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Identification of hydroxyquinazoline alkaloids from *Justicia adhatoda* L. leaves, a traditional natural remedy with NF-κB and AP-1-mediated anti-inflammatory properties and antioxidant activity

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ABSTRACT

Ethnopharmacological relevance: *Justicia adhatoda* L. is used as traditional medicine in Nepal to treat cough, asthma, and inflammatory disorders, and is indicated as "Asuro". Leaves are used worldwide as herbal medicine due to cardiotonic, expectorant, anti-asthmatic, and bronchodilatory properties. The aim of this work was to study the phytochemical composition of leaves of Nepalese *J. adhatoda* and assess their anti-inflammatory and antioxidant properties *in vitro*.

Materials and methods: Secondary metabolites were extracted from dried leaves using methanol (JAME: *J. adhatoda* methanol extract). They were analysed by means of liquid chromatography coupled with multiple-stage mass spectrometry (LC-MS^n). Anti-inflammatory potential was determined by the NF-κB and AP-1 inhibition assay, and DPPH, ABTS, and β-carotene bleaching assays were performed to assess its antioxidant properties.

Results: JAME is a rich source of secondary metabolites, especially quinazoline alkaloids such as vasicine, vasicinone, vasicoline, and adhatodine. 7-Hydroxy derivatives of peganidine, vasicolinone, and adhatodine were also identified by means of MS^n data and are here reported in *J. adhatoda* for the first time. JAME inhibited NF-κB and AP-1 expression in THP-1 cells to a greater extent than the positive control prednisolone. A moderate radical-quenching property was observed in DPPH and ABTS assays, but the anti-carotene bleaching activity was significantly higher than the reference BHT.

Conclusions: To the best of our knowledge, this is the first insight into the phytochemical composition of Asuro leaves from Nepal and their bioactivity. Our results will contribute to the valorisation of this medicinal species still widely used in the traditional and complementary medicine.

1. Introduction

The genus *Justicia* belongs to the Acanthaceae family and comprises more than 600 species widely distributed in pantropical and tropical regions of both hemispheres (Ezcurra, 1993). These herbaceous plants are still used in the current days as natural remedies for several ailments, as for example respiratory and gastrointestinal diseases, arthritis, depression, and anaemia (Corrêa and de Alcântara, 2012). Usually, the whole plant or aerial parts are used, but also extracts made from only the leaves or the roots (Corrêa and de Alcântara, 2012). Recent studies on extracts from *Justicia* plants indicate that these can also exert other activities such as anti-inflammatory (Basit et al., 2022), antimutagenic (Arvinder et al., 2015), anticancer (Kumar et al., 2022), and antimicrobial (Pa and Mathew, 2012), among others. Regarding the chemical composition, *Justicia* plants have been indicated as valuable sources of

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Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AP-1	Activator protein 1 transcription factor
BHT	butylhydroxytoluene
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
ESI	Electrospray ionization
JAME	*Justicia adhatoda* methanol extract
LC-MS^n	liquid chromatography coupled with multiple-stage mass spectrometry
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
TDDS	turbo detection data scanning

secondary metabolites, mainly alkaloids, steroids, and polyphenols such as flavonoids and tannins, as well as terpenes and terpenes (Carneiro et al., 2023).

Among the identified *Justicia* spp., only a few have been exhaustively characterized for their chemical composition and biological activities (Carneiro et al., 2023). *J. adhatoda* L. (syn, *Adhatoda vasica* Nees) is distributed throughout the South Asia and Indo-China region and is natively found in several Regions, among which East Himalayas, India, and Nepal (Kumar et al., 2022). This plant represents an important remedy in several traditional medicine systems of Asia (Ali and Hakeem, 2020), where it is used to cure asthma, colds, coughs, malaria, and venereal infections, and to alleviate rheumatism and pain. Leaves have anti-inflammatory properties and are also used as cardiotonic, expectorant, anti-asthmatic, and bronchodilator (Kumar et al., 2022). In Nepal, the plant is known by its vernacular name "Asuro" (Joshi et al., 2020). Here, different plant parts and plant preparations are used to treat various ailments (Claeson et al., 2000). The whole plant is used for bronchitis and cough (Singh et al., 2012), and to treat inflammatory swelling and rheumatism (Joshi et al., 2021). The fresh leaves juice obtained is used to treat cough, bronchitis, asthma (Joshi, 2004), diarrhoea, dysentery, and skin diseases (Chudamani and Kunwar, 2008), while the paste is applied topically to relieve rheumatic pain (Joshi, 2004). Pulverized dried leaves are used mainly for cough and asthma, but also to treat fever (Ambu et al., 2020). Recently, their use for counteracting COVID-19 infection has been reported (Khadka et al., 2021). In some regions of the Country, the plant is used also as food. The fruit, for example, is consumed as pickle in case of body-ache in Chitwan District (Rajbhandari, 2001). The composition of *J. adhatoda* in potentially bioactive secondary metabolites resembles that of other species of the same genus, being alkaloids, polyphenols, and saponins indicated as major group of constituents (Jamwal et al., 2023). Nevertheless, the plant has been explored mainly for its content in alkaloids up to now, specifically quinazoline derivatives. Vasicinone and vasicine are reported as the main constituents belonging to this chemical class, together with vasicol, adhatodine and anisotine (Bhanukiran et al., 2023). Among polyphenols, the flavonoids kaempferol, quercetin, luteolin, apigenin, and several glucoside derivatives have been reported (Nawaz et al., 2018; Singh et al., 2015).

Although several articles dealing with the chemical composition and bioactivities of *J. adathoda* have been already published over the last 10 years, many knowledge gaps still remain to be filled. Specifically, these works usually focus on the main chemical constituents of this plant, i.e. a bouquet of typical alkaloids that have also been associated with some bioactivities of *J. adathoda*. Few information regards the identification of other secondary metabolites such as polyphenols. Furthermore, several of these studies focus on plant specimens collected from India, while, to the best of our knowledge, almost no reports deal with *J. adathoda* growing in Nepal [e.g. (Velichkova et al., 2022)]. For these reasons, in this work we aimed to explore the chemical composition of the leaves of *J. adathoda* collected from the South-Eastern region of Nepal, by using liquid chromatography coupled to multi-stage mass spectrometry (LC-MS^n) at both negative and positive ionization modes, in order to cover the broadest range of metabolites. The antioxidant and anti-inflammatory properties of the same plant material were also assessed by assaying its capability to quench radicals and prevent carotene bleaching, and to inhibit NF-κB and AP-1 pathways, respectively. This article represents a further step in the characterization and valorisation of Nepalese plants of potential biomedical interest, in line with previous articles published by our group (Dall'Acqua et al., 2011; Peron et al., 2020a, 2020b, 2019; Shrestha et al., 2021).

2. Materials and methods

2.1. Collection of plant material

Fresh leaves of *J. adhatoda* were collected in the Bardibas municipality, Mahottari District, Province No. 2, Nepal (26°58′6.8″ N 85°50′42.7" E; Fig. 1) by Mr. G. Prasad Phuyal during mid-spring season. Botanical identification was performed by Dr. Suresh Kumar Ghimire of the Department of Botany of the Tribhuvan University (Kathmandu, Nepal). Plant material was washed with tap water and then dried at room temperature for 7 days, avoiding direct contact with sunlight. A voucher specimen (Fig. 1) was mounted on an herbarium sheet and was deposited at the Central Herbarium of Tribhuvan University (Kathmandu, Nepal) with the code: 24-332.

