 300.266                  | 5,8,2'-Trihydroxy-7-methoxyflavone (C$_{16}$H$_{12}$O$_{6}$) | Flavone                      | 301.1 (M+1)                | ![Structure 5](image5.png) |
| 12     | 12.01    | 1                      | 298.29                   | 7-O-Methylwogonin (C$_{17}$H$_{14}$O$_{5}$) | Flavone                      | 299.1 (M+1)                | ![Structure 6](image6.png) |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|------------------|-------------------------------|---------------------------|------------------|
| 13     | 13.18    | 3                     | 318.457                  | Andrograpanin (C_{20}H_{30}O_{3}) | Diterpenoid                  | 319.3 (M+1)          |                  |
| 14     | 13.18    | 9                     | 480.598                  | Neoandrographolide (C_{26}H_{40}O_{8}) | Diterpenoid                  | 481.3 (M+1)          |                  |
| 15     | 13.18    | 1                     | 480.598                  | Neoandrographolide (C_{26}H_{40}O_{8}) (diastereomers) | Diterpenoid                  | 499.3 (M+1+H_{2}O)  |                  |
| 16     | 13.67    | 2                     | 318.457                  | Andrograpanin (C_{20}H_{30}O_{3}) (diastereomers) | Diterpenoid                  | 584.3, 319.2 (M+1)  |                  |
| 17     | 13.96    | 3                     | 334.456                  | 14-Deoxyandrographiside (C_{20}H_{30}O_{4}) | Diterpenoid                  | 669.4 (2M+1)         |                  |
| 18     | 14.07    | 5                     | 664.88                   | Bisandrographolide A (C_{40}H_{60}O_{8}) | Diterpenoid                  | 665.4 (M+1)          |                  |
Table 1 LC-MS/MS identified compounds present in anti-malarial active methanol extract of *Andrographis paniculata* (Continued)

| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|-------------------|-------------------------------|---------------------------|-------------------|
| 19     | 16.29    | 1                     | 300.266                  | 5,8,2’-Trihydroxy-7-methoxyflavone (C_{16}H_{12}O_{6}) (diastereomers) | Flavone                     | 301.1 (M+1)                 |                   |
| 20     | 16.66    | 1                     | 358.346                  | 5-Hydroxy-7,8,2’5’tetramethoxyflavone (C_{19}H_{18}O_{7}) (diastereomers) | Flavone                     | 359.2 (M+1)                 |                   |
| 21     | 20.58    | 1                     | 350.455                  | Andrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid                  | 723.6 (2M+Na)               |                   |
| 22     | 20.74    | 1                     | 611.70                   | 19-O-[β-D-Apiofuranosyl(1f2)-β-D-glucopyranosyl]-3,14-dideoxyandrographolide (C_{31}H_{47}O_{12}) | Diterpenoid                  | 634.5 (M+Na)                |                   |
| 23     | 29.70    | 1                     | 351.20                   | 3,15,19-trihydroxy-ent-labda-8(17),13-dien-16-oic acid (C_{20}H_{30}O_{5}) | Diterpenoid                  | 352.3 (M+1)                 |                   |
| 24     | 33.78    | 1                     | 352.42                   | 7-Hydroxy-14-deoxyandrographolide (C_{20}H_{30}O_{5}) | Diterpenoid                  | 353.4 (M+1)                 |                   |
| 25     | 33.99    | 1                     | 352.42                   | 7-Hydroxy-14-deoxyandrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid                  | 353.3 (M+1)                 |                   |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|------------------------|--------------------------|-------------------|-----------------------------|---------------------------|-------------------|
| 1      | 1.12     | 1                      | 342.347                  | Tetramethoxyflavone (C₁₉H₁₈O₆) | Flavone                     | 341.2 (M-1)              |                   |
| 2      | 1.12     | 2                      | 342.347                  | Tetramethoxyflavone (C₁₉H₁₈O₆) (diastereomers) | Flavone                     | 377.1 (M+Cl)             |                   |
| 3      | 1.43     | 3                      | 364.47                   | 14-deoxy-12-methoxyandrographolide (C₂₁H₃₂O₅) (diastereomers) | Diterpenoid                 | 363.2 (M-1)              |                   |
| 4      | 2.94     | 4                      | 476                      | Skullcapavone I 2’-O-b-D-glucopyranoside | Flavone                     | 443.3 (M-1-MeOH)         |                   |
| 5      | 3.84     | 1                      | 352.46                   | 14-Deoxy-17β-hydroxyandrographolide (C₂₀H₃₂O₅) (diastereomers) | Diterpenoid                 | 351.1 (M-1)              |                   |
| 6      | 4.29     | 1                      | 484.6                    | Ent-Labda-8(17), 13Z-diene-15, 16, 19-triol 19-O-glucoside (C₂₆H₄₄O₈) | Diterpenoid                 | 505.0 (M+Na-2)           |                   |
| 7      | 4.29     | 1                      | 484.6                    | Ent-Labda-8(17), 13Z-diene-15, 16, 19-triol 19-O-glucoside (C₂₆H₄₄O₈) (diastereomers) | Diterpenoid                 | 451.2 (M-1-MeOH)         |                   |
| 8      | 5.35     | 2                      | 388.115                  | Serpyllin (C₂₀H₂₀O₈) | Flavone                     | 433.2 (M+formic acid-1)  |                   |
| 9      | 5.97     | 3                      | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C₂₂H₂₂O₈) (diastereomers) | Diterpenoid                 | 367.1 (M+Cl)             |                   |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|------------------|-------------------------------|---------------------------|-------------------|
| 10     | 6.34     | 4                     | 368.46                   | 12S-Hydroxyandrographolide (C_{20}H_{32}O_{6}Na) | Diterpenoid                  | 413.1 (M+formic acid-1) | ![structure](image1) |
| 11     | 6.97     | 3                     | 522.63                   | 6-acetylneoandrographolide (C_{28}H_{42}O_{9}) | Diterpenoid                  | 557.3 (M+Cl)        | ![structure](image2) |
| 12     | 7.09     | 15                    | 350.455                  | Andrographolide (C_{20}H_{16}O_{9}) (diastereomers) | Diterpenoid                  | 349.2 (M-1)         | ![structure](image3) |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|-------|---------|-----------------------|--------------------------|------------------|-------------------------------|----------------------------|-------------------|
| 13    | 7.80    | 2                     | 350.455                  | Andrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid                   | 349.2 (M-1)         | ![Structure 1]     |
| 14    | 8.11    | 1                     | 388.15                   | Serpyllin (C_{20}H_{30}O_{5}) (diastereomers)      | Flavone                       | 423.2 (M+Cl)        | -                 |
| 15    | 8.31    | 3                     | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C_{20}H_{30}O_{4}) (diastereomers) | Diterpenoid                   | 331.2 (M-1)         | ![Structure 2]     |
| 16    | 9.04    | 5                     | 480.598                  | Neoandrographolide (C_{26}H_{40}O_{8}) (diastereomers) | Diterpenoid                   | 515.2 (M+Cl)        | ![Structure 3]     |
Table 1  LC-MS/MS identified compounds present in anti-malarial active methanol extract of *Andrographis paniculata* (Continued)

| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|------------------------|--------------------------|-------------------|-------------------------------|---------------------------|-------------------|
| 17     | 9.25     | 1                      | 350.455                  | Andrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid                | 395.2 (M+formic acid-1) | ![Structure 1](image1) |
| 18     | 9.74     | 3                      | 480.598                  | Neoandrographolide (C_{26}H_{40}O_{8}) (diastereomers) | Diterpenoid                | 461.1 (M-1-H2O)      | ![Structure 2](image2) |
| 19     | 9.85     | 5                      | 364.43                   | Andrographic acid (C_{20}H_{28}O_{6}) | Diterpenoid                | 363.1 (M-1)         | ![Structure 3](image3) |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|------------------------|--------------------------|-------------------|-----------------------------|---------------------------|-------------------|
| 20     | 10.06    | 1                      | 350.455                  | Andrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid | 363.2 (M+formic acid-1) | ![Predicted structure](image1) |
| 21     | 2        | 664.88                 | 685.5 (M+Na-2)           | Bisandrographolide A (C_{40}H_{56}O_{8}) (diastereomers) | Diterpenoid |                       | ![Predicted structure](image2) |
| 22     | 10.24    | 1                      | 494.57                   | 14-deoxy-11,12-dihydroandrographiside (C_{26}H_{38}O_{9}) | Diterpenoid | 529.3 (M+Cl)          | ![Predicted structure](image3) |
| 23     | 10.53    | 1                      | 497.60                   | Deoxyandrographiside (C_{26}H_{38}O_{9}) | Diterpenoid | 542.2 (M+formic acid-1) | ![Predicted structure](image4) |
| 24     | 10.72    | 2                      | 350.455                  | Andrographolide (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid | 745.5 (2M+formic acid-1) | ![Predicted structure](image5) |
Table 1 LC-MS/MS identified compounds present in anti-malarial active methanol extract of *Andrographis paniculata* (Continued)

| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|-------------------|--------------------------------|---------------------------|---------------------|
| 25     | 11.15    | 1                     | 497.60                   | Deoxyandrographide (C_{26}H_{41}O_{9}) (diastereomers) | Diterpenoid                | 542.3 (M+formic acid-1) - |                     |
| 26     | 11.53    | 2                     | 334456                   | 14-Deoxyandrographolide (C_{29}H_{30}O_{4}) | Diterpenoid                | 333.2 (M-1), 539.3    |                     |
| 27     | 11.76    | 2                     | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C_{29}H_{28}O_{4}) (diastereomers) | Diterpenoid                | 331.2 (M-1)            |                     |
| 28     | 12.00    | 1                     | 522.63                   | 6-acetylneoandrographolide (C_{38}H_{42}O_{9}) (diastereomers) | Diterpenoid                | 567.5 (M+formic acid-1) |                     |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|-------------------------|-------------------|-----------------------------|--------------------------|-------------------|
| 29     | 12.32    | 1                     | 332.44                  | (C_{20}H_{28}O_{4}) (diastereomers) | Diterpenoid | 331.2 (M-1) | ![Molecular Structure](image1) |
| 30     | 12.71    | 1                     | 350.455                 | (C_{20}H_{30}O_{5}) (diastereomers) | Diterpenoid | 349.2 (M-1) | ![Molecular Structure](image2) |
| 31     | 13.19    | 2                     | 480.598                 | (C_{26}H_{40}O_{8}) (diastereomers) | Diterpenoid | 525.3 (M+formic acid-1) | ![Molecular Structure](image3) |
Table 1 LC-MS/MS identified compounds present in anti-malarial active methanol extract of *Andrographis paniculata* (Continued)

| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|-------------------|-------------------------------|-----------------------------|-------------------|
| 32     | 13.33    | 1                     | 330.42                   | 19-hydroxy-3-oxo-ent-labda-8(17),11,13-trien-16,15-olide (C20H26O4) | Diterpenoid                  | 329.3 (M-1)               | -                 |
| 33     | 13.66    | 1                     | 522.63                   | 6-acetylneoandrographolide (C28H42O9) (diastereomers) | Diterpenoid                  | 521.3 (M-1)               | -                 |
| 34     | 13.85    | 2                     | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C20H28O4) (diastereomers) | Diterpenoid                  | 331.3 (M-1)               | -                 |
| 35     | 14.06    | 1                     | 332.44                   | 14-Deoxy-11,12-didehydroandrographolide (C20H28O4) (diastereomers) | Diterpenoid                  | 709.5 (2M+formic acid-1) | -                 |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|-------|----------|-----------------------|--------------------------|-------------------|-------------------------------|-----------------------------|-------------------|
| 36    | 14.39    | 1                     | 522.63                   | 6-acetylneoandrographolide (C_{28}H_{42}O_{9}) (diastereomers) | Diterpenoid                  | 567.4 (M+formic acid-1) |                   |
| 37    | 15.34    | 2                     | 664.88                   | Bisandrographolide A (C_{40}H_{56}O_{8}) (diastereomers)     | Diterpenoid                  | 663.5 (M-1)           |                   |
| 38    | 15.63    | 1                     | 268.39                   | 13,14,15,16-tetranor-ent-labd-8(17)-ene-3,12,19-triol (C_{16}H_{28}O_{3}) | Diterpenoid                  | 267.2 (M-1)           |                   |
| 39    | 15.90    | 1                     | 364.43                   | Andrographic acid (C_{20}H_{28}O_{6}) (diastereomers)       | Diterpenoid                  | 727.6 (2M-1)          |                   |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|-------|----------|-----------------------|--------------------------|------------------|-------------------------------|-----------------------------|---------------------|
| 40    | 16.40    | 2                     | 520.482                  | 5-Hydroxy-7,8,2',5'-tetra-Methoxyflavone 5-O-glucoside (C_{25}H_{28}O_{12}) | Flavone                      | 555.4 (M+Cl)             | ![Flavone structure](image1) |
| 41    | 16.81    | 1                     | 352.42                   | 7-Hydroxy-14-deoxyandrographolide (C_{19}H_{28}O_{6}) (diastereomers) | Diterpenoid                  | 397.3 (M+formic acid-1) | ![Diterpenoid structure](image2) |
| 42    | 16.96    | 1                     | 294.38                   | 3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid (C_{17}H_{26}O_{6}) (diastereomers) | Diterpenoid                  | 293.3 (M-1)              | ![Diterpenoid structure](image3) |
| 43    | 17.50    | 1                     | 36443                    | Andrographic acid (C_{20}H_{28}O_{6}) (diastereomers) | Diterpenoid                  | 325.1 (M-K)              | ![Andrographic structure](image4) |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|-------------------|-----------------------------|---------------------------|---------------------|
| 44     | 18.10    | 2                     | 318.457                  | Andrograpanin (C_{20}H_{30}O_{3}) | Diterpenoid | 339.2 (M+Na-2)            | ![Predicted structure](image1.png) |
| 45     | 18.56    | 1                     | 296.41                   | Andrographolactone (C_{20}H_{24}O_{2}) (diastereomers) | Diterpenoid | 295.3 (M-1)               | ![Predicted structure](image2.png) |
| 46     | 21.60    | 2                     | 334.456                  | 14-Deoxyandrographolide (C_{20}H_{30}O_{4}) (diastereomers) | Diterpenoid | 311.3 (M-Na)              | ![Predicted structure](image3.png) |
| 46     | 24.55    | 1                     | 300.266                  | 5,8,2'-Trihydroxy-7-methoxyflavone (C_{16}H_{12}O_{6}) | Flavone | 277.3 (M-Na)              | ![Predicted structure](image4.png) |
| S. No. | RT (Min) | Purity percentage (%) | Molecular weight (g/mol) | Molecular formula | Class of identified compounds | LC-MS (Obtained mass, m/z) | Predicted structure |
|--------|----------|-----------------------|--------------------------|------------------|------------------------------|---------------------------|-------------------|
| 47     | 26.99    | 1                     | 318.457                  | Andrograpanin (C_{20}H_{30}O_{3}) (diastereomers)  | Diterpenoid                | 279.3 (M-K)         | ![Predicted structure](image1) |
| 48     | 30.79    | 1                     | 294.38                   | 3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17), 11-dien-13-oic acid (C_{17}H_{26}O_{4}) (diastereomers) | Diterpenoid                | 255.2 (M-K)         | ![Predicted structure](image2) |
| 49     | 31.20    | 1                     | 368.46                   | 12S-Hydroxyandrographolide (C_{20}H_{32}O_{6}Na)  | Diterpenoid                | 367.1 (M-1)         | ![Predicted structure](image3) |
Fig. 7 Structure of some essential Diterpenoids identified by LC-MS

Fig. 8
(A) Purity percentage of identified compounds present in negative ion mode in active plant extract.
(B) Purity percentage of identified compounds present in positive ion mode in active plant extract.
Fig. 9 Fragmentation pathway of Andrographolide

Fig. 10 Fragmentation pathway of Andrographolide dimer
phenolic compounds and flavones typically lie in the range of 230–290 nm or 300–350 nm [18, 19]. UV-Vis spectrum confirmed the presence of phenolic, flavones and their derivatives in the methanol extract.

The characteristic absorption band at the 1026.87 cm$^{-1}$ and 894.75 cm$^{-1}$ (C-N- and -C-H- out of plane stretch) suggested that the functional groups are amines (RNH$_2$) and aromatic (C-C ring) in nature. The absorption band at 1377.80 cm$^{-1}$ (-CH$_3$-) indicated the presence of alkanes with weak intensity. Results confirmed that ethers, hydroxyl, alkanes, amines, aromatic, esters, aromatic nitro, and phosphine compounds were presented in the methanol extract of $A$. paniculata. The –OH group indicates the presence of flavonoids, alcoholic, and phenolic compounds. $A$. paniculata is an aromatic plant, which is evident by the presence of aromatic and oxidized nitrogen functional groups. Occurrence of alkanoids in the methanol extract also confirms the presence of alkanes, primary and secondary amines, and aromatic compounds. Previously, there have been several reports on the presence of alkanes, amines, ethers, hydroxyl, aromatic, esters group aromatic nitro, and phenolic groups [2, 20], but not the presence of phosphine groups in $A$. paniculata plant extracts, while in our study we report the presence of phosphine group based on FTIR spectrum.

