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Diterpenoids as potential anti-malarial compounds from *Andrographis paniculata*

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Abstract

Background: The objectives of the current study are to evaluate the traditionally used medicinal plants *Andrographis paniculata* for in vitro anti-malarial activity against human malarial parasite *Plasmodium falciparum* and to further characterize the anti-malarial active extract of *A. paniculata* using spectroscopic and chromatographic methods.

Results: The chloroform extract of *A. paniculata* displayed anti-malarial activity with IC_{50} values 6.36 μg/ml against 3D7 strain and 5.24 μg/ml against K1 strains respectively with no evidence of significant cytotoxicity against mammalian cell line (CC_{50} > 100 μg/ml). LC-MS analysis of the extract led to the identification of 59 compounds based on their chromatographic and mass spectrometric features (a total of 35 compounds are present in positive ion and 24 compounds in negative ion mode). We have identified 5 flavonoids and 30 compounds as diterpenoids in positive ion mode, while in the negative mode all identified compounds were diterpenoids. Characterization of the most promising class of compound diterpenoids using HPLC-LC-ESI-MS/MS was also undertaken.

Conclusions: The in vitro results undoubtedly validate the traditional use of *A. paniculata* for the treatment of malaria. The results have led to the identification of diterpenoids from IGNTU_06 extract as potential anti-malarial compounds that need to be further purified and analyzed in anti-malarial drug development programs.

Keywords: *Andrographis panicualta*, Diterpenoid, In vitro anti-malarial activity, Liquid chromatography, Traditional medicine

1 Background

Malaria, caused by *Plasmodium* spp., is a major cause of global mortality [1, 2]. Anti-malarial drugs like chloroquine, quinine, artemisinin, arteether, and their derivatives have been used as major drugs against this dreadful disease [3]. The emergence of resistance against currently used anti-malarial drugs has become a critical problem for malaria control programs [4]. There is an urgent requirement for new anti-malarial drug development that is well-tolerated, affordable, and more efficient in tackling the global problem of drug resistance [5]. The majority of anti-malarial drugs like quinoline based drugs, artemisinin, and its derivatives have been derived from medicinal plants [4]. In malaria-endemic countries, traditional medicine can open new avenues for novel molecules to be developed as anti-malarial drugs [1]. For the duration of the precedent 1–2 decades, the researcher budge spotlight from synthetic drugs to alternative plant-based drugs has occurred. Currently, isolated pure compounds from medicinal plants are extensively used in modern medicine; approximately 120 active compounds are presently used [6]. Among the major 100 plants, about 40 tropical plants account as the source of more than 120 pharmacologically active metabolites [7]. Apart from the pharmaceutical importance, these plants are being extensively utilized as herbal products for nutritional and medicinal purposes that help in disease prevention and health improvement [8]. We have earlier documented 19 plants being used by traditional medicinal healers that they use for treating malaria. Geographical Information System (GIS) maps of all 19 anti-malarial plants were also prepared [9, 10]. Based on the maximum usage index, *Andrographis*

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paniculata was selected for carrying out in vitro anti-malarial assays (IC_{50} values 6.79 μg/ml against 3D7 strain and 4.26 μg/ml against K1 strains), and we obtained promising results in the chloroform whole plant extract (unpublished results).

A. paniculata is a magnificent tropical traditional medicinal plant that belongs to the family *Acanthacea* and is usually well-known as "Kalmegh" or "King of Bitter" [11]. *A. paniculata* is an herb having erect branches growing up to 1–2 m in length. It is traditionally used for medicinal purpose in Hong Kong, Philippines, Taiwan, China, India, Malaysia, Indonesia, and many South Asian countries [12]. Medicinal properties of *A. paniculata* have also been highlighted by WHO in its 2002 monograph [13]. Humans are using medicinal plants in traditional medicine since ancient times for treating a wide variety of diseases. The extracts contain several small molecules that are structurally and functionally diverse in nature and are known as secondary metabolites or phytochemicals [14–16]. It is generally used to treat different diseases such as liver and bowel disorders, colic pain, influenza [17, 18], respiratory infections, rheumatism, hypertension, diabetes, and arthritis, and used as an analgesic, protozocidal, antioxidant, anti-HIV, immune-stimulant, antimicrobial, antipyretic, and anticancer [12, 19, 20]. *A. paniculata* has been used for the treatment of dyspepsia, dysentery, snakebite, insect sting, influenza, and respiratory infections [19].

Several drugs have been obtained from natural products, and the synthesis of their derivatives has led to the improvement in drug-like properties of such molecules. The plant kingdom presents us with a diverse and large array of chemical compounds making it imperative to design reliable and rapid plant extract screening assays. Liquid chromatography (LC-MS) has emerged as a potent tool for the characterization and purification of phytoconstituents [21]. In light of these observations, this current study aims to validate the traditionally used medicinal plant *A. paniculata* for evaluating its in vitro anti-malarial potential. We have also developed a novel method to profile and identify phytochemicals from the potential anti-malarial active whole plant extract by using HPLC-LC-MS/MS. We have also identified active compounds present in chloroform extracts of *A. paniculata* by thin-layer chromatography, UV-Visible spectroscopy, FTIR, and HPLC. LC-MS method was developed to identify and characterize the major constituents (diterpenoids) present in the chloroform extract of *A. paniculata*. In the positive ion mode, the characterized diterpenoids were based on their retention time and MS spectra data.

2 Methods

2.1 Chemicals and reagents

Analytical grade (AR) or liquid chromatography (LC) grade chemicals and reagents were used in the study. Methanol (AR and LC grade), acetonitrile (HPLC and LC grade), ammonium (NH_4), and chloroform (AR) were purchased from Central Drug House (CDH (P) Ltd.). Ultra-pure water was used in preparing all buffers and reagents (Millipore, USA).

2.2 Extraction of crude extracts

Based on the relative frequency of citation obtained from the field survey *A. paniculata*, whole plants were selected and dried (air) for 30 days in the shade at room temperature (RT) (25–28 °C). The dried plant (500 g) of *A. paniculata* was powdered mechanically using a steel blender. The powder samples were stored in airtight bottles used for the extraction process using a Soxhlet apparatus (60–80 °C) for 4–8 h. Various extracts were prepared based on polar to non-polar solvents such as methanol, chloroform, n-hexane, and aqueous solvent. We classified plant extracts of *A. paniculata* as follows: IGNTU_05_methanol, IGNTU_06_chloroform, IGNTU_07_n-Hexane, and IGNTU_08_aquous. All the extracts were concentrated with the help of a rotary evaporator (22–26 mmHg; 45–60 °C). The concentrated residual extracts were weighed, and yield percentage was calculated for each and stored at 4 °C till further use.

2.3 In vitro anti-malarial activity

2.3.1 Culture of Plasmodium falciparum

Evaluation of in vitro anti-malarial activity used plant extracts against chloroquine-sensitive strain 3D7 and multidrug-resistant K1 strain (resistant to chloroquine, sulfadoxine-pyrimethamine, chlorproguanil) of *P. falciparum*. Chloroquine sensitive (3D7) strain of *P. falciparum* is routinely cultured at CSIR-Central Drug Research Institute, Lucknow, India, following the method of Trager and Jensen [22] Culture of the parasite was maintained at 6–8% parasitemia and 2% hematocrit in complete RPMI medium (RPMI 1640 supplemented with D-glucose (0.2%), HEPES, sodium bicarbonate (0.2%), albumax (0.5%), hypoxanthine (45 mg/l), fungizone (0.25 mg/l), and gentamycin (50 mg/l) at 37 °C in a humidified CO_2 incubator [23]. Stock solutions 5 mg/ml of extracts prepared in 100% di-methyl sulfoxide (DMSO) for IGNTU_05, IGNTU_08, in tetrahydrofolic acid (THF) for IGNTU_06, and in water for IGNTU_07. The extracts were diluted in RPMI-1640 culture medium. The malaria SYBR Green I-based fluorescence assay was carried out for evaluating 50% inhibitory concentration (IC_{50}) of the extracts tested [24].

2.3.2 Evaluation of anti-malarial activity

Chloroquine (C-6628, Sigma) was used as a standard drug, and stock concentration was prepared in water. Stock concentrations of plant extracts (50 μg/ml) were prepared in dimethyl sulphoxide (DMSO) or

tetrahydrofuran (THF). Experiments for parasite inhibition were conducted at 0.8% parasitemia maintained at 1% hematocrit in complete RPMI medium. The highest concentration of test samples was 50 μg/ml. Dual serial dilutions of test samples were prepared in triplicates in 96-well microtiter plates and were used for the treatment of the ring stage of the parasite. Untreated parasite (infected-RBCs) and non-infected RBCs were used positive and negative control, respectively. In parallel, parasite culture was maintained in 60 mm dish without any drug to monitor the parasite growth (37 °C, 72 h). After the 72 h, 100 μl lysis buffer [20 mM Tris-HCl (pH 7.5), saponin (0.008%), and Triton X-100 (0.08%), 5 mM EDTA] containing 2× SYBR Green dye (S7585) was added to the individual well of the 96-well plate and incubated at 37 °C for 1 h. With excitation at 480 nm and emission at 520 nm, the fluorescence was recorded (FLX800, BIOTEK instrument). Data was analyzed to obtain inhibitory concentration (IC_{50}) values for plant extracts. The extracts displaying $IC_{50} \leq 10.0$ μg/ml have promising anti-malarial activity.

