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Four new carbazole alkaloids from *Murraya koenigii* that display anti-inflammatory and anti-microbial activities†

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In our present study, four new, designated as murrayakonine A–D (**1–4**), along with 18 known carbazole alkaloids were isolated from $CHCl_3$: MeOH (1 : 1) crude extracts of the stems and leaves of *Murraya koenigii* (Linn.) Spreng. The structures of the all isolated compounds were characterized by analysis of HR-ESI-MS and NMR (1D and 2D spectroscopy) results, and comparison of their data with the literature data. For the first time, all the isolates were evaluated for their anti-inflammatory activities, using both *in vitro* and *in vivo* experiments, against the key inflammatory mediators TNF-α and IL-6. The new compound murrayakonine A (**1**), *O*-methylmurrayamine A (**13**) and mukolidine (**18**) were proven to be the most active, efficiently inhibiting TNF-α and IL-6 release in a dose-dependent manner and showing decreased LPS induced TNF-α and IL-6 production in human PBMCs. Furthermore, all the isolates were screened for their antimicrobial potential, and the compounds girinimbine (**12**) (IC_{50} 3.4 μM) and 1-hydroxy-7-methoxy-8-(3-methylbut-2-en-1-yl)-9*H*-carbazole-3-carbaldehyde (**19**) (IC_{50} 10.9 μM) displayed potent inhibitory effects against *Bacillus cereus*. Furthermore, compounds murrayamine J (**7**) (IC_{50} 11.7 μM) and koenimbine (**14**) (IC_{50} 17.0 μM) were active against *Staphylococcus aureus*. However, none of the compounds were found to be active against *Escherichia coli* or *Candida albicans*.

Introduction

Murraya koenigii (Rutaceae) is a most valuable Indian medicinal plant that is extensively used as a condiment. In the traditional system of medicine, the leaves of this plant were widely used as an analgesic, carminative and febrifuge, and for the treatment of diarrhoea and dysentery, whereas the root and stem were applied externally for skin eruptions.[1] In modern research, the leaf extracts of *M. koenigii* are widely used for antidiabetic,[2] antifungal,[3] antineoplastic,[4] nitric oxide scavenging,[5] anti-amnesic,[6] antioxidant,[7] anti-inflammatory[8] and immunomodulatory activity.[9] The therapeutic efficacy of *M. koenigii* is due to the presence of carbazole alkaloids, which demonstrate various biological potentialities such as anticancer,[10] anti-oxidative,[11] anti-TB,[12,13] and memory enhancing activities,[14] *etc*. At present seventy-seven distinct carbazole alkaloids have been reported from the various parts of *M. koenigii*,[15–17] but still the isolation of bioactive metabolites and their biological and pharmacological efficacies need to be explored further to meet the growing requirements of modern medicine.

Here, we report part of our ongoing research into the isolation of bioactive carbazole alkaloids from *M. Koenigii* to achieve new biological knowledge. We investigated compounds (**1–22**) for their activity against immunological factors that mediate inflammatory and autoimmune disorders. Among them, Tumour Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) are the two most important pro-inflammatory cytokines which mediate autoimmune diseases, and they are associated with outcomes such as pain and joint destruction in rheumatoid arthritis (RA).[18] Over-expression of TNF-α causes severe damage to the host and has been correlated with many pathological conditions like diabetes,[19] ulcerative colitis,[20] Crohn's disease, multiple sclerosis,[21] cirrhosis,[22] atherosclerosis,[23] and

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strokes.[24] IL-6, another type of pro-inflammatory cytokine present in the serum and synovium of RA patients, is also present in the ascitic fluid (AF) of cirrhotic patients,[22,25] inducing many different physiological and cellular responses during these chronic inflammation processes. On the other hand, IL-6 inhibitors have been proven to be potentially useful for the treatment of Alzheimer's disease, psychiatric disorders, cancer, diabetes, and depression.[26,27] These days, a large effort has been made regarding TNF-α inhibition, but no small molecules have been approved yet as reliable inhibitors of this cytokine.[28] So far, the TNF-α inhibitor drugs in clinics are proteins with undesirable side effects. Hence, finding natural products that can be used as IL-6 and TNF-α inhibitory agents is a major hope in the area of anti-inflammatory drug development. Herein, we have isolated four new, murrayakonines A–D (**1–4**), and 18 known (**5–22**) carbazole alkaloids (Fig. 1) from