2.2. Plant extraction and LC-MS^n analysis of extract

Dried plant material (50 g) was grinded to a fine powder and extracted by ultrasound-assisted extraction, using methanol as solvent (250 mL). The detailed protocol has been reported elsewhere (Peron et al., 2019). Liquid extract was dried under vacuum at 40 °C to constant weight, and the dried residue (*J. adhatoda* methanol extract: JAME) was stored at −20 °C until analysis. The whole extraction procedure was performed in triplicate. The mean yield was 12.5 ± 2.2%.

The sample for analysis was prepared suspending 500 mg of JAME in 10 mL of methanol. The mixture was vortexed and then centrifuged at 13,300 rpm for 10 min. The supernatant was collected (1 mL), transferred in a glass vial and analysed by means of LC-MS^n. The instrumentation was composed of an Agilent 1260 binary pump equipped with an autosampler and coupled with a Varian MS 500 mass spectrometer with ion trap analyser. Electrospray (ESI) was employed as ion source, operating in both negative (ESI-) and positive (ESI+) ionization modes. An Agilent Eclipse plus C18 column (2.1 × 150 mm, 3.5 μm) was used as stationary phase, and a mixture of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) as the mobile phase. Further details on chromatographic and MS parameters have been reported in previous publications (Peron et al., 2020a, 2020b).

For the semi-quantification of flavonoids and phenolic acids, calibration curves of rutin and chlorogenic acid, respectively, were obtained using concentrations ranging from 10 to 120 ppm. The calibration curves were y = 6415.8x – 3448.6 ($R^2 = 1$) for rutin, and y = 6945.8x – 12624 ($R^2 = 0.999$) for chlorogenic acid. Limits of detection (LODs) were 1 and 2 ppm for chlorogenic acid and rutin respectively, while limits of quantification (LOQs) where estimated by multiplying the LODs by 3.3 (Lister, 2005). Alkaloids were revealed in positive ion mode, and their semi-quantification was performed using galantamine as standard. The calibration curve was: y = 6475900x + 51328 ($R^2 = 0.999$). LOD and LOQ were 0.2 ppm and 0.6 ppm, respectively. Quantitative results were expressed as mg/g of dry weight (DW).

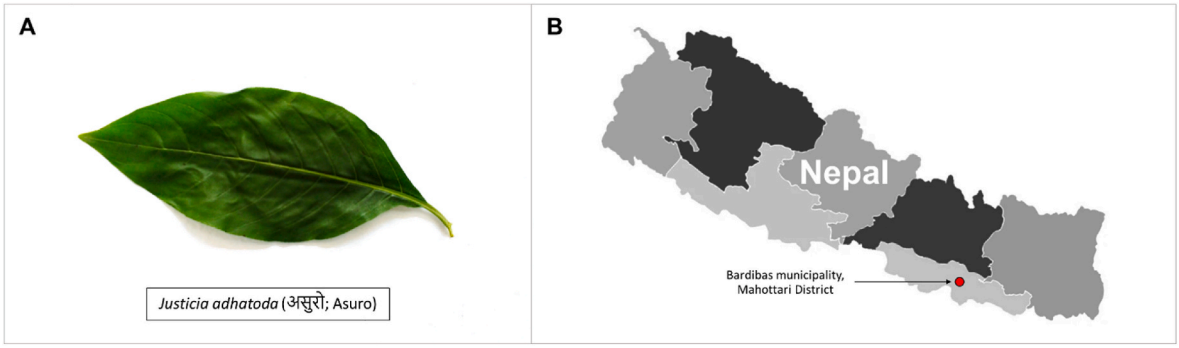

Fig. 1. Panel A: fresh leaf of *J. adhatoda*. Traditional names are also shown; Panel B: map of Nepal where the geographical position of the plant collection site is indicated.

2.3. Antioxidant activity

2.3.1. DPPH radical scavenging activity

Radical scavenging properties of JAME were first assessed on the stable 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet+}$). A protocol already published in the literature (Lopes-Lutz et al., 2008) was used. Briefly, different solutions of JAME in ethanol were prepared at increasing concentrations (from 0.1 to 0.4 g/mL). These were mixed with 2.44 mL of a DPPH solution (6×10^{-5} M) prepared in ethanol. Samples were incubated at room temperature for 1 h in absence of light. Afterwards, their absorbance was measured at 517 nm against a "white" sample. The anti-radical activity was estimated by the percentage inhibition of DPPH according to the following equation:

$$I\% = A_C - A_E/A_C \times \qquad 100$$

Where, I%: percentage of inhibition; A_C: absorbance of the DPPH solution; A_E: absorbance units of the sample.

The percentage inhibition was used to calculate the IC_{50} values, defined as the effective concentration of the extract required to reduce the concentration of initial DPPH. The IC_{50} was determined graphically by linear regression of plots where the abscissa represented the concentration of the samples and the ordinate the antiradical activity in percentage. All measurements were performed in triplicate ($n = 3 \pm$ SD). Ascorbic acid and butylhydroxytoluene (BHT) were used as positive controls.

2.3.2. ABTS radical scavenging activity

The capacity of JAME to inhibit $ABTS^{+}$ radical was tested by adopting the method of El Omari et al. (El Omari et al., 2019), with minor changes. In brief, $ABTS^{\bullet+}$ was generated by reacting 1.25 mL of ABTS stock solution and 1.25 mL of potassium persulfate $K_2S_2O_8$ solution at 2.45 mM. Then, the obtained solution was kept in the dark at 25 °C for 14 h. Before the experiment, this mixture was diluted with methanol until attending an optical density of 0.70 ± 0.09 at 734 nm. A volume of 100 μL of sample at various concentrations was mixed with 1 mL of ABTS solution, and the reaction was incubated at 25 °C for 2 min. The absorbance was established at 734 nm and the antioxidant activity was provided in term of ABTS scavenging and expressed as IC_{50} (μg/mL). Ascorbic acid was used as positive control.

2.3.3. Carotene bleaching assay

The assay was performed following a previously published protocol (Moure et al., 2000), with minor modifications. In brief, 4 mg of β-carotene was dissolved in 10 mL of chloroform by keeping the mixture in an ultrasound bath. Then, 120 mg of linoleic acid and 1200 mg of Tween 80 were added to 10 mL of this solution, which, after mixing by vortexing, was dried under vacuum at 40 °C. The residue was added of 300 mL of oxygenated ultra-pure water (MilliQ, 18.2 MOhm), and then the emulsion was vigorously shaken. 5 mL aliquots were added to 15 mL Falcon tubes containing 0.2 mL of the JAME solution at different concentrations (i.e., 200, 400, 600, 800, and 1200 μg/mL). The obtained emulsions were incubated in a water bath at 50 °C for 120 min and their absorbance was measured at 470 nm at 0, 30, 60, 90, and 120 min. The antioxidant activity of JAME was evaluated in terms of β-carotene bleaching using a formula that was previously reported elsewhere (Moure et al., 2000). A blank solution where JAME was substituted with ethanol was used as a negative control, while emulsions containing either BHT or ascorbic acid were used as positive controls.

2.4. Cell viability determination

The influence of JAME on cell viability was determined as previously described (Peron et al., 2020a). Briefly, cells [THP1-XBlue™-MD2-CD14 (Invivogen, San Diego, CA, USA)] were incubated for 24 h in the serum-free RPMI 1640 medium with increasing concentrations (1.25–20 μg/mL) of JAME dissolved in DMSO. The solvent did not exceed the concentration of 0.1% (v/v). After 24 h incubation, the amount of metabolically active (i.e., live) cells was evaluated by Cell Proliferation Reagent kit WST-1 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's manual. Relative cell viability was calculated as the ratio of absorbance values of JAME-treated to DMSO-only-treated cells. For further analysis, the concentration of JAME 2 μg/mL was selected as it was not affected by relative cell viability.

