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LC-MS/MS Molecular Networking-based Chemical Profiling and Bioactivity-Guided Isolation of Polymethoxyflavonoids from *Gardenia sokotensis*

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Abstract

Gardenia sokotensis is a medicinal plant traditionally used in West Africa for the treatment of several ailments, including malaria. While its biological activity has previously been reported, its chemical composition remains poorly investigated. This study aims to characterize the chemical composition of the antiplasmodial and antileishmanial ethyl acetate leaf extract of *G. sokotensis* through an integrated approach combinng chemical profiling and bio-guided fractionation. The extract was profiled by LC-MS/MS and molecular networking, fractionated on silica gel into eighteen fractions (**3af1–3af18**), which were all evaluated for antiplasmodial and antileishmanial activities. Selected fractions were then purified by preparative HPLC. LC-MS/MS profiling and molecular networking revealed a chemical profile dominated by triterpenoid and polymethoxyflavonoid derivatives. Several fractions (**3af7–3af15**) inhibited *P. falciparum* growth

by more than 70% at 10 µg/mL, and purification of selected fractions led to the isolation and structural identification of three polymethoxyflavonoids: 3,5,4′-trihydroxy-6,7,8-trimethoxyflavone (**19**), 3′,4′,5-trihydroxy-6,7,8-trimethoxyflavone (**24**), and 5,6-dihydroxy-7,8,4′-trimethoxyflavone (**25**), all identified for the first time from *G. sokotensis*. This study expands the limited phytochemical knowledge of *G. sokotensis* and illustrates how combining LC-MS/MS profiling with bio-guided fractionation can support the chemical characterization of a complex medicinal plant extract. Further work is needed to determine the individual contribution of the isolated compounds to the observed antiplasmodial activity.

Keywords: *Gardenia sokotensis*; molecular networking; bio-guided fractionation; polymethoxyflavonoids; triterpenoids; antiplasmodial activity; antileishmanial activity

Introduction

The chemical complexity of medicinal plant extracts, together with the structural diversity of their metabolites, complicates the discovery of bioactive natural products. Traditional bioassay-guided fractionation remains an effective approach for discovering bioactive constituents. However, its iterative separation and bioassay steps can be laborious and time-consuming; moreover, activity may be lost during fractionation when synergistic or additive interactions between constituents are disrupted. In addition, when applied to extensively studied plant families, bioassay-guided fractionation can lead to the re-isolation of already well-characterized compounds, whose biological activity is often already documented or could be assessed more directly. These limitations have stimulated the development of dereplication and metabolomics-based approaches to reduce the chemical complexity of crude extracts and to assess the relevance of subsequent isolation efforts.

LC-MS/MS-based molecular networking has emerged as a powerful tool for the exploration of complex natural product mixtures. By grouping MS/MS spectra according to fragmentation similarities, molecular networking enables the visualization of chemical families and facilitates metabolite annotation and dereplication. However, MS-based dereplication alone does not necessarily identify the metabolites responsible for the biological activity of a complex extract, particularly when compounds remain unannotated because of limited reference spectral data. In such cases, combining chemical profiling with classical bio-guided fractionation can be a relevant strategy to both obtain a preliminary overview of the metabolite content of the extract and characterize constituents of its active fractions.

Gardenia sokotensis Hutch. (Rubiaceae) provides a suitable case study for applying such an integrated workflow. This indigenous plant of the

Sudano-Sahelian savannas of West Africa is traditionally used in Niger, where its leaves are prepared as a decoction for the treatment of malaria, diarrhoea and haemorrhoids, and are also valued for their analgesic properties. The stem bark is used in traditional preparations as a tonic and for the treatment of jaundice (Jazy et al., 2017). Several pharmacological studies have reported biological activities of *G. sokotensis*. Dichloromethane and methanolic leaf extracts showed *in vitro* antiplasmodial activity against *Plasmodium falciparum* 3D7, with IC_{50} values of 14.01 µg/mL and 27.62 µg/mL, respectively (Jansen et al., 2010). A dichloromethane-methanol (7:3, v/v) leaf extract also inhibited the growth of *Plasmodium berghei in vivo* with an ED_{50} of 115 mg/kg (Traoré et al., 2006). In addition, an ethanolic leaf extract exhibited cytotoxic activity against HCT116 human colon cancer cells, with an IC_{50} of 7.5 µg/mL after 48 h of incubation (Fornari et al., 2014), while an acute toxicity study of a root extract reported an LD_{50} of 2400 mg/kg body weight following oral administration in rabbits (Jodi et al., 2008). Ouedraogo et al. (2025) recently assessed the safety of the aqueous leaf extract of *G. sokotensis*. A single oral dose of 2000 mg/kg caused no mortality or apparent signs of toxicity in mice over 14 days. In the 90-day subchronic study in rats, the authors reported no toxicity-related changes at 100 and 200 mg/kg, whereas the significant increase in serum ALT and AST at 400 mg/kg, which was no longer significant after a 14-day recovery period.