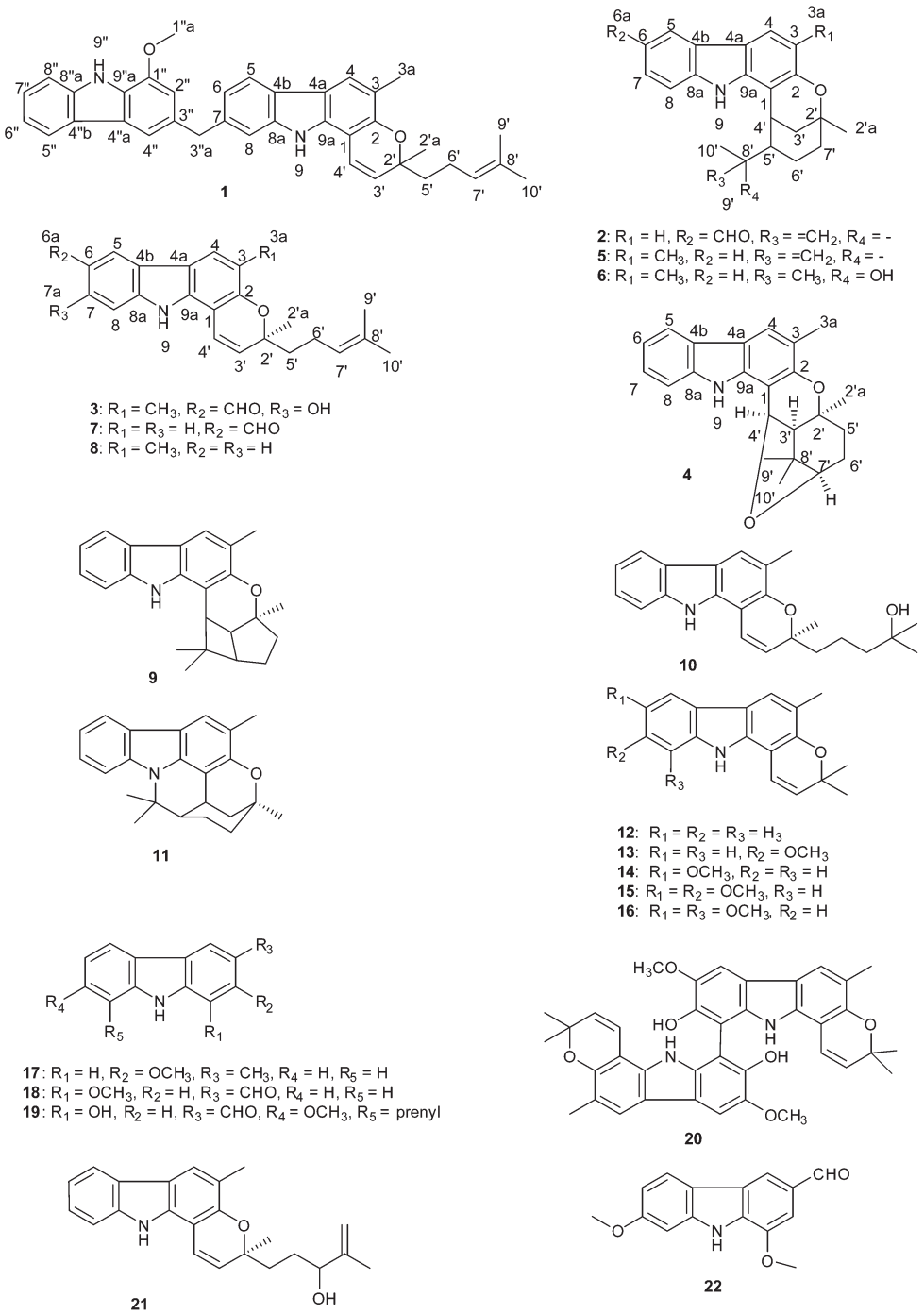

Fig. 1 Chemical structures of the isolated alkaloids **1–22**.

$CHCl_3$: MeOH (1 : 1) crude extracts of the leaves and stem of *M. koenigii*. The structures of the new compounds (**1–4**) were determined using spectroscopic methods, including extensive 2D NMR and HR-ESI-MS experiments. All these compounds (**1–22**) were screened for the first time for their anti-inflammatory and antimicrobial activity.

Results and discussion

Murrayakonine A (**1**) was obtained as a pale yellow amorphous powder. The IR spectrum displayed an intense absorption band at 3436 cm^{-1} indicating the presence of a NH group. The HR-ESI-MS spectrum showed a $[M + H]^+$ peak at *m/z* 541.2847 and, considering this in combination with the ^1H and ^{13}C NMR data, the molecular formula of **1** was determined to be $C_{37}H_{36}N_2O_2$. The ^{13}C NMR spectrum showed resonances for 35 carbons but using a combination of ^{13}C, HSQC and HMBC correlations along with the mass (HRESIMS) data of **1** suggested overlapped resonances for two carbons (C-8a with C-8a and C-1 with C-9″a). The data further revealed the presence of 37 carbons comprising four methyl, one methoxy, three methylene, thirteen methine and sixteen quaternary carbons (see ESI Fig. S1–S12;† Table 1). This above data, in addition to a literature search, indicated that **1** is a binary carbazole alkaloid which is a positional isomer of chrestifoline-C.[29] This was further confirmed by the interpretation of 1D and 2D (COSY, HMBC and NOESY) NMR data. The ^1H NMR spectrum indicated the presence of one $-CH_2CH_2CH{=}C(CH_3)_2$ unit, as evidenced by the resonances for H-7′ [δ 5.10 (t, J = 7.1 Hz, 1H)], H-5′ [δ 1.75 (m, 2H)], H-6′ [δ 2.15 (dd, J = 14.7, 6.8 Hz, 2H)] and two methyl singlets (H-9′, H-10′), and one AB-type doublet

Table 1 ^1H and ^{13}C NMR spectroscopic data for **1–4** in $CDCl_3$

	1		**2**		**3**		**4**	
No.	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	—	104.22	—	106.21	—	104.70	—	105.03
2	—	149.84	—	156.18	—	149.99	—	149.01
3	—	118.27	6.82 d (8.5)	110.45	—	119.62	—	118.53
3a	2.30 s	16.02	—	—	2.32 s	16.04	2.25 s	16.31
4	7.61 s	121.26	7.83d (8.9)	119.65	7.57 s	120.76	7.67 s	121.29
4a	—	124.14	—	115.15	—	118.38	—	115.94
4b	—	116.64	—	124.47	—	115.38	—	123.99
5	8.00 d (7.8)	120.58	8.44 s	122.62	7.98 s	125.76	7.85 d (7)	119.32
6	7.21 d (7.8)	125.48	—	129.23	—	116.08	7.09 t (7.4)	119.21
6a	—	—	10.06 s,	192.00	9.89 s	195.18	—	—
7	—	138.09	7.88–7.84 m	125.83		160.14	7.22 d (8.0)	123.99
–OH	—	—	—	—	11.42 s	—	—	—
8	7.77 s	119.43	7.41 d (8.4)	110.37	6.83 s	96.93	7.31 d (8.0)	110.62
8a[a]	—	139.47	—	143.19	—	140.60	—	139.66
9	7.84 s (NH)		8.11 s (NH)	—	8.14 (NH)		8.21 (NH)	—
9a	—	135.25	—	140.23		135.25	—	138.38
2′	—	78.12	—	74.48		78.41	—	77.81
2′a	1.44 s	25.82	1.45 s	28.83	1.45 s	25.93	1.67 s	30.40
3′	5.64 d (9.7)	128.39	1.94 m	37.26	5.70 d (9.8)	129.47	1.91 d (4.2)	51.01
4′	6.64 d (9.8)	117.56	3.42 s	36.01	6.60 d (9.9)	116.94	5.22 d (4.2)	70.39
5′	1.75 m	40.77	2.67–2.52 m	48.45	1.76 m	40.84	1.48–1.42 m, 1.88–1.80 m	27.63
6′	2.15 dd (14.7, 6.8)	22.75	1.60 m	22.93	2.16 m	22.76	1.55 s	27.08
7′	5.10 t (7.1)	124.19	2.13 m, 1.65 m	39.60	5.11 t (7.0)	124.04	3.82 d (3.9)	82.16
8′	—	131.65	—	149.76	—	131.81	—	42.73
9′	1.65 s	25.67	4.80 d (39.6)	112.41	1.66 s	25.66	1.31 s	28.11
10′	1.57 s	17.57	1.52 s	21.65	1.58 s	17.58	1.25 s	23.05
1″	—	145.58						
1″a	3.93 s	55.50						
2″	6.77 s	107.58						
3″	—	134.05						
3″a	4.30 s	42.52						
4″	7.56 s	112.84						
4″a	—	133.19						
4″b	—	123.68						
5″	7.29 d (8.3)	110.29						
6″	7.17 m	119.19						
7″	7.37 t (7.6)	125.49						
8″	7.43 d (8.1)	110.90						
8″a[a]	—	139.47						
9″	8.19 s (NH)	—						
9″a[a]	—	128.39						

[a] Overlapped resonances (C-8a with C-8″a and C-3′ with C-9″a) in **1** (see ESI Fig. S1–S13); all assignments are based on HSQC and HMBC experiments and were measured in $CDCl_3$.