2.5. Determination of NF-κB and AP-1 activity

THP-1-XBlue™-MD2-CD14 cell line was used as a model for determination of the effect of JAME on the activation of transcription factors NF-κB and AP-1 (key pro-inflammatory transcription factors). This genetically modified cell line has been specially designed for testing the activity of these transcription factors. The ability of JAME to suppress the activity of lipopolysaccharide (LPS)-activated NF-κB/AP-1 was analysed as previously described (Peron et al., 2020a). Briefly, cells were pre-treated with JAME at the non-toxic concentration of 2 μg/mL or with control drug prednisone 1 μM (Sigma-Aldrich, Milan, Italy) dissolved in DMSO for 1 h. Subsequently, cells were stimulated by LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich) dissolved in serum-free RPMI 1640 medium (1 μg/mL). 24 h later, the activity of NF-κB/AP-1 was determined by Quanti-Blue™ medium (Invivogen, Toulouse, France) spectrophotometrically on FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) at 650 nm, according to manufacturer's manual. Results were compared with a control incubated only with DMSO and stimulated with LPS (100% NF-κB/AP-1 activity).

2.6. Statistical analysis

Chemical characterization of JAME was performed in triplicate, and results are reported as mean ± standard deviation (SD).

All the biological experiments were performed in triplicate. The results are presented as mean values, with the error bars representing the standard error of the mean (SEM). A one-way ANOVA test was used for statistical comparisons, followed by Fisher's LSD multiple comparison test. D'Agostino-Pearson omnibus and Shapiro-Wilk tests were used for analysis of data normality distribution. GraphPad Prism 8.01 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Chemical characterization of J. adhatoda methanol extract

Overall, the amount of compounds identified in JAME by LC-MS^n and quantified was 13.90 mg/g. Chromatograms obtained from analyses in ESI- and ESI + showed several peaks attributable mainly to secondary metabolites (Fig. 2), 35 of which were identified by means of MS^n data. Identified compounds and their characteristic MS signals are reported in Table 1. Three compounds were annotated as unknown, although their fragmentation pattern allowed to identify part of their structure. Compound 2, eluted at 3.3 min and presenting adduct with chlorine $[M + Cl–H]^-$ at *m/z* 377 and protonated ion $[M-H]^-$ at m/z 341, was identified as a glucose-glucose disaccharide, as already reported in the literature (Zhu and Cole, 2001). Compounds 26 and 27, eluted at 8.1 and 8.7 min respectively, and revealed $[M-H]^-$ at *m/z* 470 and 308, were partially identified as hexoside and pentoside derivatives, considering the neutral losses of 162 uma and 132 uma observed in their respective MS2 spectra.

Several flavonoids were detected and quantified in JAME, mainly kaempferol, vitexin, apigenin. Identification was achieved by ESI- following the aglycone behaviour, as previously described (Fabre et al., 2001). Vitexin and other C-glycosides were easily identified on the basis of the diagnostic loss of −90 and −120 uma (Geng et al., 2016) and by comparison of authentic standards. Furthermore, the O-glucoside derivatives of flavonoids were deduced on the basis of the literature (March et al., 2006) and comparison with available reference compounds. Caffeic acid hexoside, dihydrocaffeoylglycerol, eucomic acid, and the lignan medioresinol were also identified by ESI- MS. Metabolites detected after 14 min in the ESI- chromatogram were identified as lipids, namely hydroxyl fatty acids (peaks 13–16) and sterols (peaks 17, 18). β-Sitosterol was confirmed by comparison with a reference standard.

Analysis of JAME by ESI + MS allowed to associate several MS features to quinazoline alkaloids. Many of these are known metabolites of *J. adhatoda* such as vasicine, vasicinone, vasicoline, vasicinol, and adhatodine (Biharee et al., 2023). Other alkaloids were identified as hydroxyl derivatives of peganidine, adhatodine, vasicolinone, and anisotine. Except for 3-hydroxyanisotine, they are here reported in *J. adhatoda* for the first time. The compound eluting at 6.2 min presented $[M+H]^+$ at *m/z* 261 and was tentatively identified as 7-hydroxypeganidine on the basis of its fragmentation pattern. The MS2 spectrum, reported in Fig. 3, shows a base peak ion at *m/z* 187, corresponding to the loss of water (18 uma) and the propan-2-one moiety that was assigned in C9. The fragmentation of this species in MS3 yields a base peak ion at *m/z* 159 due to the loss of radical CH_2N moiety, and this species, upon the same loss, yields in MS4 the ion at *m/z* 131. This fragmentation pattern is characteristic of peganidine (Khashimov et al., 1969) and vascinol (Liu et al., 2015a), which presents the same tetrahydropyrroloquinazoline backbone.

The compound eluted at 10.89 min presented $[M+H]^+$ at *m/z* 322 and was identified as 7-hydroxyvasicolinone. In the MS2 spectrum, signals at *m/z* 308, 290 (base peak) and 262 are observed (Fig. 4). The ion at *m/z* 308 is formed due to the loss of a N-methylene (14 uma), which upon loss of a water molecule from the aromatic ring yields the fragment at *m/z* 290. The loss of CO (28 uma) from this ion yields in the MS3 spectrum the one at *m/z* 262, which represents the base peak ion. Upon loss of a methyl radical from the aromatic amine, the specie at *m/z* 247 is formed, while the loss of dehydroaniline (91 uma) followed by rearrangement is supposed to lead to the base peak ion in MS4 where the base peak is observed at *m/z* 171.

Compound 34, eluted at 11.83 min showing $[M+H]^+$ at *m/z* 352, was tentatively identified as 7-hydroxyadhatodine. Its fragmentation pattern was comparable to that observed for adhatodine (Fig. 5), one of the most characteristic secondary metabolites of *J. adhatoda* and one of the most abundant constituents identified in JAME, together with vasicine (1.55 mg/g and 2.76 mg/g, respectively). The ion presenting $[M+H]^+$ at *m/z* 352 in MS2 shows signals at m/z 306 and 302 (Fig. 6), the former corresponding to the loss of water and a radical CH_2N, the latter yielded upon rearrangement of the fragment formed from the loss of water and CH_3OH from the parent compound. This latter ion forms fragments at *m/z* 274 and 246 in MS4, losing CO and radical CH_2N moieties.

3.2. Antioxidant activity of JAME

The antioxidant potential of JAME was evaluated as its ability to quench radicals such as DPPT and ABTS, and to prevent carotene bleaching. As shown in Fig. 7, the antioxidant power of JAME was significantly lower than the two positive controls, i.e. ascorbic acid and BHT. IC_{50} measured for JAME in the DPPH assay was 42.62 ± 0.42 μg/mL, compared to 2.20 μg/mL and 4.80 μg/mL of ascorbic acid and BHT,

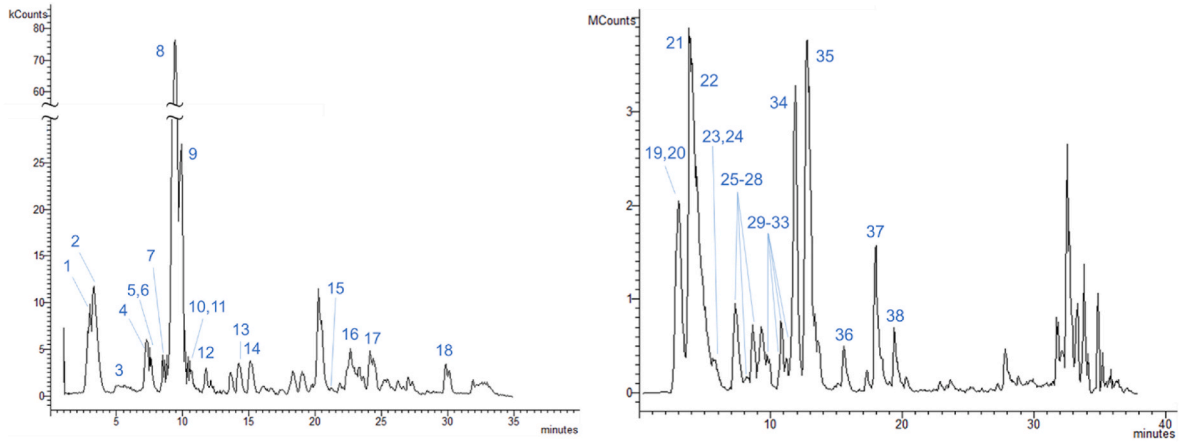

Fig. 2. Exemplificative chromatograms of JAME obtained by LC-MS (left: ESI- MS; right: ESI+ MS).