2.3.3 Determination of in vitro cytotoxic activity

The MTT assay was performed for cytotoxicity evaluation in VERO cells (C1008, epithelial cells from monkey kidney). VERO cells were maintained in RPMI media supplemented with HEPES, 0.2% sodium bicarbonate, 0.2% D-glucose, 10% FBS, fungizone (0.25 mg/l), and gentamycin (50 mg/l) in a humidified CO_2 incubator maintained at 37 °C. Cells (10^4) were seeded in 96-well plates and treated with the test compounds in different dilutions (16–18 h post-seeding). Podophyllotoxin (P4405, Sigma) was used as the positive control. After 72 h of incubation, 25 μl of MTT (M2128, Sigma) (stock 5 mg/ml) dye was added to each well of the plate that was re-incubated in CO_2 incubator for another 2 h. Cell supernatant was removed carefully without disturbing the cells. DMSO (150 μl) was added to each well for dissolving the purple precipitate. Absorbance was recorded at 540 nm (ELISA reader) and data was analyzed to determine 50% cytotoxic concentration (CC_{50}).

2.4 Thin-layer chromatography (TLC)

TLC was undertaken at room temperature (RT) and 60% relative humidity at lab conditions for analysis of the plant extracts. The solvent system chloroform: methanol (90:10) was used as the mobile phase. The TLC plates (Merck-silica gel 60 F_{254}) were kept in the UV chamber (254 nm) for a few minutes to visualize various spots in the samples under analysis. The Rf values were calculated using standard formula.

2.5 UV-Visible spectroscopy

The plant extracts were scanned between the 200–800 nm wavelength ranges (Shimadzu, UV-1800 UV-Vis-Spectrophotometer). Chloroform (analytical grade) was used as a blank and the plant extract was prepared in methanol 0.10 mg/ml concentration.

2.6 Fourier-transform infrared spectroscopic (FTIR) analysis

The FTIR spectroscopic analysis was performed to identify the characteristic peaks and the functional groups present in *A. paniculata* whole plant chloroform extract (IGNTU_06) using Thermo scientific iD7 ATR Spectrophotometer system. The plant extract was diluted in chloroform before scanning the FTIR spectrum at 400–4000 cm^{-1}. The analysis was performed in duplicate for the confirmation of the spectrum.

2.7 High-performance liquid chromatography

About 100 mg of plant extract was taken separately into a round bottom flask with the addition of 50 ml of methanol. After sonication in a water bath for 5 min, the mixture was passed through a 0.45-μm membrane filter. The C18 column was used for chromatographic separation C18 column (250 mm × 4.6 × 5 μm) (Water-HPLC Model no. waters 2489 UV/Visible). Isocratic elution was carried out with 1 ml/min flow rate at 1600 psi using mobile phase acetonitrile:water (60:40 v/v). The plant extract separation was detected with a D2 lamp at 223 nm wavelength. Ten-microliter samples were injected, and run time was kept at 30 min. Empower 3 software was used for integration, calibration, and analysis.

2.8 Liquid chromatographic mass analysis

Liquid chromatography (LC) analysis was undertaken using a Aqua C18, 150 × 2.1, 2.6 μm column (Waters ACQUITY QSM -TQD#QBB1152). Gradient elution (Gradient Table 1) was performed using solvent A: 95% (H_2O: ACN); solvent B: acetonitrile; solvent C: methanol; and solvent D: 5 mM NH_4 in 95:5 (H_2O: ACN) at a constant flow rate of 0.250 ml/min with flow ramp rate of 0.45 min. Column temperature was set to 30 °C at run time of 40 min. The sample volume injected was set at 1 μl and the high-pressure limit was set at 15,000 psi. Both positive and negative ion modes (+Ve and −Ve) were used for acquiring the MS spectra using electrospray ionization (ESI) source. For the MS analysis, capillary voltage was kept to 3.50 kV in +Ve and −Ve ion modes, collision gas flow was 0.23 ml/min in +Ve ion mode and 0.10 ml/min in −Ve ion mode, while the source temperature was 120 °C for both modes. The ion energy I (0.50) and energy II (3.0) were used for positive and negative ion modes while the mass range 100 to 1000 m/z was used for both ion modes. The instrument

Table 1 Yield analysis, anti-malarial, and cytotoxic activities of *Andrographis paniculata* whole plant extracts

S. No.	Plants	Extract code	Parts used	Solvent	Yield (% w/w) (W1 × 100/W2)*	IC_{50} (μg/ml) against		CC_{50} (μg/ml)
						3D7	K1	
1	*Andrographis paniculata*	IGNTU_05	Whole plants	Methanol	4.85	10.75	10.64	63.03
2		IGNTU_06	Whole plants	Chloroform	7.01	6.36	5.24	> 100
3		IGNTU_07	Whole plants	n-Hexane	6.97	> 50	> 50	> 100
4		IGNTU_08	Whole plants	Distil water	4.11	9.35	10.87	> 100
Ref. drug	CQ-diphosphate*					0.005	0.259; 0.251	0.005
Ref. drug	Podophyllotoxin*					na	na	3.13

Parasite inhibition experiment was performed in 3D7 and K1 strains and cytotoxicity experiment was performed in VERO cells. IC_{50} and CC_{50} are the half-maximal inhibitory and cytotoxic concentrations, respectively. IC_{50} and CC_{50} values were determined 72 h after treatment of plant extract. Chloroquine (CQ), a 4-aminoquinoline derivative, was used as a reference compound in the parasite inhibition assay. IC_{50} obtained in PF3D7 and K1 was 5 nM and 255 nM, respectively. For cytotoxicity evaluation, podophyllotoxin was used as a reference compound. CC_{50} obtained for podophyllotoxin was 3.13 μM. Selectivity index is a ratio of CC_{50}/IC_{50} and found IGNTU_06 exhibits most promising anti-malarial activity and minimal cytotoxicity

*W1 = is the weight of the extract residue obtained after solvent removal

*W2 = is the weight of the plant parts in powder form taken

was externally calibrated daily for achieving acceptable accuracy with a bunch threshold of 0.1459 before sample analysis.

2.9 Mass spectrometric conditions

Positive electrospray ionization (ESI) mode was used for operating the mass spectrometer and recording the spectra in the mass range from 50 to 355 *m*/*z* for daughters of 333, 50 to 375 *m*/*z* for daughters of 351, 50 to 505 *m*/*z* for daughters of 481, and 50 to 690 *m*/*z* for daughters of 665 used for MS/MS. Collision energy was set at 25 eV for both masses. The accurate mass data of the molecular ions (M+1) and MS/MS product ions was processed using the Software Version: 1.50.1481 (Waters technology).

2.10 Statistical analysis

All the data analysis was done using Microsoft Excel. The inhibitory concentration values (IC_{50}) of the plant extracts were obtained by transferring the data into an Excel graphical program and expressed as a percentage of the untreated controls and then evaluated by Logit regression analysis using a preprogrammed Excel spreadsheet.

3 Results

3.1 Yield calculation and in vitro anti-malarial activity

Plant extracts were analyzed for yield namely IGNTU_06 (7.01% w/w), IGNTU_07 (6.97% w/w), IGNTU_05 (4.85% w/w), and IGNTU_08 (4.11% w/w), respectively. In the chloroform extracts, the highest yield was observed, and in aqueous extracts, the lowest yield was observed (Table 1). To validate the use of traditional plants by tribal communities against malaria, different plant extracts were in vitro evaluated for anti-malarial potential against both drug-sensitive and resistant strains of *P. falciparum*. We observed that plant extracts exhibited wide spectrum parasite inhibition concentration (IC_{50}) values. In chloroquine-sensitive strain, promising anti-malarial activities (IC_{50}) were observed in IGNTU_06 (6.79 μg/ml), IGNTU_08 (9.35 μg/ml), and IGNTU_ 05 (9.85 μg/ml) extracts, while IGNTU_07 had poor anti-malarial activity respectively. Similar inhibitory concentrations were obtained in multidrug-resistant K1 strain (resistant to chloroquine, sulfadoxine-pyrimethamine, chlorproguanil). Further, these plant extracts were tested for their cytotoxic effects (CC_{50}) in VERO cells, and these plant extracts had variable CC_{50} values. Comparison of IC_{50} and CC_{50} values suggests that these plant extracts specifically inhibit parasite growth and have a minimal toxic effect on mammalian cells. Out of the tested extracts, IGNTU_06 showed the most promising anti-malarial activities against both drug-sensitive and resistant strains (Fig. 1 and Table 1). It would be interesting to further identify the bioactive scaffolds in these extracts and perform a comprehensive SAR to identify the potential lead molecule.

3.2 TLC and UV-Visible spectroscopy

The TLC analysis of chloroform extract of *A. paniculata* revealed the presence of five spots in the visible region having Rf values of 0.55, 0.64, 0.78, 0.82, and 0.92, and in the UV region with Rf values of 0.44, 0.55, 0.64, 0.78, 0.82, and 0.92 (Fig. 2a, b) when a mobile phase of chloroform: methanol (90:10) was used. These Rf values when compared with the available literature data suggested them to be related with that of the diterpenoids.

The qualitative UV-Visible spectrum of chloroform extract of *A. paniculata* was scanned at 200 to 800 nm wavelength range to achieve peak sharpness and proper baseline. The spectrum peaks displayed seven bands at 235, 416, 458, 540, and 667 nm (Fig. 2c) with absorption values of 4.000, 3.212, 2.307, 0.303, and 1.469, respectively.