[at 5.64 (d, J = 9.7 Hz, H_1-3′) and δ 6.64 (d, J = 9.8 Hz, H_1-4′)] was indicative of the presence of one (2*H*)-pyran ring. The HMBC spectrum exhibited interactions of the H_2-5′ with C-3′, C-2′, C-2′a, C-6′ and C-7′; whereas the H_3-2′a showed HMBC cross-peaks with C-3′, C-2′ and C-5′. This HMBC data suggests attachment of the $-CH_2CH_2CH{=}C(CH_3)_2$ moiety to the C-2′ of the (2*H*)-pyran ring. From the signals for one aryl methyl group H-3a (δ 2.30 (s, 3H), the ABX pattern of aromatic signals for H-5 [δ 8.00 (d, J = 7.8 Hz, 1H)], H-6 [δ 7.21 (d, J = 7.8 Hz, 1H)] and H-8 [δ 7.77 (s, 1H)], one singlet for H-4 [δ 7.61 (s, 1H)] and one broad singlet at δ 7.84 (s, –NH) the presence of a 3-methyl-2-oxygenated-1,7-alkyl carbazole unit was inferred. The location of the methyl group H-3a substituent at C-3 was inferred from the HMBC interactions of H-4 (δ 7.61) with C-3a. This was further confirmed from the NOESY spectrum (Fig. 3), which exhibited the association of H-4 with H-3a. From the HMBC correlation of H-4′ with C-9a, C-1 and C-2, we inferred the presence of a 3-methyl-2-oxygenated-1,7-alkyl carbazole unit fused to the (2*H*)-pyran ring at C1–C2, forming a 7-alkylated mahanimbine. Furthermore, the ^{1}H NMR spectrum also indicated the presence of one 1-methoxy-3-alkyl-carbazole unit, as evidenced by the resonances at δ 6.77 (s, H_1-2″), 7.56 (s, H_1-4″), 7.29 (d, J = 8.3 Hz, H_1-5″), 7.17 (m, H_1-6″), 7.37 (t, J = 7.6 Hz, H_1-7″) and 7.43 (d, J = 8.1 Hz, H_1-8″), and the resonances for one methoxy group (δ 3.93, s, H_3-1″a) and a –NH (δ 8.19, s). This was confirmed from the key HMBC correlations of –NH/C-8″a, C-9″a and C-4″b, of H-2″/C-1″, C-4″ and C-9″a, of H-4″/C-2″, C-9″a and C-4″b, of H-5″/C-7″ and 4″a, of H-6″/C-8″ and C-4″b, of H-7″/C-8″a, and of H-8″/C-6″ and C-4″b (Fig. 2). The HMBC correlation between the OCH_3 (δ 3.93) and C-1″ (δ 145.59) suggests attachment of the OCH_3 group at C-1″. Finally, that the 7-alkylated mahanimbine and 1-methoxy-3-alkyl-carbazole units are connected through a methylene group H_2-3″a (δ 4.30) was indicated by the HMBC interactions of H_2-3″a/C-2″, C-3″, C-4″, C-8 and C-6, and of H-6, H-8, H-2 and H-4″/C-3′a (Fig. 2). This was further confirmed from the NOESY correlations of H_2-3″a (δ 4.30) with H-6, H-8, H-2″ and H-4″ (Fig. 3). The absolute configuration at the C-2′ position of compound **1** for the (2*H*)-pyran ring remains undefined.[30] Based on this spectroscopic evidence, a new molecular structure was assigned as shown in Fig. 1 and designated as murrayakonine A (**1**).

Murrayakonine B (**2**) was obtained as a colourless oil. The molecular formula was determined to be $C_{23}H_{23}NO_2$ using the ^{13}C NMR data and the (+)-HR-ESI-MS ion peak at m/z 346.1805 $[M + H]^+$ (calcd 346.1802). The IR spectrum displayed absorptions characteristic of amino (3369 cm^{-1}) and carbonyl (1674 cm^{-1}) groups. The ^{1}H NMR spectrum showed signals due to one formyl group, one –NH, five aromatic protons, two vinylidene protons that appeared as typical broadened singlets, two methine groups, and three methylene groups including one diastereotopic group and two methyl groups. ^{13}C NMR and DEPT, along with HSQC, experiments for **2** revealed the presence of 23 carbons including one formyl (C-6a), eight sp^2 quaternary (C-1, C-2, C-4a, C-4b, C-6, C-8a, C-9a and C-8′), one sp^3 quaternary (C-2′), five sp^2 methine (C-3, C-4, C-5, C-7 and C-8), one sp^2 methylene (C-9′), two sp^3 methine (C-4′ and C-5′), three sp^3 methylene (C-3′, C-6′and C-7′) and two methyl (C-10′, C-2′a) carbons. The above-mentioned data, together with biogenetic considerations, suggests that **2** is a carbazole alkaloid that is similar to murrayamine G[31] and murrayazolidine (**5**), which has also been reported for the same plant. The main difference between **2** and murrayamine G is the aldehyde (–CHO) group, which is replaced by a methyl group ($-CH_3$) for murrayamine G, at the C-6 position, which was further authenticated by the HMBC correlations of H-6a/C-5, C-7 and C-6

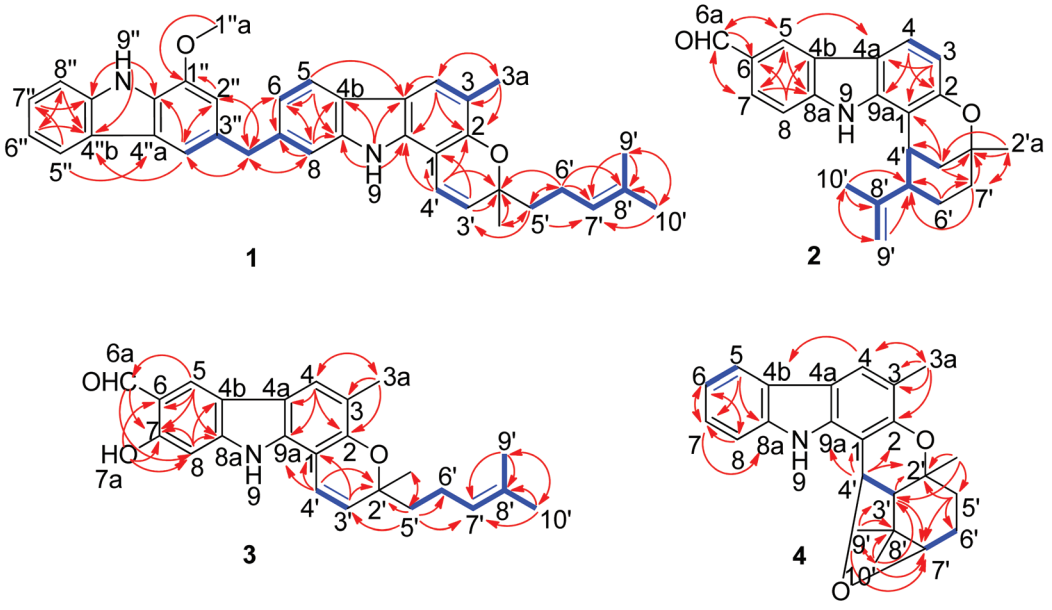

Fig. 2 Key COSY (▬) and HMBC (⌒) correlations for compounds **1–4**.