Table 1
Secondary metabolites tentatively identified in JAME by means of LC-MS^n data. Results are presented as mean values of n = 3 independent measurements ± SD. Peaks corresponding to single metabolites are shown in the chromatograms in Fig. 2.

N.	R.T. (min)	m/z	Fragments	Tentative identification [a]	Class	mg/g
ESI(−)						
1	2.9	387	341 179 153	Caffeic acid hexoside ([M-H + FA]$^-$)	PA	1.17 ± 0.07
2	3.3	377	341 179 161	Glucose-glucose disaccharide ([M-H + Cl]$^-$)	Other	NQ
3	5.2	255	181 163	Dihydrocaffeoylglycerol	PA	0.81 ± 0.10
4	7.3	239	179 149 133	Eucomic acid	PA	1.17 ± 0.07
5	7.52	431	341 311	Vitexin [c]	FL	0.13 ± 0.01
6	7.6	387	207 163	Medioresinol [b]	PA	0.44 ± 0.01
7	8.55	563	503 473 443 383 353	Apigenin C-hexoside-C-pentoside	FL	0.91 ± 0.16
8	9.45	533	473 443 383 353	Apigenin-6, 8-C-dipentoside	FL	0.13 ± 0.01
9	9.83	533	473 443 383 353	Apigenin-6, 8-C-dipentoside (asymmetric)	FL	0.17 ± 0.03
10	10.4	577	503 473 383	Apigenin C-rhamnoside-C-glucoside	FL	0.06 ± 0.01
11	10.6	269	225 151	Apigenin [c]	FL	0.05 ± 0.01
12	11.7	285	255 227	Kaempferol [c]	FL	0.06 ± 0.02
13	14.3	327	291 211	Oxo-dihydroxyoctadecenoic acid	Other	NQ
14	15.1	329	229	Trihydroxyoctadecenoic acid	Other	NQ
15	21.2	311	229	Dihydroxyoctadecenoic acid	Other	NQ
16	22.6	293	275	Hydroxyoctadecatrienoic acid	Other	NQ
17	24.2	295	277 195 171	Phytol	TRP	1.12 ± 0.09
18	29.79	211	ND	Stigmasterol [c]	TRP	1.06 ± 0.12
				Total identified and quantified		7.28 ± 0.70
				Total phenolic acids		3.59 ± 0.25
				Total flavonoids		1.52 ± 0.24
				Total terpenes		2.17 ± 0.21
ESI(+)						
19	2.54	104	60	Choline	Other	NQ
20	2.8	118	59 58	Betaine	Other	NQ
21	3.6	205	187 169 159	Vasicinol	ALK	0.12 ± 0.01
22	3.9	189	171 154 118	Vasicine	ALK	2.76 ± 0.35
23	5.65	219	201 186 174 148	Vasicinolone	ALK	0.18 ± 0.01
24	**6.2**	**261**	**243 187 159**	**7-Hydroxypeganidine**	ALK	0.12 ± 0.02
25	7.5	203	185	Vasicinone	ALK	0.27 ± 0.04
26	8.1	470	308 290	UNK hexoside	Other	NQ
27	8.67	308	176 158 143	UNK pentoside	Other	NQ
28	8.77	233	218 187	Adhavasinone	ALK	0.02 ± 0.00
29	9.84	219	204 187	Adhavasicine	ALK	0.01 ± 0.00
30	10.65	187	159 131	Deoxyvasicinone	ALK	0.04 ± 0.00
31	**10.89**	**322**	**308 290 262**	**7-Hydroxyvasicolinone**	**ALK**	0.02 ± 0.00
32	11.37	306	288 276	Vasicolinone	ALK	0.11 ± 0.03
33	11.6	292	277 247 171 154	Vasicoline	ALK	0.20 ± 0.02
34	**11.83**	**352**	**334 302 274**	**7-Hydroxyadhatodine**	ALK	0.69 ± 0.03
35	12.9	336	304 291	Adhatodine	ALK	1.55 ± 0.01
36	15.6	366	348 316 288	3-Hydroxyanisotine	ALK	0.10 ± 0.01
37	17.97	350	318 305	Anisotine	ALK	0.29 ± 0.01
38	19.4	336	304 183	Vasnetine	ALK	0.14 ± 0.02
				Total alkaloids		6.61 ± 0.53

R.T.: retention time; NQ: not quantified; PA: phenolic acid; FL: flavonoid; TRP: terpene; ALK: alkaloid.
[a] All metabolites identified as pseudomolecular ions, except 1 and 2 that were identified as formic acid (FA) and chlorine adducts, respectively.
[b] Lignan quantified using the calibration curve of chlorogenic acid.
[c] Identification confirmed by comparison with reference standard.

respectively. In the ABTS assay, measured IC_{50} values were 33.62 ± 1.52 μg/mL for JAME, 1.79 μg/mL for ascorbic acid, and 3.13 μg/mL for BHT. A higher antioxidant activity compared to BHT ($p = 0.0015$) was observed in the carotene bleaching assay, were IC_{50} for JAME was 45.64 ± 0.66 μg/mL, compared to 49.48 μg/mL measured for BHT.

3.3. Cytotoxicity

The viability of THP-1 cell line was not significantly changed after treatment with JAME. Moreover, JAME at the concentration of 20 μg/mL increased the relative cell viability twice (Fig. 8). It could be explained by the presence of some kind of nutrient in the raw extract (such as vitamins, amino acids, saccharides, etc.). The relative cell viability decreased back to values comparable with control untreated cells at the concentration of 5 μg/mL (data not shown). The subsequent *in vitro* cell-based experiments were performed with JAME at 2 μg/mL which did not affect the relative cell viability.

3.4. Inhibition of NF-κB and AP-1 activities

The anti-inflammatory potential of JAME was evaluated as the ability to suppress the activation of transcription factors NF-κB and AP-1 on LPS-stimulated THP-1-XBlue™ MD2-CD14 cells. JAME significantly decreased the activation of both NF-κB and AP-1 at the concentration of 2 μg/mL. The observed NF-κB/AP-1 activities were reduced by 35% in comparison to untreated LPS-stimulated control (Fig. 9).