In our previous study, the ethyl acetate leaf extract of *G. sokotensis* showed *in vitro* antiplasmodial and antileishmanial activities, with IC_{50} values of 4.3 ± 0.5 µg/mL against *Plasmodium falciparum* NF54, and 4.3 ± 2.6 µg/mL against *Leishmania donovani* MHOM/ET/67/L82, respectively (Damé et al., 2025). However, the extract showed no selectivity between the parasites and L6 mammalian cells. Despite these biological findings, the chemical composition of *G. sokotensis* remains poorly documented. Previous phytochemical investigations of different plant organs and extracts have reported broad classes of metabolites, including triterpenes, flavonoids, tannins, saponins and alkaloids (Traoré et al., 2006; Jodi et al., 2008; Sama et al., 2022; Ouedraogo et al., 2025). Information on the individual constituents of *G. sokotensis* remains limited. Sama et al. (2022) detected rutin by TLC in a leaf decoction, and Nadembega (2010) tentatively identified chlorogenic acid and rutin in a lyophilized leaf decoction by HPTLC and HPLC, by comparison with reference standards. After hydrolysis, a peak corresponding to quercetin was observed, while the ^{1}H NMR profile showed sugar signals and aromatic resonances tentatively attributed to chlorogenic acid and flavonoids. From the dichloromethane extract, Jansen (2013) isolated four terpenes by bio-guided fractionation, including a new 3,4-seco-cycloartane

triterpene, sokotensine A. To our knowledge, polymethoxylated flavonoids have not previously been reported from this species.

In this study, the ethyl acetate leaf extract of *G. sokotensis* was first profiled by LC-MS/MS and molecular networking to group its metabolites into clusters, visualize their relative peak areas, and attempt their annotation and dereplication. The extract was then fractionated by normal-phase open column chromatography, and the resulting fractions were evaluated for antiplasmodial and antileishmanial activities. Three fractions were subsequently purified to characterize potentially bioactive compounds.

Methods

Plant material

Leaves of *Gardenia sokotensis* Hutch. were purchased at Harobanda market (13°29′31.0″N 2°05′51.2″E), Niamey, Niger, in August 2022. The plant was identified by Arzika Tanimoune (Garba Mounkaila Laboratory, Abdou Moumouni University), and a voucher specimen (n° 219) is available at the Herbarium of the Biological Department, Abdou Moumouni University. Details of the plant material preparation and of the HPLC-PDA-HRMS/MS data acquisition for the extract are available in the previous publication (Damé et al., 2025).

MS/MS data treatment

The HRMS data file (.d) obtained in negative mode was converted to an .mzML file using MSConvert 3.0 software and then processed using MZmine 2.53 free software (Pluskal et al., 2010). The noise level was set at 1.5×10^2 and 10 for MS1 and MS2 mass detection, respectively. The ADAP chromatogram generator was used with a minimum group size of 5 scans, a group intensity threshold of 1.0×10^3, the highest minimum intensity of 1.0×10^3 and an m/z tolerance of 10 ppm. The S/N intensity window was used as the S/N estimator to deconvolve the chromatogram by the ADAP wavelet algorithm with a signal-to-noise ratio set at 15, a minimum feature of 1.0×10^3, a peak duration ranging from 0.02 to 0.6 min, a coefficient threshold at 10, and an RT wavelet range of 0.01-0.2 min. "Isotope peak grouper was used to detect peaks forming an isotopic pattern and to eliminate additional minor isotopic peaks using an *m*/*z* tolerance of 10 ppm, an RT tolerance of 0.5 min (absolute), and a maximum load set at 1. The most intense isotope was selected as the representative isotope. The resulting mass list was filtered using the ‘duplicate peak filter’ and ‘feature list rows filter’ to eliminate all duplicates and retain only the MS/MS scans..