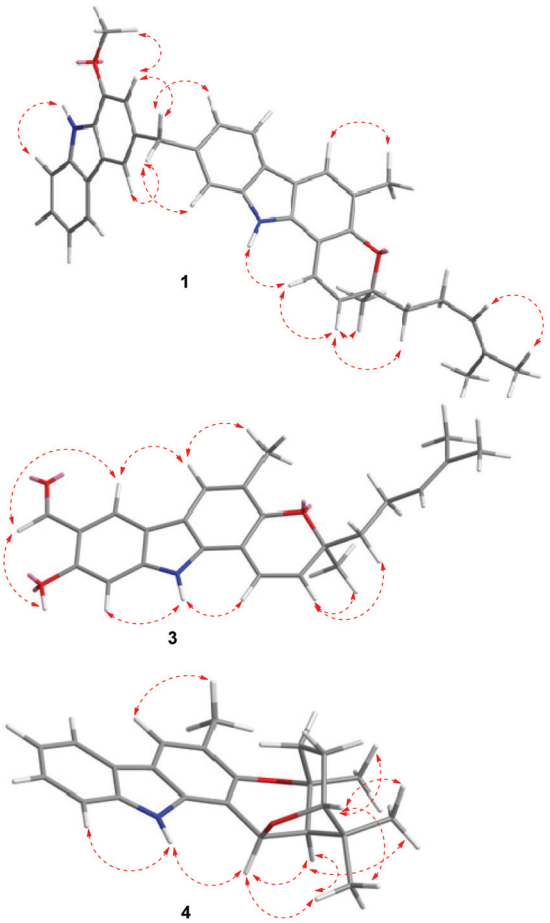

Fig. 3 Key NOESY (↔) correlations for compounds **1**, **3** and **4**.

(Fig. 2). On the basis of this spectral evidence the new molecule was determined as being 5-methyl-2-(prop-1-en-2-yl)-1,2,3,4,5,13-hexahydro-1,5-methanoxocino-[3,2-*a*]carbazole-10-carbaldehyde, which was designated as murrayakonine B (**2**).

Compound **3** was isolated as a light-greenish gum, and its molecular formula was established as $C_{24}H_{25}NO_3$ from the HR-ESI-MS ion peak at *m*/*z* 376.1909 $[M + H]^+$. The UV spectrum of **3** showed absorptions at λ_{max} values of 320, 268 and 246 nm, which are typical for an angular pyrano-carbazole moiety.[32] The IR spectrum displayed an intense absorption band at 3436 cm^{-1}, indicating the presence of a –NH group. Close inspection of the ^1H and ^{13}C NMR data ($CDCl_3$, 400 MHz) (Table 1) for **3** using DEPT and HSQC experiments indicated the presence of one carbonyl (C-6a), ten sp^2 quaternary (C-1, C-2, C-3, C-4a, C-4b, C-6, C-7, C-8a, C-9a and C-8′), six sp^2 methine (C-4, C-5, C-8, C-3′, C-4′ and C-7′), one sp^3 quaternary (C-2′), two sp^3 methylene (C-5′and C-6′), and four methyl (C-3a, C-2′a, C-9′ and C-10′) carbons. This data indicated that **3** is a pyrano-carbazole alkaloid that is similar to mahanimbine (**8**)[30] except for the disappearance of two aromatic protons, instead compound **3** exhibited downfield proton signals at δ_H 9.89 (–CHO) and 11.42 (–OH). These characteristics implied that compound **3** is a mahanimbine (**8**) derivative with –CHO and –OH groups on the aromatic ring. This was further authenticated by 2D (COSY, HMBC and NOESY) NMR spectral data analysis. The ^1H–^1H COSY analysis of **3** led to three partial structural units; a 2-methyl pentenyl unit was apparent (H-5′ ↔ H-6′ ↔ H-7′ ↔ (allylic) H-9′ and H-10′), one AB-type doublet was observed which was indicative of the presence of a (2*H*)-pyran ring (H-3′ ↔ H-4′), and a benzylic unit was apparent (H-4 ↔ H_3-3a), as shown by the bold-faced lines in (Fig. 2). The HMBC spectrum exhibited the key correlations of H-3′ (δ 5.70)/C-2′ (δ 78.41), of H_3-2′a (δ 1.45)/C-2′ (δ 78.41), C-3′ (δ 129.47) and C-5′ (δ 40.84), and of H_2-5′ (δ 1.76)/C-2′ (δ 78.41) (Fig. 2). This HMBC data suggests attachment of the 2-methyl pentenyl unit at the C-2′ of the (2*H*)-pyran ring.

The signals for one aryl methyl group at δ 2.32 (s, H_3-3a), one AX-type of aromatic protons [δ 7.98 (s, H-5) and 6.83 (s, H-8)], one aromatic proton singlet at δ 7.57 (s, H-4), a formyl proton at δ 9.89 (s, H-6a), a phenolic proton at δ 11.42, and one amine proton at δ 8.14 indicated the presence of a carbazole alkaloid skeleton, with formyl (–CHO) and –OH groups, in compound **3**. The methyl group substitution at C-3 was inferred from the HMBC interactions of H-4 (δ 7.57) with C-3a, C-2, C-9a, and C-4a and of H_3-3a with C-2, C-3 and C-4. This was further confirmed from the NOESY correlations of H-4 and H_3-3a (Fig. 3). The HMBC correlations between –OH and C-6, C-7 and C-8 suggest the presence of a –OH group at C-7. The presence of a formyl group at position C-6 was confirmed from the HMBC correlations of H-6a with C-7, and C-8 and of H-5 with C-6a, C-7, C-8a, and C-6. This was further confirmed from the NOESY correlations of H-6a with H-5 and –OH (Fig. 3). From the above data it was inferred that compound **3** has a 7-hydroxy-3-methyl-9*H*-carbazole-6-carbaldehyde unit. Finally, the HMBC correlations of H-4′ with C-2′, C-1, C-2 and C-9a show that the (2*H*)-pyran ring is fused to the 7-hydroxy-3-methyl-9*H*-carbazole-6-carbaldehyde unit at C1–C2. The CD curve of **3** displayed a positive Cotton effect in the 265–276 nm region (Fig. S33, ESI†), so the absolute configuration of **3** at C-2′ was determined as *S*.[33,34] On the basis of this spectroscopic evidence, the new compound **3** was assigned as 9-hydroxy-3,5-dimethyl-3-(4-methylpent-3-en-1-yl)-3,11-dihydropyrano-[3,2-*a*]carbazole-8-carbaldehyde, and it was designated as murrayakonine C (**3**).

Compound **4** was isolated as a bluish gum, and its molecular formula was established as $C_{23}H_{25}NO_2$ from the HR-ESI-MS ion peak at 348.1956 $[M + H]^+$, indicating a hydrogen deficiency index of 12. The UV spectrum of **4** showed absorptions at λ_{max} values of 304, 238 and 214 nm, which are typical for an angular pyrano-carbazole moiety. The IR spectrum displayed an intense absorption band at 3436 cm^{-1} indicating the presence of a –NH group. The ^{13}C NMR showed resonances for 22 carbons, but using a combination of ^{13}C NMR, HSQC and HMBC correlations along with the mass (HR-ESI-MS) data of **4** suggested an overlapped resonance for one carbon at δ_c 123.9 [C-4b with C-7]. Data analysis further revealed the presence of 23 carbons comprising four methyl, two methylene, eight methine and nine quaternary carbons.