4. Discussion

Plants of the *Justicia* genus have a long-term use in the folk medicine of Southeast Asia and the Indian subcontinent, and are widely studied for their beneficial effects on health. Insights into the chemical composition of these plants indicate them as valuable sources of bioactive secondary metabolites (Kumar et al., 2022). The most representative belong to the classes of alkaloids, lignans, flavonoids, and terpenoids, and are distributed in the different parts of the plants (Corrêa and de Alcântara, 2012). Regarding more specifically

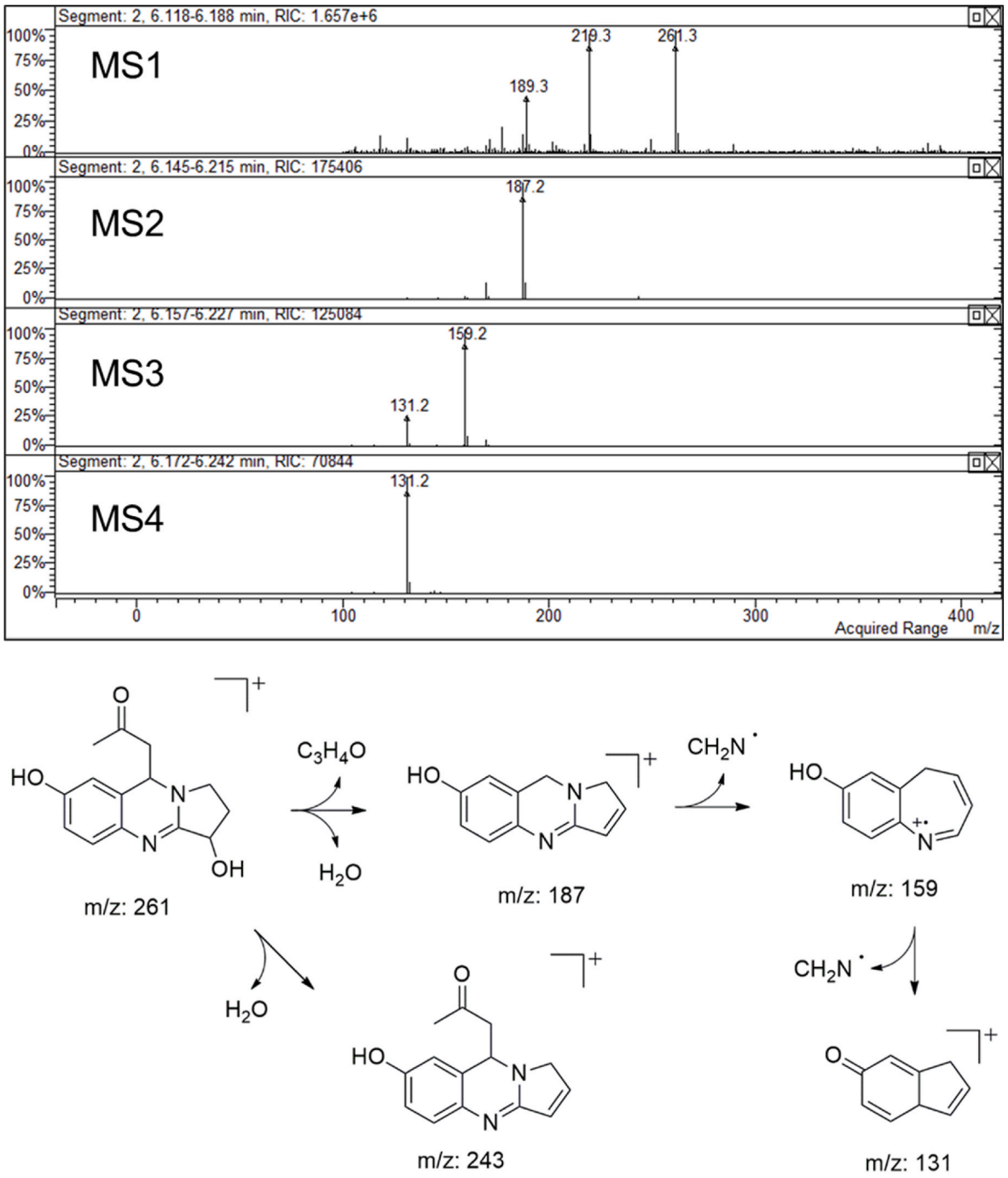

Fig. 3. MS spectra obtained from the fragmentation of the $[M+H]^+$ ion with m/z 261, tentatively identified as 7-hydroxypeganidine. In each panel the signals of the main fragments are shown. The proposed fragmentation scheme of the compound is reported below.

J. adathoda, a couple of recent works exhaustively summarize its content in secondary metabolites (Biharee et al., 2023; Singh, B. and Sharma, 2020). The most abundant and most studied constituents of this plant species are pyrroloquinazoline alkaloids, mainly represented by vasicine, vasicinone, vasicoline, vasicinol, and adhatodine. Among other constituents, few flavonoids such as apigenin, astragalin, kaempferol, quercetin, and vitexin, and triterpenes like α-amyrin and taraxerone have been reported in the aerial parts. Other different secondary metabolites have been identified in leaf extracts and described in a recent study (Kumar et al., 2022). Nevertheless, in this latter article the identifications are not supported by MS/MS data or other structural information (e.g., NMR), hence have to be considered preliminary putative annotations. In JAME, low amounts of flavonoids such as kaempferol and vitexin were detected. The main representative constituents belonging to this chemical class were identified as apigenin and its derivatives apigenin-6,8-C-dipentoside and its asymmetric isomer, and apigenin C-hexoside-C-pentoside. To the best of our knowledge, these compounds are here described in the leaves of *J. adhatoda* for the first time, considering that only the aglycone has been reported previously (Memon et al., 2023). Phytol and β-stigmasterol, two terpenes with anti-inflammatory, anticancer, antioxidant, and antimicrobial properties (Morgan et al., 2021; Saha and Bandyopadhyay, 2020) previously reported in *J. adhatoda*, were also identified in JAME. More importantly, all the representative pyrroloquinazoline alkaloids of *J. adathoda* were