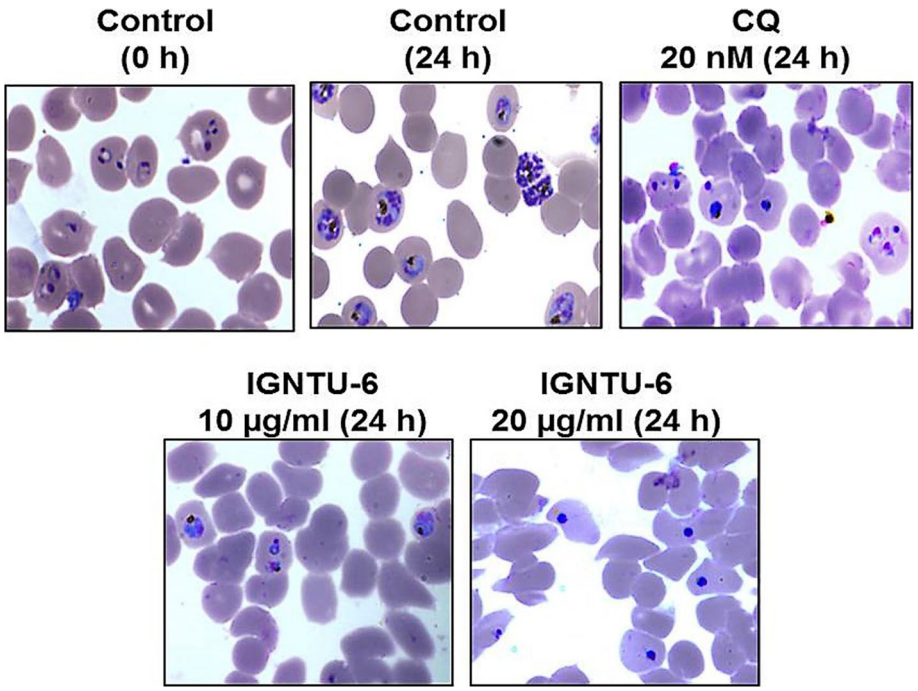

Fig. 1 Microscopic examination of Giemsa-stained *Plasmodium falciparum* (PF-3D7) strain maintained in human RBCs. Slides were prepared at different time-points before and after compound/plant extract treatment. Compound/plant extract treatment is given at ring-stage. Chloroquine (CQ) is used as a positive control

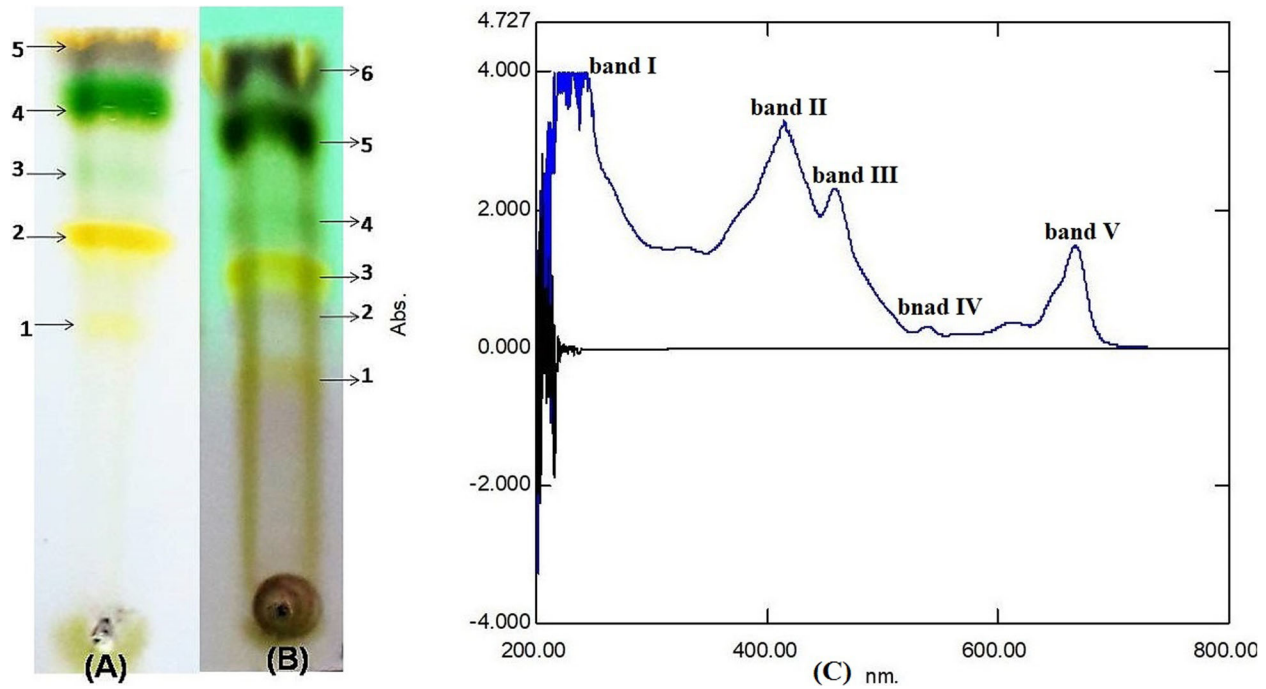

Fig. 2 Thin-layer chromatograph of chloroform extracts of *Andrographis paniculata* whole plant. **a** Visualization in visible regions. **b** Visualization in UV-regions (254 nm). **c** UV-visible spectrum of chloroform extract of *Andrographis paniculata* whole plant

3.3 FTIR analysis

The FTIR spectrum was recorded for the identification of the major functional groups present in the active compounds based on the peak value observed in the infrared region. The observed peak values and functional groups are listed in Table 2 and Fig. 3. The spectrum displayed major peaks at 3350.1168, 3079.7370, 2917.4682, 2848.9200, 1738.9023, 1643.9741, 1616.5713, 1497.4885, 1450.7719, 1376.2922, 1346.5665, 1214.3886, 1162.4174, 1077.3798, 1033.4677, 892.8850, 828.7537, 754.9412, and 719.7369 cm^{-1}, respectively (Fig. 3).

3.4 HPLC analysis

The HPLC chromatogram of extract depicted nine compounds with different retention times using solvent system acetonitrile:water (60:40 v/v). The peaks which could be identified in the chromatogram were unknown (2.543, 2.39%), unknown (3.101, 3.32), unknown (3.760, 7.82), unknown (4.761, 23%), andrographolide (5.922, 49.50%), unknown (7.081, 2.06%), unknown (10.040, 1.11%), neoandrographolide (13.678, 9.05%), and unknown (18.532, 1.76%) (Fig. 4).

3.5 LC-MS analysis of active compounds

We were able to identify a total of 59 compounds and based on chemical skeleton majority of which belonged to the diterpenoids class and a few were flavonoids.

3.5.1 Identification of diterpenoids

Diterpenoids were analyzed in both +Ve and −Ve ion modes, and thus, the method was optimized for both ionization modes. A total of nineteen diterpenoids were detected in the positive ion mode, the base peak chromatogram is shown in Fig. 5a, and compounds are displayed in Table 3. Similarly, sixteen diterpenes were detected in negative ion mode along with an analysis of their structures. The base peak chromatogram of negative ion mode is shown in Fig. 5b and Table 3. The chemical structure of diterpenoids is displayed in Fig. 10.

3.5.2 Identification of flavones

The base peak chromatogram in positive ionization mode of the active extract (IGNTU_06) is represented in Fig. 5a. Based on MS data, five flavones were detected in positive ion mode while not a single compound was present in negative ion mode (Fig. 5b and Table 3).

Table 2 FTIR analysis of anti-malarial active extract of *Andrographis paniculata*

Wavenumber (Cm^{-1})	Functional group	Class/bond	Intensity
3436.0894	–OH– broad	Hydroxyl	Medium
3079.7370	–C–H– stretch	Aromatic	Strong
2917.4682	–CH– stretch	Alkanes	Strong
2848.9200	–CH2–	Alkanes	Medium
1738.9023	–C=O– stretch	Esters	Strong
1643.9741	–C=C– stretch (3-ring structure)	Alkanes	Weak
1616.5713	–C=C– stretch (5-ring structure)	Alkanes	Weak
1497.4885	–C–C– stretch	Aromatic	Weak
1450.7719	–CH2 and CH3–	Alkanes	Medium
1376.2922	–CH3–	Alkanes	Weak
1346.5665	N–O–Aromatic Nitro.	Aromatic Nitro	Weak
1214.3886	–C–N– stretch	Amines	Weak
1162.4174	–P–H– bending	Phosphine	Weak
1077.3798	–C–O– stretch	Ethers	Weak
1033.4677	C–N– stretch	Amines	Medium
892.8850	=C–H–out of plane	Alkanes	Medium
828.7537	–C–H–out of plane	Aromatic	Medium
754.9412	–S–OR–	Esters	Strong
719.7369	–C–H–out of plane (trans RCH=CHR)	Aromatic	Weak