For the ^{1}H NMR spectrum, the signals for one aryl methyl group at δ 2.25 (s, H_3-3a), one aromatic proton singlet at δ 7.67 (s, H-4), one 1,2-disubstituted benzene ring at δ 7.85 (d, 7.7 Hz, H-5), 7.09 (t, J = 7.4 Hz, H-6), 7.22 (d, 8.0 Hz, H-7), and 7.31 (d, 8.0 Hz, H-8), and one amine proton at δ 8.21 indicated the presence of a 3-methyl-2-oxygenated-1-alkyl carbazole unit. COSY correlations between H-3′ and H-4′ and between H-6′ and H-7′ indicated the presence of two spin systems (Fig. 2). The HMBC correlations of H-3′/C-2′, of H-4′/C-1, C-2, C-9a, and C-2′, and of H-2′a/ C-2′, C-3′ clearly indicated that the first spin system is attached (at C4′–C1) to the 3-methyl-2-oxygenated-1-alkyl carbazole unit, forming a pyran ring (1 ↔ 2 ↔ 1′ ↔ 2′ ↔ 3′ ↔ 4′). The HMBC correlations of H-9′/C-3′, C-7′, C-8′ and C-10′, of H-10′/C-3′, C-7′, C-8′ and C-9′, and of H-3′/C-7′ indicated that the C-3′ and C-7′ are linked through quaternary carbon C-8′ (δ 42.73). Furthermore, the HMBC correlations of H-5′/C-2′, C-6′ and C-7′, and of H-2′a/C-2′, C-3′ and C-5′ indicated that C-6′ is linked to C-2′ through methylene carbon C-5′ (δ 40.84), forming a fused six-membered ring. Even though no HMBC correlations were found for H-4′ to C-7′ and H-7′ to C-4′, an ether linkage was proposed in order to satisfy the ring residues and mass of the compound **4**. The relative configuration of **4** was determined as 2′*S**, 3′*S**, 4′*R**, 7′*R** using the NOESY cross peaks of H-2′a/H-3′, H-7′ and H-9′, and of H-3′/H-4′ (Fig. 3). From the above experimental data, the new compound **4** was deduced to be (2*R*,4a*S*,13*R*,13a*S*)-1,1,4a,6-tetramethyl-1,2,3,4,4a,12,13,13a-octahydro-2,13-epoxychromeno[3,2-*a*]-carbazole, and designated as murrayakonine D (**4**).

The known carbazole alkaloids murrayazolidine (**5**),[35] murrayazolinine (**6**),[35] murrayamine J (**7**),[36,37] mahanimbine (**8**),[35] bicyclomahanimbine (**9**)[35] mahanimbinine (**10**),[38] currayangine (**11**),[35] girinimbine (**12**),[39–42] *O*-methylmurrayamine-A (**13**),[33,40,43] koenimbine (**14**),[43–45] koenigicine (**15**),[45,46] mukonicine (**16**),[45,47] 2-methoxy-3-methyl-9*H*-carbazole (**17**),[48,49] murrayanine (**18**),[50] 1-hydroxy-7-methoxy-8-(3-methylbut-2-en-1-yl)-9*H*-carbazole-3-carbaldehyde (**19**),[51] 8,8″-biskoenigine (**20**),[52,53] murrayamine C (**21**)[54] and clauraila A (**22**)[55,56] were characterized by comparing their NMR spectral data with the literature data.

Anti-inflammatory activity

The release of pro-inflammatory cytokines is an important mechanism by which immune cells regulate the inflammatory response and contribute to various inflammatory and autoimmune disorders. All the isolated compounds were assayed for their ability to decrease lipopolysaccharide (LPS) induced TNF-α and IL-6 production in human peripheral blood mononuclear cells (PBMCs).[57–59] LPS, a component of the Gram-negative bacterial cell wall, is one of the major causative agents of Gram-negative sepsis. LPS is a potent inducer of monocytes and macrophages, which are key mediators of the innate immune response. Stimulation of cells with LPS leads to a cascade of intracellular signalling events that ultimately result in the production and secretion of cytokines and other inflammatory mediators that constitute the pro-inflammatory response. The potential anti-inflammatory properties of the compounds were evaluated using PBMCs because this selected macrophage model produces high concentrations of IL-6 and TNF-α in culture upon activation with LPS, and reduces the need for the additional animals required to obtain and use primary cultures. In the *in vitro* experiments, incubation of the compounds with lipopolysaccharide (LPS) for 5 h showed a considerable inhibition of TNF-α and IL-6 production over three concentrations (10, 5 and 1 μM), when compared to LPS-induced control cells and a rolipram standard (Table 2). The compounds **1**, **5**, **12**, **13**, **15**, **20**, **19** and **18** (Table 2) showed significant inhibitory effects, having a % inhibition of >40 at 10 μM concentration, against both TNF-α and IL-6. The compounds **2**, **7**, **8**, **11**, **14** and **17** exhibited very poor % inhibition, whereas for **3**, **4**, **6**, **9**, **10** and **16** inhibition activity was not detected (nd) against both TNF-α and IL-6. In the *in vitro* experiments, compounds **1** (IC_{50} 10 μM), **13** (IC_{50} 9.4 μM) and

Table 2 *In vitro* cytokine (TNF-α and IL-6) inhibition assay results for the isolated compounds

	In vitro cytokine assay					
	TNF-α (%age inhibition)			IL-6 (%age inhibition)		
Compound code	10 μM	5 μM	1 μM	10 μM	5 μM	1 μM
1	50.7 ± 2.2	25.8 ± 5.4	4.9 ± 1.4	48.6 ± 2.0	27.8 ± 3.0	12.3 ± 1.4
2	37.8 ± 1.6	15.8 ± 2.5	7.6 ± 1.8	34.9 ± 3.0	21.1 ± 7.0	11.6 ± 3.0
5	44.3 ± 2.4	19.4 ± 9.5	4.2 ± 0.9	40.9 ± 1.1	26.0 ± 3.1	13.5 ± 3.1
7	33.1 ± 0.4	10.1 ± 0.7	nd	23.4 ± 2.7	8.6 ± 0.9	4.3 ± 1.8
8	30.1 ± 0.6	8.8 ± 2.9	nd	21.7 ± 1.6	11.5 ± 5.2	4.4 ± 1.1
11	21.2 ± 0.4	11.9 ± 1.1	nd	14.9 ± 2.0	7.6 ± 1.2	5.2 ± 1.2
12	41.5 ± 0.6	16.5 ± 4.0	nd	48.7 ± 1.4	17.5 ± 2.2	10.7 ± 2.9
13	53.9 ± 6.4	30.4 ± 5.4	8.0 ± 1.9	59.5 ± 0.6	18.9 ± 3.3	5.0 ± 2.2
14	28.3 ± 2.1	10.2 ± 1.7	4.4 ± 3.1	25.2 ± 1.9	6.7 ± 1.9	4.5 ± 1.6
15	40.9 ± 1.7	18.2 ± 4.2	3.8 ± 2.0	28.6 ± 0.6	18.2 ± 1.6	4.8 ± 3.6
17	31.1 ± 0.6	12.1 ± 1.1	1.8 ± 1.4	30.2 ± 0.6	8.4 ± 1.8	5.1 ± 0.5
18	66.0 ± 3.9	20.7 ± 1.7	6.2 ± 4.2	59.4 ± 4.0	26.7 ± 2.6	15.1 ± 5.2
19	43.2 ± 5.4	28.2 ± 3.0	nd	36.1 ± 1.3	24.6 ± 3.0	11.6 ± 0.6
20	48.9 ± 1.0	22.2 ± 2.4	9.3 ± 2.7	32.6 ± 2.0	15.4 ± 5.6	2.1 ± 1.3
Rolipram (0.3 μM)	77			70		