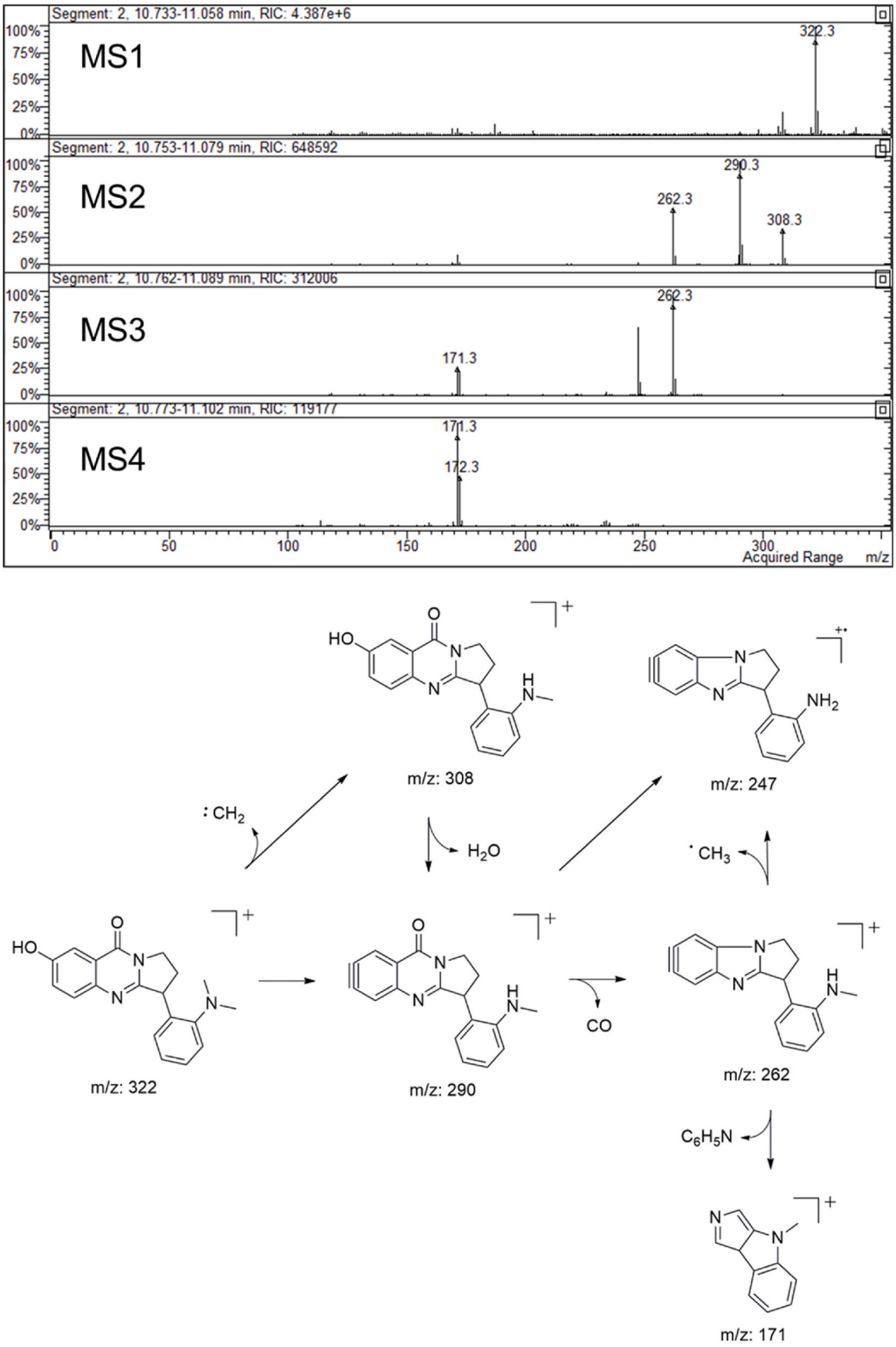

Fig. 4. MS spectra obtained from the fragmentation of the $[M+H]^+$ ion with m/z 322, tentatively identified as 7-hydroxyvasicolinone. In each panel the signals of the main fragments are shown. The proposed fragmentation scheme of the compound is reported below.

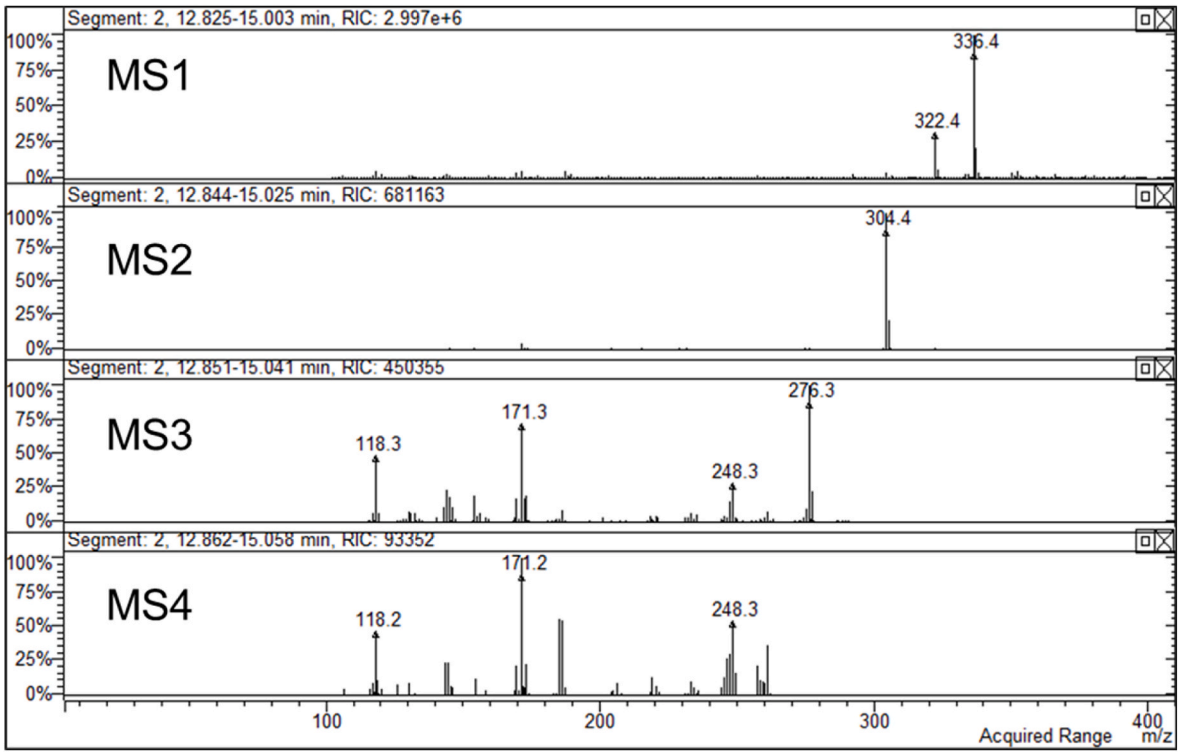

Fig. 5. MS^n (n = 4) spectra of the metabolite eluting at 12.9 min with m/z 336, identified as adhatodine.

detected and identified by means of MS^n data. Quantitative analysis revealed that the amount of identified alkaloids in JAME is comparable with that generally reported in literature for alcoholic extracts of *J. adhatoda* leaves [e.g. (Kumar et al., 2013),]. However, it is to note that wide variations of those amounts have been observed in different plant materials and in extracts obtained using different methods [e.g. (Gautam and Pitchaimuthu, 2023; Shukla et al., 2017; Soni et al., 2008),]. Three novel hydroxyquinazoline alkaloids were also identified in leaf extract of Nepalese *J. adhatoda*, namely 7-hydroxypeganidine, 7-hydroxyadhatodine, 7-hydroxyvasicolinone. To the best of our knowledge, ours is the first work to report these compounds in *J. adhatoda*, as well as the first to identify them in plants. Although our results are preliminary because identifications by MS^n should be confirmed with further analyses (e.g., structural NMR) on isolated compounds, we speculate that they represent chemical markers of a novel *J. adhatoda* chemotype growing in Nepal.

Characteristic pyrroloquinazoline alkaloids of *J. adhatoda* such as vasicine and vasicinone have been previously associated to the biological and medicinal properties of this natural product, especially those on inflammation. The alkaloid fraction isolated from the leaves exerts potent anti-inflammatory effects (Chakraborty and Brantner, 2001). Other studies have reported the significant contribution of single alkaloids to the anti-inflammatory properties of the plant. For instance, vasicine showed potent anti-inflammatory effects in rats after carrageenan injection, and vasicinone after complete Freund's adjuvant injection (Singh and Sharma, 2013). In silico, the same compounds have demonstrated inhibitory properties on COX-1 (vasicinone) and COX-2 (vasicine) (Ghanta et al., 2022). Apart from anti-inflammatory, these alkaloids have also expectorant and bronchodilator (Liu et al., 2015b), antimicrobial (Pa and Mathew, 2012; Singh and Sharma, 2013), and anti-cholinesterase (vasicinone) (Bhanukiran et al., 2023) properties. Overall, these data indicate that the most representative alkaloids of *J. adhatoda* are responsible of some of its main medicinal properties. However, several other characteristic alkaloids of this natural product have never been investigated in depth, and can potentially contribute to its bioactivity. Among these, the 7-hydroxy derivatives reported in this work, which should be further analysed in future studies. To note, semisynthetic derivatives of vasicine have been developed as bronchial mucolytic, anti-inflammatory and antioxidant compounds for use in chronic pulmonary disease, while vasicinone was used as scaffold for the development of semi-synthetic compounds with anti-allergic properties (Sharafkhaneh et al., 2007). In a similar way, 7-hydroxypeganidine, 7-hydroxyvasicolinone, and 7-hydroxyadhatodine should be investigated in the future to assess their usefulness as scaffolds for the development of novel bioactive compounds.