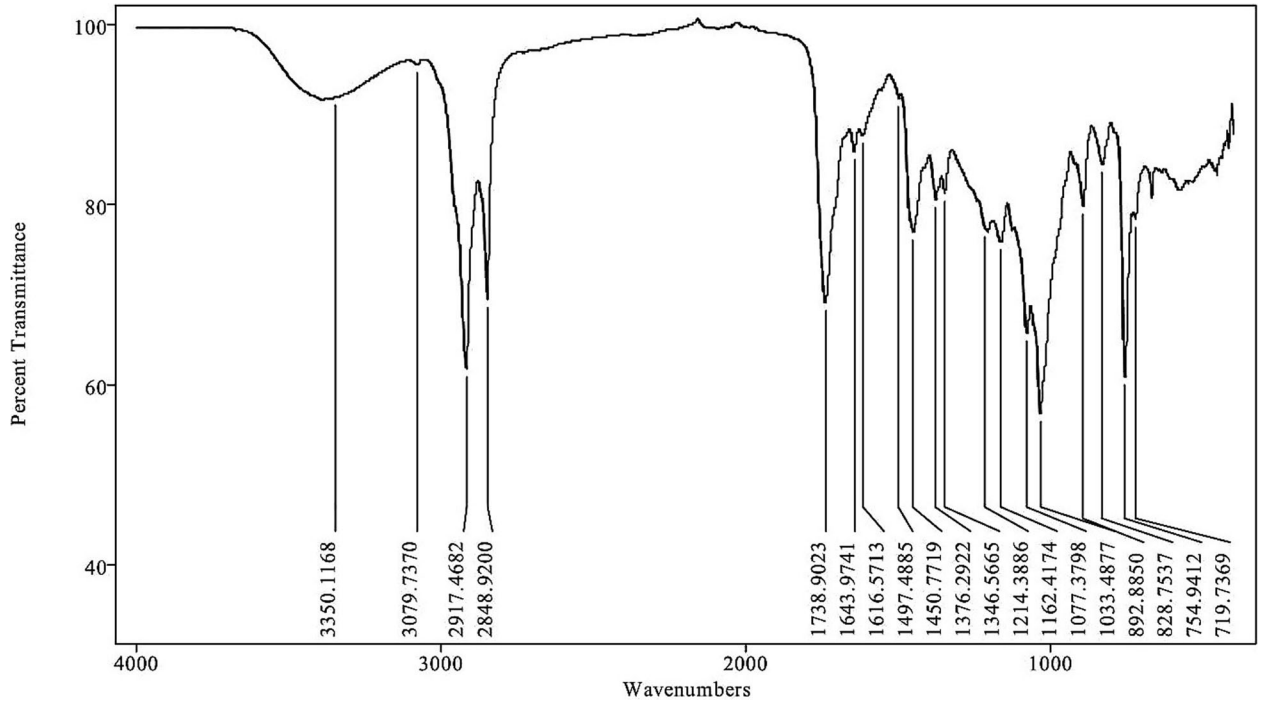

Fig. 3 FTIR spectrum of chloroform extract of *Andrographis paniculata* whole plant

3.6 MS/MS characterization of diterpenoids

Both low and high molecular weight molecules from plant extracts can effectively be characterized structurally using LC-MS/MS. IGNTU_06 was found to contain 41 compounds with a maximum number of diterpenoids (36 compounds). To authenticate the current findings, we carried out MS/MS analysis of four different molecules with different chemical skeleton and mass. The fragmentation pathways and diagnostic product ions of andrographolide, neoandrographolide, and bisandrographolide A and 14-deoxy-11,12-didehydroandrographolide were studied by LC-ESI-MS/MS spectra of the $(M+H)^+$ ions as shown in Figs. 6, 7, 8, and 9.

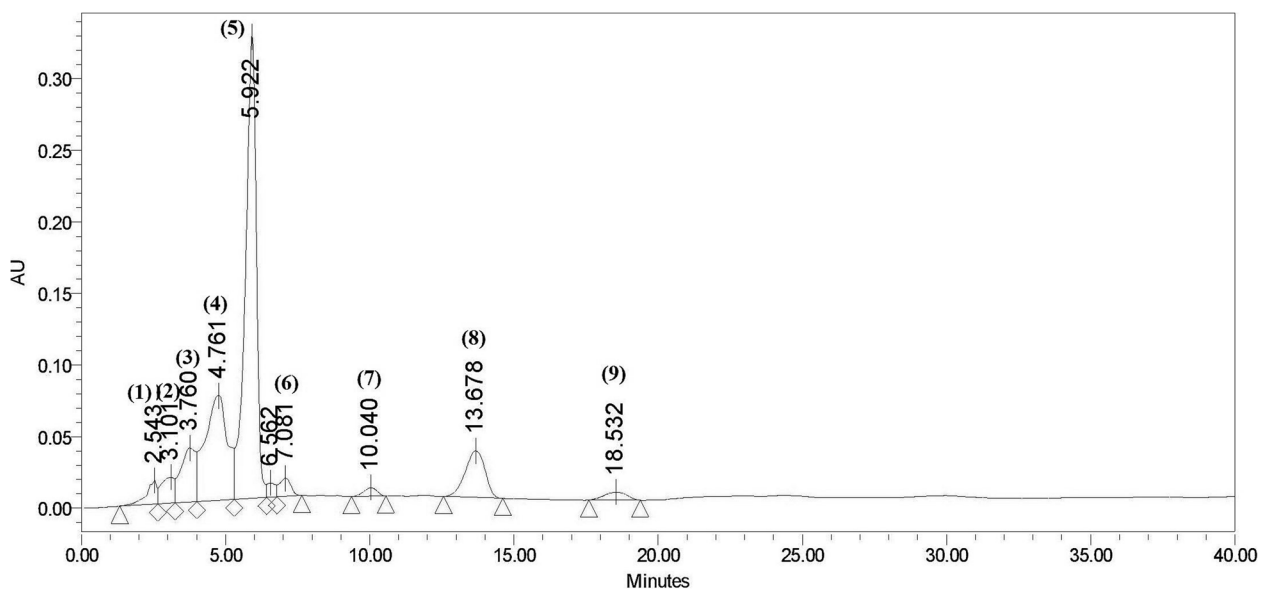

Fig. 4 HPLC chromatogram of chloroform extract of *Andrographis paniculata* whole plant

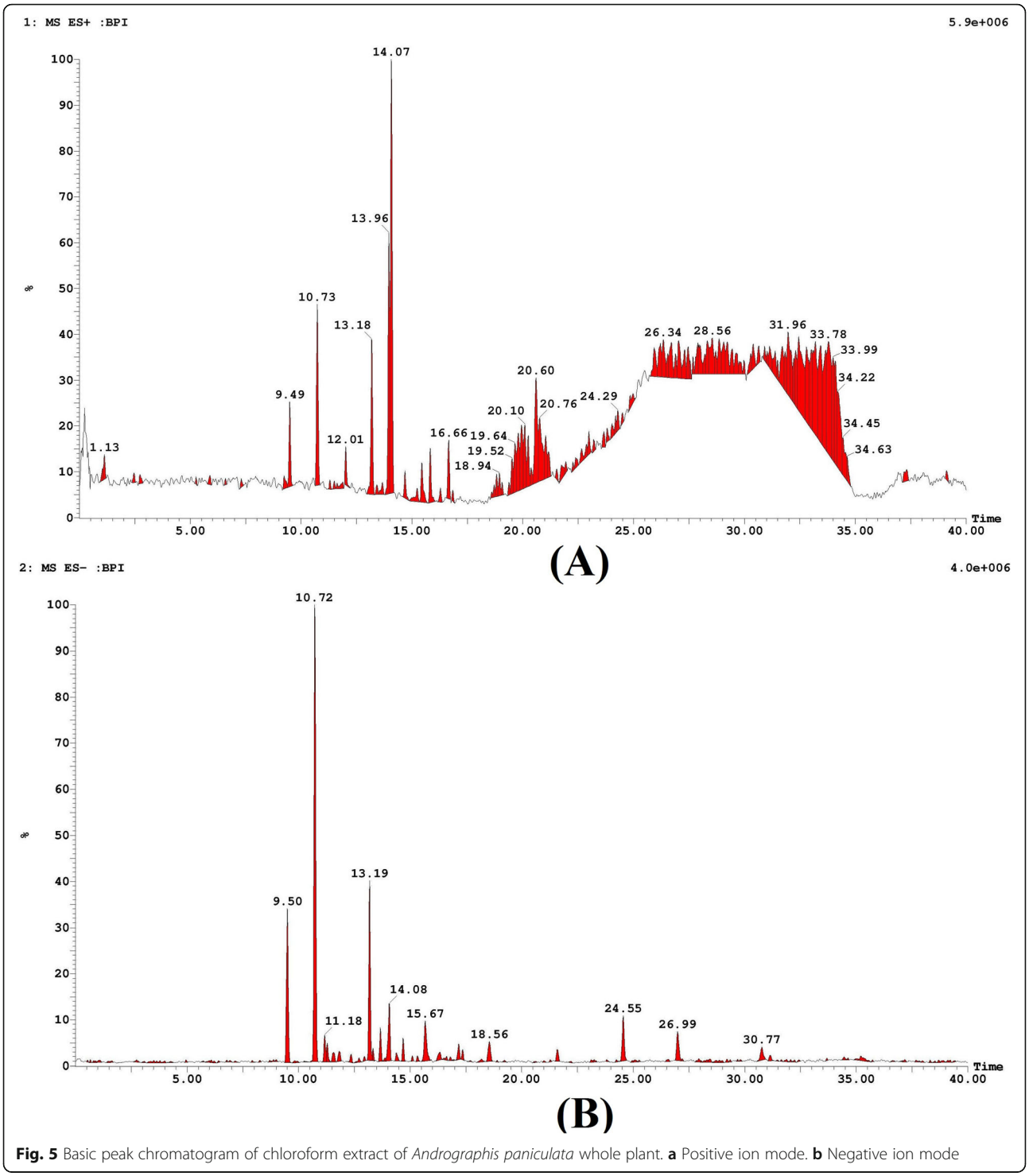

Fig. 5 Basic peak chromatogram of chloroform extract of *Andrographis paniculata* whole plant. **a** Positive ion mode. **b** Negative ion mode

The MS/MS spectrum and fragmentation pathways of 14-deoxy-11, 12-didehydroandrographolide ([M+H] + ion detected at m/z 333) are shown in Fig. 6. The MS/MS spectrum showed product ion at *m*/*z* 197and 183 due to loss of $C_9H_{12}O$ from 333 *m*/*z* and CH_2 (demethylation) from 197 *m*/*z*. Product ions at 169 and 133 *m*/*z* were observed due to fragmentation of CH_2 (demethylation) and $2H_2O$ (hydrolysis) from 183 and 169 *m*/*z*, respectively. Similarly, ions at 119 and 105 *m*/*z* were formed due to loss of CH_2 (demethylation) from 133 and 119 *m*/*z* in that order. The ions at 91 and 77 *m*/*z* were produced by consecutive loss of CH_2 (demethylation) from 105 and 91 *m*/*z*.