Table 3 *In vivo* cytokine (TNF-α and IL-6) inhibition assay with the compounds

	In vivo cytokine assay					
	TNF-α (% age inhibition)			IL-6 (% age inhibition)		
Compound code	10 mg kg^{-1}	5 mg kg^{-1}	1 mg kg^{-1}	10 mg kg^{-1}	5 mg kg^{-1}	1 mg kg^{-1}
1	51.8 ± 2.4	10.8 ± 1.3	1.9 ± 0.2	31.3 ± 9.9	11.0 ± 4.8	3.9 ± 2.5
2	46.7 ± 1.2	25.7 ± 7.9	8.7 ± 1.1	39.8 ± 2.1	26.3 ± 7.1	8.1 ± 1.3
5	49.7 ± 1.5	8.6 ± 5.1	2.0 ± 1.4	48.7 ± 3.8	21.5 ± 4.9	6.0 ± 3.0
7	34.3 ± 2.5	13.7 ± 2.9	7.0 ± 2.2	28.0 ± 5.0	11.6 ± 1.8	5.6 ± 1.9
8	40.4 ± 8.4	8.6 ± 1.7	3.7 ± 0.5	35.9 ± 2.9	11.1 ± 5.4	4.5 ± 1.6
11	20.5 ± 1.1	11.8 ± 2.2	4.7 ± 0.9	11.9 ± 3.8	5.7 ± 2.6	nd
12	38.7 ± 4.4	20.1 ± 6.4	5.4 ± 0.3	35.6 ± 2.1	10.9 ± 1.9	3.5 ± 2.0
13	58.9 ± 3.8	11.4 ± 1.1	2.6 ± 0.4	61.7 ± 9.9	15.4 ± 2.8	9.0 ± 2.3
14	30.1 ± 2.8	6.8 ± 2.4	3.9 ± 1.6	19.0 ± 5.5	8.4 ± 1.6	2.9 ± 1.8
15	32.7 ± 1.5	8.6 ± 5.1	2.0 ± 1.4	30.6 ± 0.8	23.4 ± 5.5	7.0 ± 3.3
17	34.7 ± 3.9	20.1 ± 5.4	10.4 ± 4.4	27.7 ± 1.7	13.3 ± 2.9	4.6 ± 0.2
18	65.3 ± 2.6	6.0 ± 5.0	3.0 ± 1.2	69.5 ± 1.8	22.4 ± 10.9	2.2 ± 1.1
19	40.6 ± 1.6	17.6 ± 8.4	7.9 ± 3.9	39.5 ± 6.4	25.5 ± 14.6	6.3 ± 4.1
20	52.8 ± 2.2	17.3 ± 4.7	2.9 ± 1.3	46.8 ± 9.8	27.6 ± 4.4	6.1 ± 1.6
Rolipram (1 mg kg^{-1})	70			68		

18 (IC_{50} 7 μM) against TNF-α, and compounds **13** (IC_{50} 8.4 μM) and **18** (IC_{50} 8.4 μM) against IL-6 were proven the most potent among all the isolated compounds (Table 2).

For all the active compounds from the above *in vitro* analysis, an *in vivo* evaluation was performed with mice to determine their ability to replicate the inhibition results in animal models (Table 3). Compounds **1** (51.8%), **13** (58.9%), **18** (65.5%) and **20** (52.8%) against TNF-α, and compounds **13** (61.7%) and **18** (69.5%) against IL-6 were proven the most potent among all the isolated compounds. In summary, the compounds **13** and **18** were inhibiting both TNF-α and IL-6; whereas, the new compound **1** is a specific inhibitor for TNF-α. The cytokines IL-6 and TNF-α were evaluated because these pro-inflammatory cytokines play a key role in the inflammatory response and can be easily quantified in supernatant and serum samples. The levels of IL-6 and TNF-α were not detectable for untreated cells and for animals that served as a control *in vitro* and *in vivo* respectively. Cell culture data does not always allow accurate prediction of the results using an animal model. Thus, the compounds under observation were studied *in vivo*. This was supported by the finding that there is considerable variation between the *in vitro* and *in vivo* results. Discrepancies between the cell culture data and animal data are not entirely surprising considering only one cell type exists in the culture studies, whereas multiple cell types capable of producing pro-inflammatory cytokines are present in a whole animal system. Additionally, other inflammatory mediators may influence the cytokine response.

Antimicrobial activity

Furthermore, all the isolated compounds were evaluated for their antimicrobial activities against a panel of important bacterial pathogens (Ciprofloxacin was used as a standard) and the fungal pathogen *Candida albicans* (Nystatin was used as a standard) (Table 4).

Table 4 Anti-microbial activity of the isolated compounds

	IC_{50} in μM			
Comp. code	S.A	B.C	E.C	C.A
1	—	49.9	—	—
2	—	—	—	—
3	—	—	—	—
4	—	—	—	—
5	—	—	—	—
6	30.5	—	—	—
7	11.7	23.2	—	—
8	22.4	—	—	—
9	—	27.5	—	—
10	—	—	—	—
11	—	—	—	—
12	—	3.4	—	—
13	—	—	—	—
14	17.0	22.5	—	—
15	20.9	41.4	—	—
16	95.56	91.9	—	—
17	—	24.1	—	—
18	—	—	—	—
19	95	10.9	—	—
20	—	—	—	—
21	—	—	—	—
22	—	—	—	—
Ciprofloxacin/Nystatin	0.27	0.12	1.6	1.6

S.A = *Staphylococcus aureus* ATCC 29213; B.C = *Bacillus cereus* IIIM 25; E.C = *Escherichia coli* ATCC 25922; C.A = *Candida albicans* MTCC 4748; — = IC_{50} > 100 μM.

Among these results, compounds **2**, **3**, **4**, **5**, **6**, **10**, **11**, **13**, **18**, **20**, **21** and **22** did not show any significant antimicrobial activity up to a concentration of 100 μM, whereas, the rest of the compounds showed significant activity against *B. cereus* and *S. aureus*. Compounds **7**, **9**, **12**, **14**, **17** and **19** showed IC_{50} values of 23.2, 27.5, 3.4, 22.5, 24.1 and 10.9 μM respectively against *B. cereus*. Furthermore, compounds **7**, **8**, **14** and **15** were active against *S. aureus* showing IC_{50} values of 11.7, 22.4, 17.0 and 20.9 μM, respectively. However, none of the

compounds were active against the Gram negative bacterium, *E. coli*, or the fungal pathogen *C. albicans*. This study revealed that girinimbine (**12**) is the most potent compound among all the isolated compounds, and that it specifically inhibited *B. cereus* with an IC_{50} value of 3.4 μM.