JAME demonstrated to inhibit the total expression of two transcription factors involved in the inflammatory process, i.e. NF-κB and AP1 (Fujioka et al., 2004), in THP-1 cells stimulated with LPS. The presence of relatively high amount of flavonoids and other phenolic compounds in JAME may explain the observed effects, being already known for their anti-inflammatory properties (Brezani et al., 2018). Other constituents such as phytol may contribute. Recent studies indicate that phytol exhibits dose-dependent anti-inflammatory effects, and in silico this compound inhibits COX-1 and 2, NF-κB, and interleukin-1β (Islam et al., 2020). Stigmasterol has been also reported to inhibit the NF-κB pathway (Jie et al., 2022). The inhibitory effect of *J. adhatoda* on NF-κB has been reported recently by other Authors, although with a different mechanisms of action and in a different cell line. In fact, Kumar et al. have showed that a methanol extract of *J. adhatoda* leaves inhibits the translocation of NF-κB subunit p65 to the nucleus of unstimulated MCF-7 cells (human breast cancer cell line) in a dose-dependent manner. Being the NF-κB pathway implied in the growth of tumors, the accumulation of the transcription factor in cytosol induced apoptosis (Kumar et al., 2022). It is worth to note that in the study of Kumar et al. concentrations of 100 and 150 μg/mL of extract were tested, whereas in our study only 2 μg/mL was used. It indicates that JAME in low concentrations could serve as anti-inflammatory agent, but in high concentrations it shows cytotoxic and anti-cancer potential.

Finally, JAME revealed a mild antioxidant activity. The extract showed a lower ability to quench DPPH and ABTS radicals compared to the positive controls, although the efficacy was higher when compared to data published by other authors on extracts of the same plant from

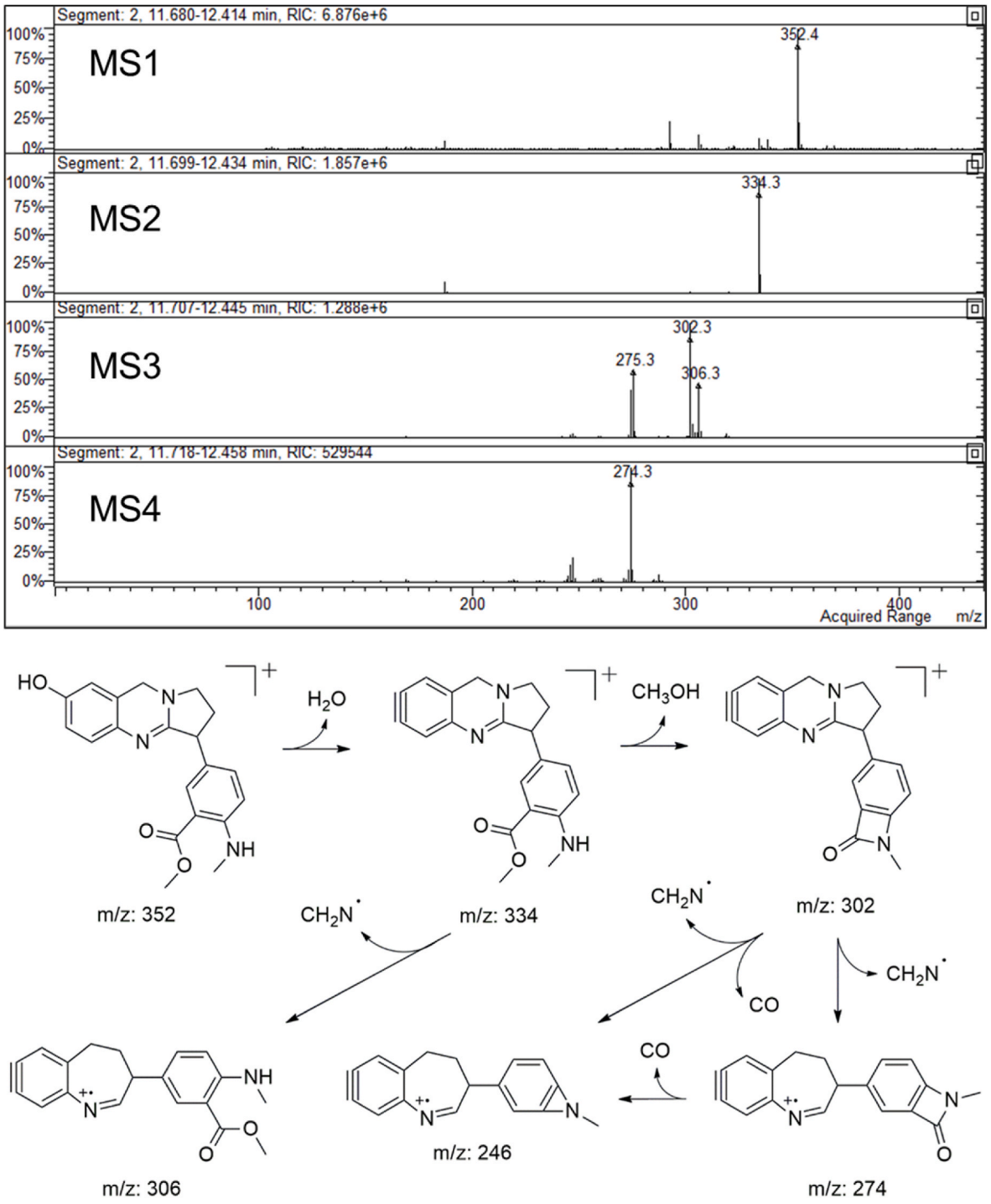

Fig. 6. MS spectra obtained from the fragmentation of the positive ion with m/z 352, tentatively identified as 7-hydroxyadhatodine. In each panel the signals of the main fragments are shown, together with their putative structures.

Nepal [IC_{50} for DPPH: 102.43 μg/mL (Oli et al., 2023)]. JAME showed a higher efficacy in inhibiting carotene bleaching, since the antioxidant effect was higher than the positive control BHT. Our results are in line with data already published on *J. adhatoda* (Lu et al., 2021), and support the potential use of JAME as antioxidant agent in nutraceutical formulations and foods. However, further studies are required to assess its safety and efficacy on different cellular models and in vivo.

5. Conclusion

Leaves of *J. adhatoda* are used in the traditional medicine of Nepal to treat several ailments, among which inflammatory disorders. Inflammation can be induced by the activation of the NF-κB/AP-1 pathway, which in turn can be activated by radicals or reactive species such as hydrogen peroxide (Hong et al., 2024). In this study, JAME revealed to exert a significant anti–NF–κB/AP-1 activity and mild antioxidant effects: these results may highlight two possible mechanisms of action of this natural product against inflammation, and contribute to rationalize its traditional use as anti-inflammatory agent.