Table 3 Identification of compounds present in anti-malarial active extract of *Andrographis paniculata* by LC-MS/MS analysis

Peak No.	RT (Min)	Molecular weight (g/mol)	Molecular formula	Class of identified compounds	LC-MS (obtained mass, *m/z*)
Positive mode (ES+)					
1	6.58	480.598	Neoandrographolide ($C_{26}H_{40}O_8$)	Diterpenoid	481.6 (M+1)
2	9.49	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	351.3(M+1)
3	10.73	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	701.5 (2 M+1)
4	11.29	368.46	12S-Hydroxyandrographolide ($C_{20}H_{32}O_6Na$)	Diterpenoid	451.2 (M+1+2ACN)
5	11.50	332.44	14-Deoxy-11,12-didehydroandrographolide ($C_{20}H_{28}O_4$)	Diterpenoid	333.5 (M+1)
6	11.62	484.6	Ent-Labda-8(17), 13Z-diene-15, 16, 19-triol 19-O-glucoside ($C_{26}H_{44}O_8$)	Diterpenoid	485.3 (M+1)
7	12.01	300.266	5,8,2′-Trihydroxy-7-methoxyflavone ($C_{16}H_{12}O_6$)	Flavone	301.1 (M+1)
8	13.18	318.457	Andrograpanin ($C_{20}H_{30}O_3$)	Diterpene	319.2 (M+1)
9	13.18	497.60	Andrographatoside ($C_{26}H_{42}O_9$)	Diterpenoid	498.3 (M+1)
10	13.66	522.63	6-acetylneoandrographolide ($C_{28}H_{42}O_9$)	Diterpenoid	523.3 (M+1)
11	13.67	520.482	5-Hydroxy-7,8,2′,5′- tetra- Methoxyflavone 5-O-glucoside ($C_{25}H_{28}O_{12}$)	Flavone	584.3 (M+Na+ACN)
12	13.96	334.456	14-Deoxyandrographiside ($C_{20}H_{30}O_4$)	Diterpenoid	691.5 (2M+Na)
13	14.07	664.88	Bisandrographolide A ($C_{40}H_{56}O_8$)	Diterpenoid	687.5 (M+Na)
14	14.07	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	433.1 (M+1+2ACN)
15	14.69	314.3	Skullcapflavone I ($C_{17}H_{14}O_6$)	Flavone	315.2 (M+1)
16	15.23	296.41	Andrographolactone ($C_{20}H_{24}O_2$)	Diterpenoid	297.2 (M+1)
17	15.44	352.42	7-Hydroxy-14-deoxyandrographolide ($C_{19}H_{28}O_6$)	Diterpenoid	353.4 (M+1)
18	15.44	664.88	Bisandrographolide A ($C_{40}H_{56}O_8$)	Diterpenoid	665.5 (M+1)
19	15.83	318.457	Andrograpanin ($C_{20}H_{30}O_3$)	Diterpenoid	659.6 (2 M+Na)
20	16.66	358.346	5-hydroxy-7,8,2′,5′tetramethoxyflavone glucoside ($C_{19}H_{18}O_7$)	Flavone	359.2 (M+1)
21	18.71	664.88	Bisandrographolide A ($C_{40}H_{56}O_8$)	Diterpenoid	665.5(M+1)
22	18.82	298.29	7-O-Methylwogonin ($C_{17}H_{14}O_5$)	Flavone	623.5 (2M+Na)
23	19.06	532.586	3-Oxo-14-deoxy-11,12-Didehydroandrographolide ($C_{28}H_{36}O_{10}$)	Diterpenoid	533.5 (M+1)
24	19.79	664.88	Bisandrographolide A ($C_{40}H_{56}O_8$)	Diterpenoid	687.5 (M+Na)
25	20.25	512.596	Andrographiside ($C_{26}H_{40}O_{10}$)	Diterpenoid	513.4 (M+1)
26	20.60	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	723.5 (2 M+Na)
27	20.76	611.70	19-O-[β-D-Apiofuranosyl(1f2)-β-D-glucopyranoyl]-3,14-dideoxyandrographolide ($C_{31}H_{47}O_{12}$)	Diterpenoid	634.5 (M+Na)
28	21.03	611.70	19-O-[β-D-Apiofuranosyl(1f2)-β-D-glucopyranoyl]-3,14-dideoxyandrographolide ($C_{31}H_{47}O_{12}$)	Diterpenoid	634.7 (M+Na)
29	21.16	522.63	6-acetylneoandrographolide ($C_{28}H_{42}O_9$)	Diterpenoid	545.4 (M+Na)
30	21.95	294.38	3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid ($C_{17}H_{26}O_4$)	Diterpenoid	311.3 (M+1+O2)
31	22.88	334.456	14-Deoxyandrographiside ($C_{20}H_{30}O_4$)	Diterpenoid	352.3 (M+NH4)
32	24.50	511.58	3-O-β-D-Glucopyranosyandrographolide ($C_{26}H_{39}O_{10}$)	Diterpenoid	528.5 (M+1+O2)
33	25.93	300.266	5,8,2′-Trihydroxy-7-methoxyflavone ($C_{16}H_{12}O_6$)	Flavone	601.5 (2M+1)
34	26.69	352.42	7-Hydroxy-14-deoxyandrographolide ($C_{19}H_{28}O_6$)	Diterpenoid	354.3 (M+2)
35	27.46	330.42	19-hydroxy-3-oxo-ent-labda-8(17),11,13-trien-16,15-olide ($C_{20}H_{26}O_4$)	Diterpenoid	678.5 (2 M+NH4)
Negative mode (ES−)					
1	9.50	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	349.2 (M−1)
2	10.72	350.455	Andrographolide ($C_{20}H_{30}O_5$)	Diterpenoid	395.3 (M+formic acid-1)
3	11.18	496.597	Andropanoside ($C_{26}H_{40}O_9$)	Diterpenoid	541.3 (M+formic acid-1)

Table 3 Identification of compounds present in anti-malarial active extract of *Andrographis paniculata* by LC-MS/MS analysis *(Continued)*

Peak No.	RT (Min)	Molecular weight (g/mol)	Molecular formula	Class of identified compounds	LC-MS (obtained mass, *m/z*)
4	11.57	494.57	19-[(b-D-Glucopyranosyl)oxy-19-oxo-entlabda-8(17),13-dien-16, 15-olide ($C_{26}H_{38}O_9$)	Diterpenoid	539.5 (M+formic acid-1)
5	11.82	494.57	19-[(b-D-Glucopyranosyl)oxy-19-oxo-entlabda-8(17),13-dien-16, 15-olide ($C_{26}H_{38}O_9$)	Diterpenoid	461.2 (M−1−MeOH)
6	12.36	332.44	14-Deoxy-11,12-didehydroandrographolide ($C_{20}H_{28}O_4$)	Diterpenoid	331.2 (M−1)
7	13.19	480.598	Neoandrographolide ($C_{26}H_{40}O_8$)	Diterpenoid	525.3(M+formic acid-1)
8	13.33	330.42	19-hydroxy-3-oxo-ent-labda-8(17),11,13-trien-16,15-olide ($C_{20}H_{26}O_4$)	Diterpenoid	329.3 (M−1)
9	13.66	522.63	6-acetylneoandrographolide ($C_{28}H_{42}O_9$)	Diterpenoid	521.3 (M−1)
10	13.87	332.44	14-Deoxy-11,12-didehydroandrographolide ($C_{20}H_{28}O_4$)	Diterpenoid	331.2 (M−1)
11	14.08	318.457	Andrograpanin ($C_{20}H_{30}O_3$)	Diterpenoid	377.3 (M+acetic acid-1)
12	14.39	334.456	14-Deoxyandrographolide ($C_{20}H_{30}O_4$)	Diterpenoid	369.1 (M+Cl)
13	14.39	532.586	3-Oxo-14-deoxy-11,12-Didehydroandrographolide ($C_{28}H_{36}O_{10}$)	Diterpenoid	567.3 (M+Cl)
14	15.32	664.88	Bisandrographolide A ($C_{40}H_{56}O_8$)	Diterpenoid	663.5 (M−1),
15	16.52	512.596	Andrographiside ($C_{26}H_{40}O_{10}$)	Diterpenoid	557.2 (M+formic acid-1)
16	17.35	294.38	3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid ($C_{17}H_{26}O_4$)	Diterpenoid	293.3 (M−1)
17	18.20	294.38	3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid ($C_{17}H_{26}O_4$)	Diterpenoid	293.3 (M−1)
18	18.56	294.38	3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid ($C_{17}H_{26}O_4$)	Diterpenoid	339.3 (M+formic acid-1)
19	21.60	296.41	Andrographolactone ($C_{20}H_{24}O_2$)	Diterpenoid	295.2 (M−1)
20	24.55	334.456	14-Deoxyandrographolide ($C_{20}H_{30}O_4$)	Diterpenoid	311.3 (M−Na)
21	26.99	454.563	19-Benzyl isoandrographolide ($C_{27}H_{34}O_6$)	Diterpenoid	453.5 (M−1)
22	27.20	454.563	19-Benzyl isoandrographolide ($C_{27}H_{34}O_6$)	Diterpenoid	453.4 (M−1)
23	30.77	368.46	12S-Hydroxyandrographolide ($C_{20}H_{32}O_6Na$)	Diterpenoid	367.1 (M−1)
24	31.14	294.38	3,19-dihydroxy-14,15,16-trinor-ent-labda-8(17),11-dien-13-oic acid ($C_{17}H_{26}O_4$)	Diterpenoid	293.1 (M−1)

Andrographolide was detected at *m/z* 351, $[M+H]^+$ ion (Fig. 7). Its MS/MS spectrum displayed product ions at 187 *m/z* as $C_{10}H_{12}O_2$ molecule was lost. The product ions at 187 *m/z* further yielded ions at 169 and 133 *m/z* due to H_2O and $2H_2O$ loss, respectively, a hydrolysis reaction. Loss of CH_2 (demethylation) (ion at 133 *m/z*) resulted to the ion at 119 *m/z*. The ion at 119 *m/z* consecutively lost CH_2 and yielded the ions at 105 and 91 *m/z* in that order.