Conclusions

In summary, herein we have reported four new, murrayakonines A–B from the stem and C–D from the leaves (**1–4**), along with 18 known carbazole alkaloids (**5–22**), which were isolated from *Murraya koenigii*. All these compounds were evaluated for their anti-inflammatory and antimicrobial activities. Compounds **1**, **13** and **18** efficiently inhibited the release of pro-inflammatory cytokines TNF-α and IL-6, and decreased the LPS induced TNF-α and IL-6 production in human PBMCs, in both *in vitro* and *in vivo* experiments. Additionally, compounds **12** and **19** exhibited prominent antimicrobial activities, with an IC_{50} value of 3.4 and 10.9 μM respectively, against *Bacillus cereus*. Furthermore, compounds **7**, **14** and **15** were active against *Staphylococcus aureus* showing IC_{50} values of 11.7, 17.0 and 20.9 μM respectively.

Experimental

General experimental procedures

High resolution mass spectra were obtained using an Agilent 6540 (Q-TOF) high resolution mass spectrometer, in the electrospray (ESI-MS) mode. The ^{1}H NMR spectra were recorded (Bruker Avance) at 400 and 500 MHz, and the ^{13}C NMR spectra were recorded at 100 and 125 MHz in $CDCl_3$. Chemical shifts values are reported in δ (ppm) units and the coupling constant values are reported in Hertz. Tetramethylsilane (TMS) was used as an internal standard. Infra-red spectra were recorded with a Perkin Elmer 65 FT-IR spectrometer and the wavelengths (λ) are given in cm^{-1}. CD spectra were recorded using a JASCO J-810 CD spectrometer at a concentration of 1.0×10^{-4} M in MeOH at 25 °C. CD spectra were measured between 200 and 600 nm using 10 mm path-length quartz cuvettes. Column chromatography was performed using silica gel (100–200, 230–400 mesh; Merck). Semi-preparative HPLC was performed using an Agilent HPLC with an Eclipse XDB-C-18; 5 μm, 250 × 9.4 mm column, a photodiode array detector and an auto-injector function (Agilent 1260 series). All the solvents were purchased from Merck. Rolipram was purchased from Sigma Chemical Co. (St. Louis, MO) and the lipopolysaccharide (LPS) (*E. coli*) from (Callbiochem, USA). Ficoll–Hypaque and Phosphate Buffer Saline (PBS) were bought from Sigma-Aldrich. Anticoagulant tubes were purchased from BD Biosciences. A Human TNF-α ELISA kit and Human IL-6 ELISA kits were purchased from Invitrogen, USA. A Mouse TNF-α ELISA kit was bought from Invitrogen, USA and the Mouse IL-6 ELISA kit was from Invitrogen, USA. All the other reagents used were of analytical grade.

Plant material

The stem of *Murraya koenigii* was collected from the Botanical garden of the Indian Institute of Integrative Medicine, Jammu (India), in April 2013. The plant was identified by a taxonomist Dr Bikarma Singh, and a voucher specimen (RRLH 22909) was deposited at the Herbarium of the IIIM, Jammu (India).

Extraction and isolation

From the stem. Air-dried *M. koenigii* stems (2.0 kg) were extracted with DCM : MeOH (1 : 1) over a period of 4 days at room temperature and the solvent evaporated under reduced pressure to provide the crude extract (62.5 g). This extract was subjected to column chromatography using silica gel (100–200 mesh) and eluted by increasing the polarity of the elution solvent system of hexanes and EtOAc (100% hexanes to 100% EtOAc) to afford five fractions (MK-I–V) based on their analytical TLC data. Fraction I (16.3 g) was further subjected to column chromatography over silica gel and was eluted with 5% EtOAc–hexanes to give pure compounds **14** (10 gm), **8** (0.4 gm), **12** (0.5 gm) and **9** (24.5 mg). Fraction MK-II (6.5 g) was subjected to column chromatography with flash silica gel (230–400 mesh) using 7% EtOAc–hexanes to afford crystalline compound **18** (4 gm) and **19** (25 mg). Fractions MK-III–IV exhibited similar chromatograms, so they were pooled together (350 mg) and chromatographed over silica gel (230–400 mesh) with 15% EtOAc–hexanes to yield compounds **17** (85 mg) and **1** (8 mg). Fraction MK-V was also re-chromatographed with silica gel using 20% CH_2Cl_2–hexanes to yield compounds **11** (1.0 gm) and **5** (10 mg), and a mixture (130 mg) which was further separated using semi-preparative HPLC with 100% acetonitrile as the mobile phase (30 min, flow rate of 1 ml min^{-1} with an Eclipse XDB-C-18; 5 μm, 250 × 9.4 mm column) to afford compounds **2** (5 mg, t_R = 15.3 min) and **7** (6 mg, t_R = 18.1 min).

From the leaves. Ground leaves of *Murraya koenigii* (2 Kg) were extracted with $CHCl_3$: MeOH (1 : 1) at room temperature to yield 90 g of crude extract. The extract was subjected to fractionation in an open column using silica (100–200 mesh) gel as the solid phase and a gradient solvent system with hexane–ethyl acetate of 9 : 1, 7 : 3, 1 : 1, and 100% ethyl acetate, resulting in five fractions (Fr.1–Fr.5). Fr.1 was subjected to silica (200–400 mesh) gel column chromatography and eluted with hexane–ethyl acetate (from 10 : 0 to 9 : 1) to give **11** (15 mg), **5** (23 mg), **8** (5 g), **12** (3 g) and **13** (2 g). Fr.2 was separated into five subfractions, Fr.2.1–Fr.2.5, using Sephadex LH-20 column chromatography and eluting with MeOH. Subfraction Fr.2.5 was separated using semi-preparative HPLC (Eclipse XDB-C-18; 5 μm, 250 × 9.4 mm; isocratic 100% ACN, 30 min; 1 mL min^{-1}) to give compounds **13** (9 mg, t_R 14.5 min), **14** (20 mg, t_R 15.2 min), **6** (35 mg, t_R 20.07 min) and **3** (4 mg, t_R 25.03 min). Fraction Fr.3 was fractionated using a silica column eluting with $CHCl_3$–MeOH (10 : 0 to 10 : 1, v/v) to give five subtractions (Fr.3.1–Fr.3.5). Fraction Fr.3.4 was purified using preparative TLC (9 : 1 $CHCl_3$: MeOH) to afford compounds **4** (5 mg) and **22** (12 mg). The fraction Fr.4 was divided into three subfractions

(Fr.4.1 to Fr.4.3) using silica gel column chromatography (hexane/EtOH from 8 : 2 to 1 : 9 and then a final elution with EtOAc). Compound **16** (35 mg) was purified from Fr 4.1 using preparative TLC ($CHCl_3$: MeOH, 9 : 1). Purification of the fraction Fr.4.2 using silica gel column chromatography (hexane/EtOAc, from 7 : 3 to 2 : 8) yielded **20** (300 mg), **15** (20 mg) and **10** (5 mg). The third subfraction Fr.4.3 was subjected to semi-preparative HPLC with an isocratic solvent of CH_3CN (Eclipse XDB-C-18; 5 μm, 250 × 9.4 mm; 100% ACN, 15 min; 1 mL min^{-1}) furnishing compounds **20** (50 mg, t_R 6.28 min), **7** (10 mg, t_R 6.71 min), **21** (11 mg, t_R 7.98 min) and **22** (9 mg, t_R 8.66 min).