Previously, Sharma and Sharma (2018) reported andrographolide (methanol leaf extract) compared to the standard and with an Rf value of 5.674 at 223 nm [21]. Andrographolide peaks were identified by comparing the obtained chromatogram with those already reported with comparison of standard and obtained result at RT 4.338 at 229 nm. A retention time of 4.8 at 223 nm has also been reported for Andrographolide [22, 23]. Comparing with available standard data, the peaks for neoandrographolide were identified at RT 14.10 at 220 nm [24]. The biological activities of plant extracts largely depends upon the employed extraction technique. Selection of the extraction method and the solvent used is of prime importance and should be optimized before final use. The process of separation, identification, and characterization of phytoconstituents from a plant extracts have always been a challenging task, but the technological advances, availability of several separation techniques, and detectors with wide range are tremendously speeding the separation and characterization process.

One of the most important techniques is the combination of liquid chromatography and mass spectrometry that symbolizes a robust, sensitive, quick, and reproducible analytical method for the identification and characterization of secondary metabolites present in bioactive extracts. Several studies have reported the presence of diverse secondary metabolites from leaves, root, stem, or from whole plants of $A$. paniculata extracts, such as andrographolide (major compound), neoandrographolide, 14-deoxy-11, 12-didehydroandrographide and isandrographolide [8, 25, 26], 14-deoxy-17-hydroxyandrographolide,
12S-hydroxyandrographolide, andrographatoside, 14-deoxy-11, 12-didehydroandrographolide, andrographolide, andrographic acid, 14-deoxyandrographolide, andrographolide derivatives, andrograpanin, bisandrographolide, skullcapflavone I, 5-Hydroxy-7,8,2′,5′-tетраметоксифлавон, 5-O-glucoside, and 7-O-methylwogon [27, 28]. It might be possible that the anti-malarial potency of the methanol extract observed in our earlier studies [7] might be due to the synergistic activity among the diterpenes and flavones present in majority in the methanol extract. Diastereomers are stereoisomers and second group of isomers that do not display mirror image and also non-super imposable on one another; however, their chemical structures are qualitatively and quantitatively similar. They possess more than one stereogenic center and differ from each other in physical and chemical properties such as solubility or melting point. Due to the presence of diastereomers, we observed two bands in TLC in the Vis region, while five bands were visible in the UV region. This could be related to the achiral environment of the TLC plates that do not yield two different solute zones.

The observed diterpenes were classified into monomers and polymers of diterpene lactones and can be classified as penta-cyclic or macrocyclic diterpenoids depending on their skeletal core [29]. The monomers possess multiple hydroxyls group that displays a characteristic fragmentation behavior by successive loss of one or more than one H$_2$O molecules [28]. The positive ion mode was much more efficient for the analysis of these kinds of compounds. Kumar et al. (2018) also reported fragmentation of Andrographolide from the methanol extract of aerial parts of A. paniculata, but the fragmentation pattern differed at m/z 303 and 275 due to loss of CO in reverse Diels-Alder (RDA) [27]. The biological function of a specific diterpenoid cannot be representative for the whole class of molecules due to their diverse nature and often restricted distribution [29]. Andrographolide, a labdane diterpenoid, found to be present in highest concentrations is known for wide range of therapeutic properties. It has been found to be effective in vitro and in vivo antiplasmodial assays demonstrating its importance in antimalarial drug discovery programs. It is known to target protein and nucleic acid synthesis acting as “transcription blocker” [30]. Andrographolide has been gaining attention of the scientific community due to its broad biological activities such as anti-angiogenic, immune-stimulating properties, anti-cancer, anti-inflammatory, cardio-protective properties and anti-HIV [31], anti-feedant, anti-viral, antibacterial, and anti-diabetic activities [32]. Neoandrographolide is anti-inflammatory, anti-oxidant, lipid lowering, and cardio protective molecule. It is known to be a chemo-sensitizer and is known to inhibit iNOS and COX-2 expression [33, 34]. Owing to these biological properties and its presence in the A. paniculata methanol extract, it should be evaluated for its anti-malarial potential. Andrographolactone has not been reported with any biological activity till date and has been recently identified and characterized [35]. 14-Deoxy-11,12-didehydroandrographolide has been reported to possess promising anti-steatohepatitis, anti-liver fibrosis, anti-oxidant, and anti-inflammatory activities [36]. 14-Dehydroxy-11,12-didehydroandrographolide is also known to inhibit biofilm formation when used in combination with azithromycin and gentamicin [37] preventing the development of multiple drug resistance [37]. Experimental results suggested the use of 14-dehydroxy-11,12-didehydroandrographolide in combination therapies to combat biofilm associated infections. To our knowledge, till date andrographic acid has not been reported for any biological activity.