Neoandrographolide was detected at *m/z* 481, and its fragmentation pathway is shown in Fig. 8. Product ions at 481 *m/z* due to loss of $C_{10}H_{10}O_2$ resulted into ions at 319 *m/z*. Product ions at 319 *m/z* lost H_2O (hydrolysis) and formed product ions at 301 *m/z* and also lost HCHO and formed product ions at 289 *m/z*. Loss of CO forms the ion at 289 *m/z* resulted in the ion at 261 *m/z*, a reverse Diels-Alder (RDA) reaction [25, 26]. The product ions at 261 *m/z* further yielded ions at 231, 205, and 191 *m/z* due to loss of HCHO, C_2H_2, and CH_2 (demethylation) in that order. Further loss of $CH_{10}O$ and H_2O (hydrolysis) from 191 and 153 *m/z* produced the ions at 153 and 135 *m/z* in that order. Consecutive losses of C_2H_2 and CH_2 (demethylation) were observed from the ion at 135 and 109 *m/z* to yield the ions at 109 and 95 *m/z*. Further, the product ions at 95 and 81 *m/z* were observed due to losses of CH_2 (demethylation) and C_2H_2 to yield the resulted ions at 81 and 55 *m/z*.

The fragmentation pathway of bisandrographolide A, detected at 665 *m/z* $[M+H]^+$ ion, is shown in Fig. 9. MS/MS product ion at 665 *m/z* due to loss of $C_{20}H_{30}O_5$ resulted into ions at 315 *m/z*. Further, product ions at 315 *m/z* due to loss of H_2O (hydrolysis) and HCHO produced the ions at 297 and 285 *m/z* in that order. The product ions at 285 *m/z* due to loss of CO formed product ions at 257 *m/z*, a reverse Diels-Alder (RDA) reaction [25, 26]. Loss of CH_4 and CH_2 (demethylation) respectively from 257 and 241 *m/z* resulted in the ions at 241 and 227 *m/z*. The ions at 203 and 177 *m/z* arose by

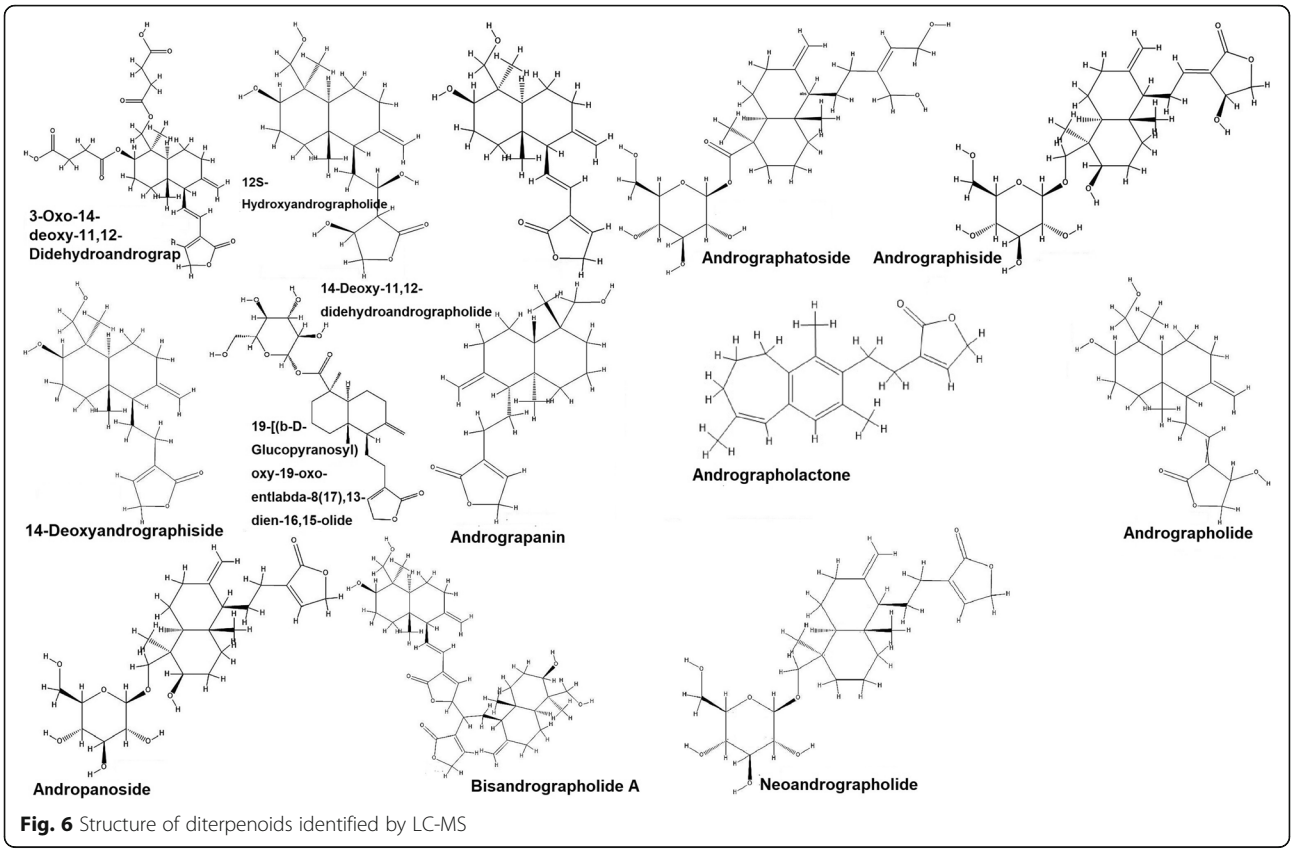

Fig. 6 Structure of diterpenoids identified by LC-MS

losses of C_2H and C_2H_2 from 227 and 203 *m/z* in that order. The ion at 177 *m/z* due to loss of C_3H_8 yielded the ion at 133 *m/z* and 133 *m/z*, further loss of C_2H resulted in the ion at 109 *m/z*. Loss of CH_2 (demethylation) from 109 and 95 *m/z* in that order, produced the ions at 95 and 81 *m/z*.

4 Discussion

The current study was undertaken to authenticate the traditional medicinal knowledge of the tribal communities of the Anuppur district, Madhya Pradesh, India. Traditional medicinal systems around the world rely mostly on the medicinal plants prevalent in particular regions of the world. The treatment of various diseases using plant products is possible due to large structural diversity of the secondary metabolites. Most of the modern drugs are isolated from plants or are synthesized based on plant metabolites.

The current study was undertaken with an aim to systematically validate the anti-malarial properties of Indian traditional medicinal plant *A. paniculata* that is known to have potential use in traditional medicine against malaria [27, 28]. However, the phytochemical constituents and the biological activity of these plants vary based on the chemotype under study, collection, and preparation methods. Mishra et al. (2011) has reported anti-malarial activity of methanol extracts of *A. paniculata* with an IC_{50} value of 9 μg/ml. Further, in vitro activity of *A. paniculata* in combination with *Hedyotis corymbosa* and curcumin has synergistic anti-malarial activity [29]. Several bioactive molecules such as 14-deoxyandrographolide, isoandrographolide, neoandrographolide, andrograpanin, andrographolide, andrographic acid, and andrographiside are known to be present in the leaf extract of *A. paniculata* [30]. In vitro activity (IC_{50} value) of andrographolide, isolated from methanol extract of *A. paniculata*, was found to be 9.1 μM [29]. Besides anti-malarial activity, *A. paniculata* plant extracts are traditionally used for treating colic pain, appetite loss, common cold, hypertension, diabetes, cancer, snakebite, liver disorders, anti-pyretic, and anti-diarrheal properties. It is being used in at least 26 ayurvedic formulations in Indian Pharmacopeia [31, 32]. Identification of the pure compounds from this extract may open a venue for scientific exploration of new scaffolds against diseases.