*Murrayakonine A (**1**).* Pale-yellow amorphous powder; for 1D and 2D NMR data, see Table 1 and Fig. S1–S13 in the ESI;† IR (neat), ν_{max} 3436.2 (–NH), 1615 and 1612 cm^{-1} (ar. system); U. V. spectrum, λ_{max} (acetonitrile) 226.0, 252.0 and 282.0 mAU (Fig. S13†); (+)-HR-ESI-MS ion peak at *m*/*z* 541.2847 $[M + H]^+$ (calcd for $C_{37}H_{37}N_2O_2$, 541.2850).

*Murrayakonine B (**2**).* Colourless oil; for 1D and 2D NMR data, see Table 1 and Fig. S14–S21 in the ESI;† IR (neat), ν_{max} 3369 (–NH), 2827 (H–CO–), 1674 (–HC═O), 1608 and 1583 cm^{-1} (ar. system); U.V. spectrum, λ_{max} (acetonitrile) 240.0 and 294.0 mAU (Fig. S21†); (+)-HR-ESI-MS ion peak at *m*/*z* 346.1805 $[M + H]^+$ (calcd for $C_{23}H_{24}NO_2$, 346.1802).

*Murrayakonine C (**3**).* Greenish gum; for 1D and 2D NMR data, see Table 1 and Fig. S24–S33;† IR (neat), ν_{max} 3345 (–NH/–OH), 2827 (H–CO–), 1705 (–HC═O), 1614 and 1565 cm^{-1} (ar. system); U.V. spectrum, λ_{max} (acetonitrile) 246.0, 268.0 and 320.0 mAU (Fig. S30†); (+)-HR-ESI-MS *m*/*z* 376.1909 $[M + H]^+$ (calcd 376.1913).

*Murrayakonine D (**4**).* Bluish gum; for 1D and 2D NMR data, see Table 1 and Fig. S34–S42;† IR (neat), ν_{max} 3434 (–NH), 1624 and 1608 cm^{-1} (ar. system); U.V. spectrum, λ_{max} (acetonitrile) 214.0, 238.0 and 304.0 mAU (Fig. S39†); (+)-HR-ESI-MS *m*/*z* 348.1956 $[M + H]^+$ (calcd *m*/*z* 348.1958).

In vitro anti-inflammatory assay

Isolation of the PBMCs. Healthy volunteers of both sexes (22-35 years old) served as blood donors. Blood was withdrawn under authorized supervision from the subjects who had given their consent. The CSIR-Indian Institute of Integrative Medicine (IIIM), institutional bio-safety guidelines were followed for collection of the blood samples from the volunteers. Heparinised venous blood was taken from the healthy human volunteers. The mononuclear cells were isolated using a Ficoll–Hypaque density gradient using standard procedures, which separated the PBMCs from the whole blood. The buffy coat containing the PBMCs was removed carefully following centrifugation and washed twice using Phosphate Buffer Saline (PBS). The cells were counted and the samples were incubated with 1 × 10^6 cells per well in triplicate in 96 well microtiter plates. All the compounds (**1–22**) were tested to check their anti-inflammatory activity against TNF-α and IL-6 at three different concentrations. The anti-inflammatory activities against TNF-α and IL-6 were analyzed using the percentage inhibition of the production of these cytokines when the human peripheral blood mononuclear cells (PBMCs) were treated with lipopolysaccharide (LPS). A dose response study was conducted using LPS (mitogen) (1 μg ml^{-1}) induced PBMCs with different concentrations *i.e.* 10, 5 and 1 μM of the compounds. The results were expressed in % inhibition with respect to the control. The experiments were done in triplicate and repeated three times. The anti-inflammatory effects shown in the compounds' dose response assays were used to determine the capacity of inhibition for these compounds with respect to production of the pro-inflammatory cytokines TNF-α and IL-6. The data represents the mean ± SEM of the representative compounds for the inhibition of TNF-α and IL-6 production, tested in three different assays.

***In vivo* anti-inflammatory assay.** Female Balb/C mice (Mus musculus) that were 8–10 weeks old and weighed 20–25 g, in groups of six, were used for the study. The experiments were designed to minimize the numbers of animals used and all the experiments were performed after approval (no. IIIM.IAEC.65/62/8/15) from the CSIR-Indian Institute of Integrative Medicine (IIIM) institutional animal ethics committee. The animals were housed under the standard laboratory conditions of 23 ± 1 °C, 55 ± 10% relative humidity and 12 h/12 h light/dark cycles, were fed with a standard pellet diet (Lipton India Ltd) and received water *ad libitum*. None of the animals were sacrificed during the study; however, as per the institute's normal procedure, the animals, after experimentation, were subjected to euthanasia using a high inhalational dose of diethyl ether and disposed off by incineration. The mice were orally treated with the test compound at different concentrations for 1 h. Then *via* i.p. administration 0.5 mg kg^{-1} LPS was given. After 2.5 h, blood was obtained from the retro-orbital plexus and allowed to clot for 30 minutes at R.T. Serum was collected and stored at 80 °C until use in an ELISA.[60] Then it was analyzed for IL-6 and TNF-α using an ELISA. The cytokine analysis for IL-6 and TNF-α was performed using mouse TNF-α ELISA and mouse IL-6 ELISA kits purchased from Invitrogen, USA. The absorbance was read at 450 nm. The results were expressed as % inhibition with respect to the control (rolipram). The experiments were done in triplicate and repeated three times. The results are expressed as the mean ± SEM.

Determination of the antimicrobial activities

The compounds isolated from *Murraya koenigii* were evaluated for their antimicrobial activities against a panel of pathogens. Stock solutions of each culture were prepared using a normal saline solution (0.85% NaCl (w/v)) at a concentration of 10^8 cells/spores per ml. The method used for bacteria by Mueller-Hinton and PD broth for fungus were supplemented with the compounds at concentrations of 100, 50, 25, 12.5 and 6.25 μg ml^{-1} dissolved in DMSO to obtain a stock solution of 10 μg ml^{-1}. Each well was inoculated with 10^4 cells/spores of the relevant test organism and incubated at 37 °C for 24 h. The absorbance was measured by spectrophotometer at 620 nm and the IC_{50} of the isolated compounds was calculated from the average percent inhibition of three replicates of each concentration.[61]

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