JAME is rich in secondary metabolites such as quinazoline alkaloids and their hydroxyl derivatives. Considering also previously published data on the bioactivity of these compounds, their involvement in the

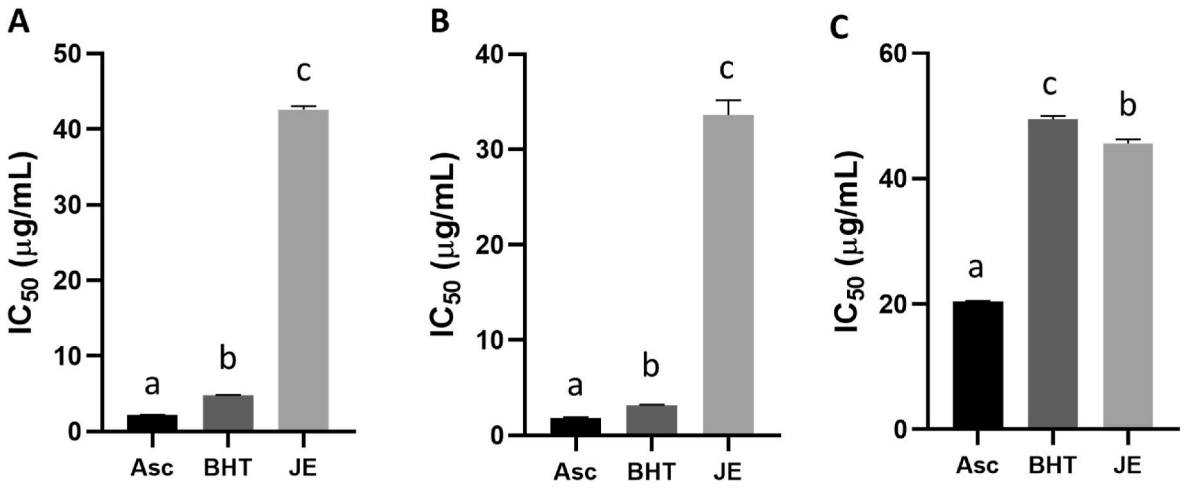

Fig. 7. Results expressed as IC_{50} obtained from the evaluation of the antioxidant activity of *J. adhatoda* methanol extract (JE). Panel A: DPPH assay; Panel B: ABTS assay; Panel C: β-carotene bleaching assay. Superscript letters indicate the significance level ($p < 0.05$): a>b > c. Asc: ascorbic acid; BHT: butylhydroxytoluene.

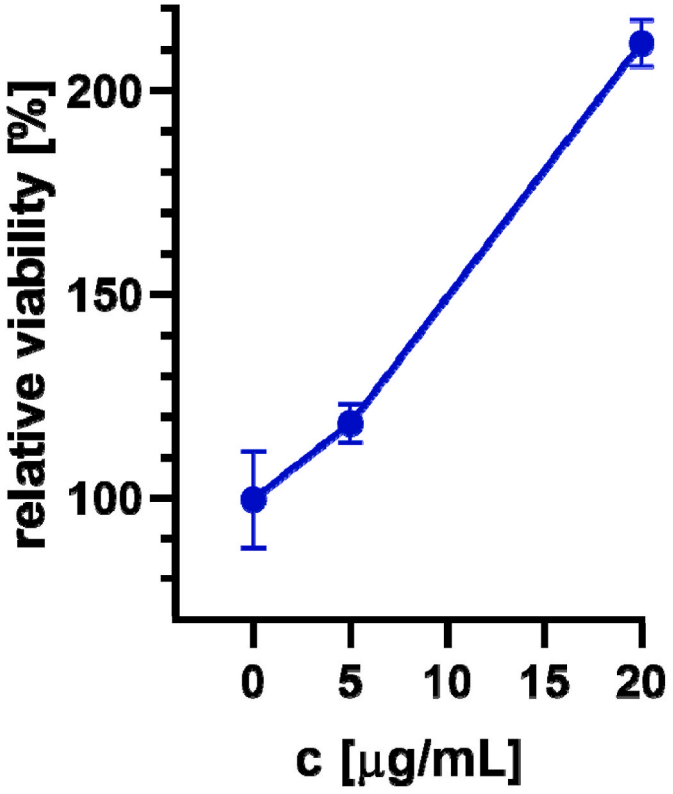

Fig. 8. Relative viability of THP-1-XBlue™ MD2-CD14 cells incubated with JAME. The viability was measured using WST-1 kit after 24h incubation.

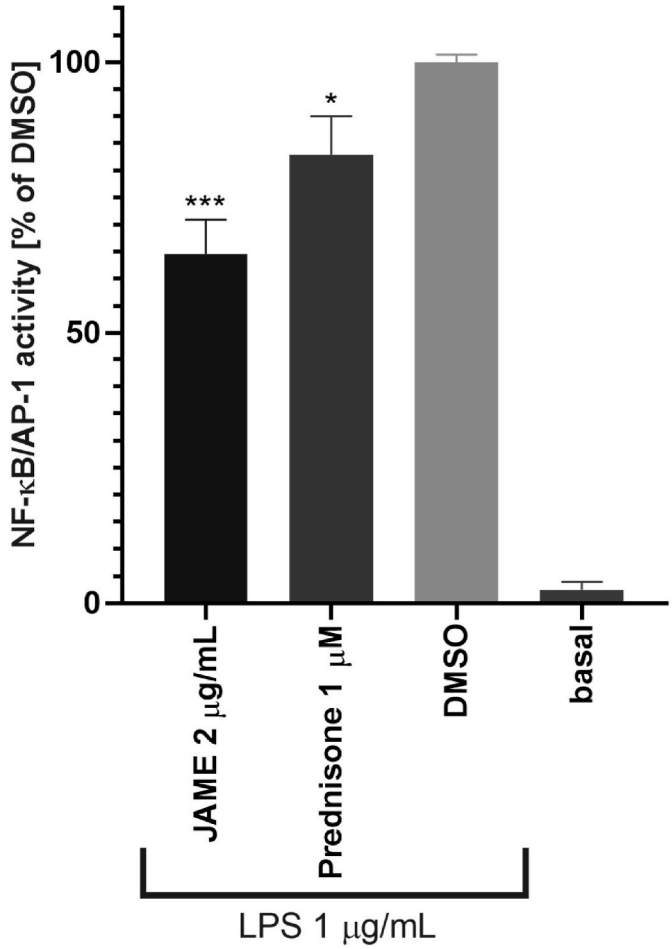

Fig. 9. Effect of *J. adhatoda* methanol extract (JAME) on the activity of NF-kB/AP-1 in LPS-stimulated THP-1-XBlue™ MD2-CD14 cells. Cells were pre-treated in JAME (2 μg/mL), prednisone (1 μM) or only solvent (DMSO) for 1 h. Afterwards, LPS was added and NF-kB/AP-1 activity was measured 24 h later. * indicates statistical significance to positive control (DMSO), $p < 0.05$; *** indicates statistical significance to positive control (DMSO), $p < 0.001$.

observed anti–NF–κB/AP-1 and antioxidant effects can be hypothesized. Furthermore, the hydroxy derivatives of peganidine, vasicolinone, and adhatodine, here reported for the first time, may contribute to differentiate Nepalese *J. adhatoda* from the same species growing in other Regions, and can be potentially considered as molecular markers of a new chemotype. Nevertheless, further research is needed to verify these hypotheses. First of all, the compounds should be isolated and completely characterized by means of NMR and high-resolution MS, for example. Afterwards, their bioactivity should be tested on different *in vitro* and in vivo models, and possible synergies with other alkaloids from *J. adhatoda* should be assessed.

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CRediT authorship contribution statement

Gregorio Peron: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Formal analysis, Conceptualization. **Ganga Prasad Phuyal:** Writing – review & editing, Resources, Investigation, Conceptualization. **Jan Hošek:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation. **Rameshwar Adhikari:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis. **Stefano Dall'Acqua:** Writing – review & editing, Supervision, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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