TLC is widely used in separation techniques due to its ease of use, low cost, high sensitivity, and quick separation of a large number of samples simultaneously. On the basis of Rf values, we observed three compounds namely 4-deoxy-11, 12-didehydroandrographolide (0.84), andrographolide (0.72), and other flavonoids and

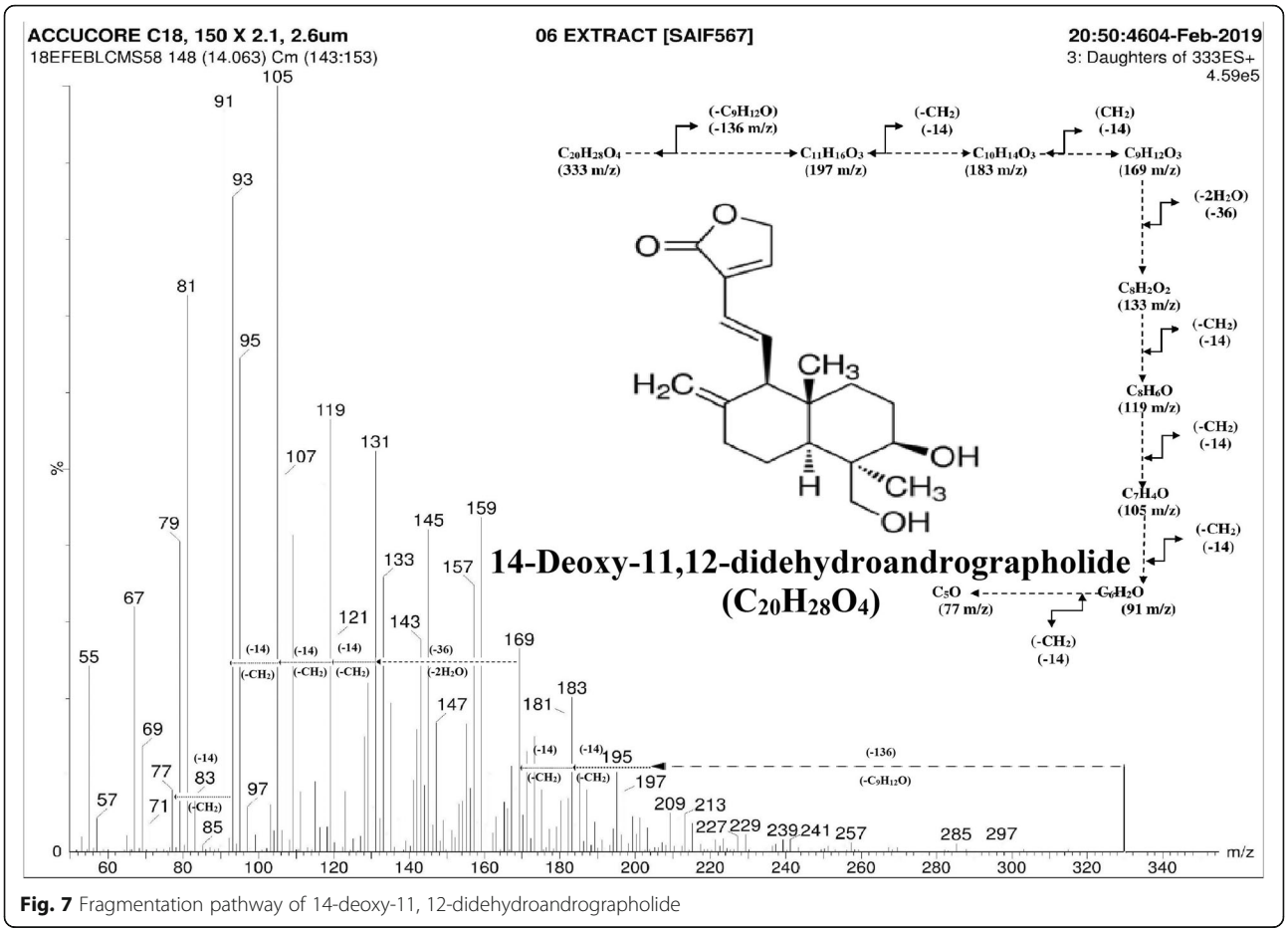

Fig. 7 Fragmentation pathway of 14-deoxy-11, 12-didehydroandrographolide

glycosides (0.78) in the IGNTU_06 extract [33, 34]. The UV-Visible spectrum was compared with reported literature, and we observed a peak at 235 nm which has been reported to be associated with andrographolide [33, 34]. The spectroscopic analysis also revealed the presence of phenolic and flavonoid compounds in the extract that is an indicator of the medicinal properties of *A. paniculata*. The spectra of phenolics and flavonoids are typically observed between 230 and 290 nm [35], and within 300–350 nm [36]. The results confirm the presence of diterpenoids, phenolic, flavonoids and their derivatives in IGNTU_06. Analysis of functional groups gives us a direct idea about the chemical properties of a given compound. Upon FTIR analysis we obtained bands at 1033.4677 cm^{-1} and 828.7537 cm^{-1} that revealed that the C–N– stretch and –C–H– were out of plane, respectively, leading to the idea that the functional groups were amines (RNH_2) and aromatic (C–C ring) respectively. We also report that the functional group at 1376.2922 cm^{-1} to be –CH3– which is a signature of the presence of alkanes with weak intensity. Based on the FTIR spectrum, the results confirm the presence of hydroxyl, aromatic, alkanes, esters, amines, ethers, aromatic nitro, and phosphine compounds in the IGNTU_06 extract. Presence of hydroxyl group (–OH) indicates the presence of flavonoids, alcoholic, and phenolic compounds thus supporting the UV-VIS spectrum data obtained for the extract. The aromatic properties of *A. paniculata* were confirmed by the presence of aromatic and oxidized nitrogen functional groups. The presence of alkanes, primary and secondary amines, and aromatic compounds confirms the occurrence of alkaloids in the IGNTU_06 extract of the plant. Previous studies have confirmed the presence of hydroxyl, aromatic, alkanes, esters, amines, ethers, aromatic nitro, phenolic, and phosphine groups in *A. paniculata* plant fresh leaves extracts [11, 37].

Based on the reported literature, the two major peaks obtained in the HPLC chromatogram were identified as those belonging to andrographolide and neoandrographolide. We were unable to identify the other six peaks which might be novel compounds not reported earlier. Andrographolide has been reported with RT 4.8 at 223 nm and RT 5.674 at 223 nm [38–40]. Neoandrographolide has been reported at RT 14.10 at 220 nm [41]. Chromatographic and spectral fingerprint analysis plays a significant role in the identification of known and unknown compounds in plant extracts. HPLC generate an