Flavones are the most common naturally occurring extensively dispersed group of low molecular weight phenolic compounds having a benzo-γ-pyrone structure. They are known to be synthesized by plant against microbial infections and their activity depends on their structural category, hydroxylation, conjugations and degree of polymerization [38-40]. The presence of functional hydroxyl group is responsible for the antioxidant activities of these polyphenolic compounds making them molecules of interest for nutraceutical, pharmaceutical and medicine industries for treating an array of human diseases such as cardiovascular, cancers, and other age-related diseases [41, 42]. Flavones have the capacity to induce protective enzymatic pathways and also plays major role in inflammation suppression by inhibiting xanthine oxidase, cyclo-oxygenase, lipoxygenase, and phosphoinositide 3-kinase [43]. There are several reports that suggest flavones to be protective against many infectious and degenerative diseases.

Skullcapflavone I, an important flavonoid, was found in high concentrations in the methanolic extract of A. paniculata that previously displayed in vitro anti-malarial activity [7]. Flavonoids are known to possess several biological activities [44, 45]; however, there are no reports on the anti-parasitic activity of Skullcapflavone I and thus, it should be evaluated for its anti-malarial potential. 5-Hydroxy-2′,7,8-trimethoxyflavone and tetramethoxyflavone are able to inhibit bacterial activity against sensitive and multidrug-resistant strains of Mycobacterium tuberculosis [46]. We hypothesize that neoandrographolide, andrographolactone, 14-dehydroxy-11,12-didehydroandrographolide, andrographic acid, skullcapflavone I, 5-Hydroxy-2′,7,8-trimethoxyflavone, and tetramethoxyflavone might be lead molecules for infectious disease drug development programs and could be an alternative to counter the emerging anti-malarial drug resistance phenomenon.
Conclusions

In the current study, a total of 74 compounds were reported from the anti-malarial active methanol extract of A. paniculata. Most of the metabolites identified on the basis of LC-MS data were diterpenes in nature. Based on the mass spectrum a diterpenes, andrographolide (15%) and a flavone, skullcapavone I (8%) were present in highest amount in both, positive and negative, ion modes. The diversity of the phytoconstituents identified presents us novel category of molecules that should be screened in anti-malarial drug development programs. It can also be inferred that the mixture of diterpenoids and flavonoids might be displaying synergetic effect against P. falciparum in the in vitro assays. The LC-MS method developed in the current study led to identification diterpenoids and thus can be utilized in quality control methods. The results of our study led to identification Neoandrographolide, andrographolactone, 14-dehydroxy-11,12-didehydroandrographolide, skullcapflavone I, and 5-Hydroxy-2′,7,8-trimethoxyflavone as the major metabolites from the methanol extract of A. paniculata. A. paniculata is widely used in the traditional medicine for malaria treatment by the tribal healers of Amarkantak region and therefore, based on current results, activity guided fractionization, purification, and characterization of the active extract is urgently required for further development of novel anti-malarial drugs.

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Plant authentication

Dr. Ravindra Shukla, Department of Botany, identified the plant specimen. The voucher specimen (AM/DOB/004) was deposited in the Department of Botany for the future references.

Authors’ contributions

MKD, SS, SM, and PS collected the plant material, performed the experiments, analyzed the data, and wrote the manuscript. PKS designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data and material are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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