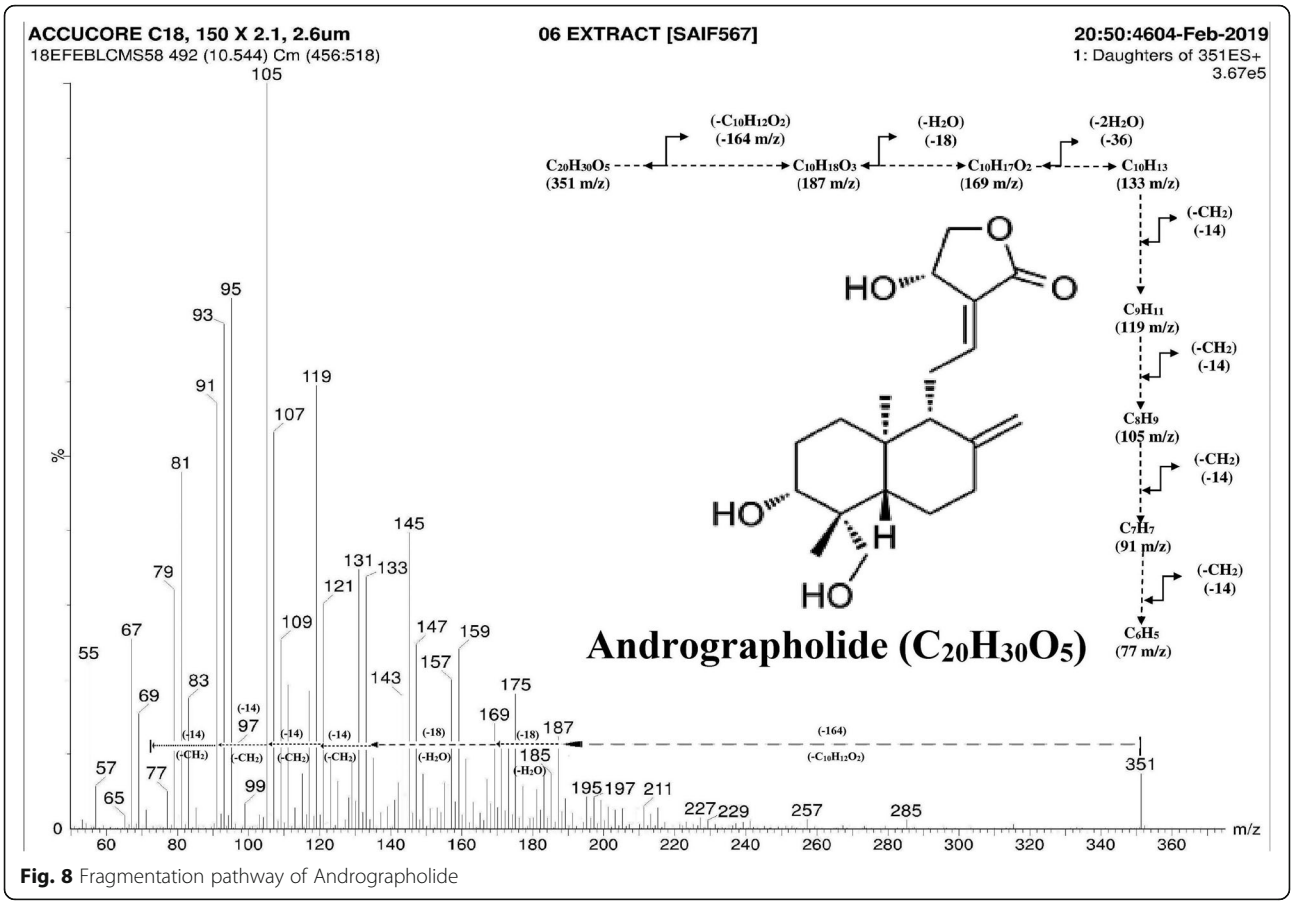

Fig. 8 Fragmentation pathway of Andrographolide

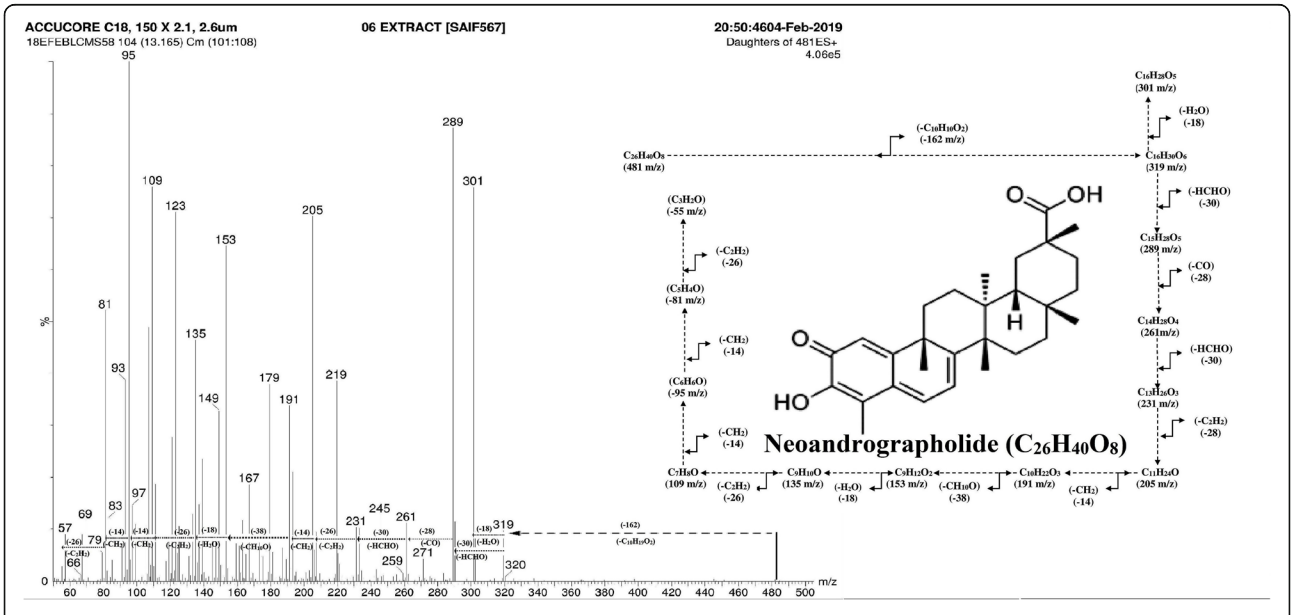

Fig. 9 Fragmentation pathway of Neoandrographolide

electronic image of the chromatographic fingerprint and a densitogram that is useful for the detection of secondary metabolites in plant samples. However, the fingerprint may vary based on the chemotype of the plant and the experimental conditions utilized. The presence of plant secondary metabolites closely depends upon the geographical location of the plant that has a direct effect on the biological activities of the plant extracts. It therefore becomes to collect the plant as a specific duration of time, select appropriate solvent for extract preparation that might least influence the properties of the secondary metabolites. Some of the identified compounds such as andrographolide have recently received increased attention due to their wide range of medicinal effects such as immunostimulating, anti-cancer, anti-bacterial, anti-inflammatory, anti-viral, anti-angiogenic, anti-HIV, cardio-protective, anti-feedant, and anti-diabetic activities [42, 43]. Neoandrographolide is known to have hypolipidemic, anti-inflammatory, and anti-oxidant effects; it protects the cardiovascular without significant liver damage and has been used as chemosensitizer [44].

In the LC-MS analysis, we were able to identify several diterpenoids in the IGNTU_06 extract in both ion modes (+V and −V). Diterpenoids are a heterogeneous class of natural compounds, occurring in many medicinal plants [45]. Natural products with medicinal activities can be directly isolated from plants and used in therapies or can act as a basic structure for the development of several derivatives for the development of more active and less toxic analogs [45]. Several diterpenoids including 14-deoxy-11, 12-di-dehydroandrographide, andrographolide, neoandrographolide, and iso-andrographolide have been earlier reported from *A. paniculata* [12, 46–48]. Kumar et al. (2018) have reported Skullcapflavone I, 5-Hydroxy-7,8,2′,5′-tetra-Methoxyflavone 5-O-glucoside and 7-O-Methylwogonin to be present in the methanolic extract of aerial parts of *A. paniculata*.

Several flavonoids were also identified in the extract analyzed that supports the traditional use of *A. paniculata* in traditional medicine. Flavonoids are known to have a variety of biochemical effects and are antioxidant properties that have been associated with several diseases such as atherosclerosis, cancer, and Alzheimer's [49–51]. Functional hydroxyl groups in flavonoids are known to scavenge free radicals, exert anti-inflammatory, and anti-mutagenic properties [52, 53]. They are reported to inhibit several enzymes such as cyclooxygenase, xanthine oxidase, lipoxygenase, and phosphoinositide3-kinase [54–56].

LC-MS/MS-based quantification and structural characterization of the secondary metabolites is the most efficient way for the analysis of plant extracts (Fig. 10). In the current analysis, we utilized both ion modes (+V and −V) for the identification of possible molecules present in the IGNTU_06 extract of *A. paniculata*. The diterpenoids were identified as monomers or polymers of diterpenoids lactones. Monomers have multiple hydroxyl groups in their structures which release one or two H_2O molecules upon fragmentation [57, 58] which was observed in our fragmentation analysis. Several biological activities have been attributed to diterpenoids

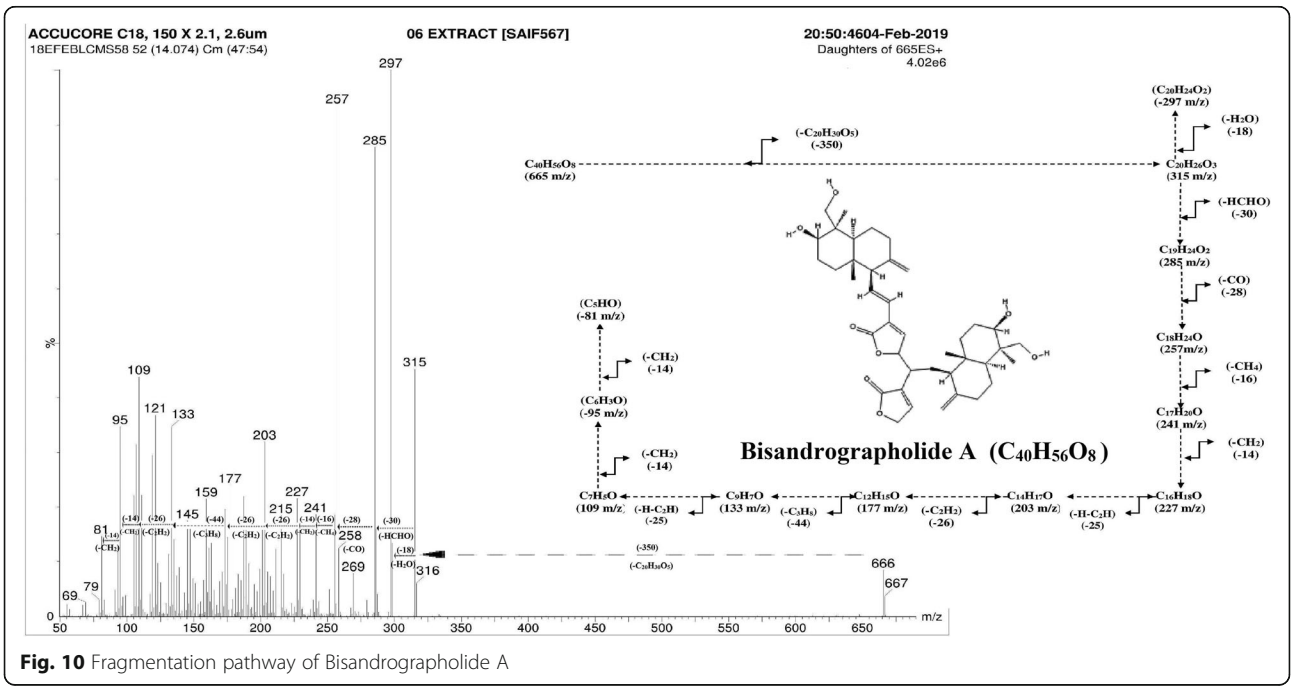

Fig. 10 Fragmentation pathway of Bisandrographolide A

due to their varied distribution [45]. A number of diterpenoids isolated from higher plants have been reported to possess an effect on insect growth regulation [59], insect anti-feedant [60], or insecticidal activity [61], insect pheromones. or defense chemicals [62]. The defensive roles of diterpenoids suggest that the compounds of this class might bear potent anti-malarial activities as compounds from this group were present in maximum number in IGNTU_06 extract. This is also validated by the fact that most of the traditional healers use this plant for the treatment of malaria in the study area.

5 Conclusions

We report a total of 59 compounds to be present in the IGNTU_06 extract. Most of the metabolites identified based on the LC-MS analysis belonged to the diterpenoids class of the compounds that were definite by LC-MS/MS analysis. The results present us with enormous structural and chemical diversity of natural products that can be utilized for anti-malaria drug development programs. This is to also mention that the LC-MS method developed by us can be uniquely used for the identification of maximum diterpenoids from *A. paniculata* as we obtained 54 diterpenoids from the 59 compounds evaluated. The results strongly validate the traditional use of *A. paniculata* by the traditional healers of the Amarkantak region. Activity-guided fractionization, purification, and characterization of the active components of the *A. Paniculata* extract are underway.

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Authors' contributions

MKD, SS, and SM 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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ICMR-DHR, New Delhi. The funder has no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Human Ethics Committee clearance number for the *P. falciparum* culture in human RBCs is CDRI/IEC/2019/A8.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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