Molecular network creation

After data processing, the .mgf file obtained from MZmine was exported to the GNPS website to build a molecular network. The precursor ion mass tolerance was set at 2.0 Da with an MS/MS fragmentation tolerance of 0.5 Da. The network was created by setting the edges to have a cosine value of 0.70 and more than 3 common peaks.

Annotation and dereplication

The network spectra were searched against the GNPS spectral libraries, under the same conditions. Additional identification of unmatched peaks was performed by comparing MS/MS fragmentation models available in the literature (http://www.reaxys.com/ ; https://lotus.naturalproducts.net/ ; https://coconut.naturalproducts.net). Data were visualised using Cytoscape 3.8.0 software. Peak area data were added to the network from the .csv file obtained from MZmine. The size of the nodes was set in proportion to the total surface area of each ion detected in the extract.

Bio-guided isolation and characterisation

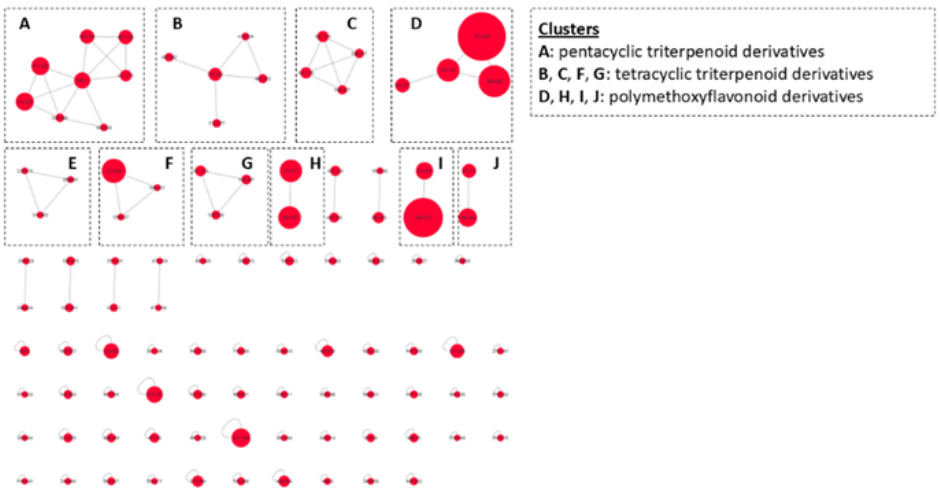
After preparative TLC using several solvent systems (petroleum ether-ethyl acetate, cyclohexane-ethyl acetate, dichloromethane-methanol), the extract was fractionated on a silica gel column in a dichloromethane-methanol system of increasing polarity. Follow-up TLC fractionation yielded eighteen fractions of the extract, coded **3af1–3af18**. The biological activity of the fractions was evaluated at 10 and 2 µg/mL against *P. falciparum* and *L. donovani* using the same protocol as previously applied to the crude extract (Damé et al., 2025). Selected fractions were then purified by preparative HPLC. Separation was carried out on an Uptisphere C18 column, 250 x 4.6 mm, particle size 5 µm. The sample solution was prepared at 10 mg/mL methanol, and the injection volume was 10 µL. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) in different gradients for each fraction of the extract. Chromatogram detection wavelengths ranged from 190 to 500 nm. MS spectra were acquired using an Agilent 1200 HPLC-PDA system coupled to an Agilent Accurate Mass QToF 6520 mass spectrometer (Agilent, USA), controlled by Mass Hunter software. ^{1}H NMR analyses were performed using a Bruker 400 MHz spectrometer and HSQC and HMBC analyses were performed using a Bruker 500 MHz spectrometer. The data were processed using Mnova 12.0.4 software.

Results

LC-MS/MS profiling and molecular networking

The first step of the workflow consisted of rapidly exploring the chemical composition of the ethyl acetate extract of *G. sokotensis*. The metabolites were analysed by tandem mass spectrometry and visualized using molecular networking (Figure 2; Table 1). The resulting molecular network organized the detected features according to similarities in their MS/MS fragmentation patterns and revealed major clusters putatively corresponding to pentacyclic triterpenoid, tetracyclic triterpenoid and polymethoxyflavonoid derivatives. Dereplication against the GNPS spectral libraries and the literature did not yield confident matches to individual structures, and the features were therefore annotated at the level of compound classes.

Figure 1 : Molecular network of the ethyl acetate leaf extract of *G. sokotensis*. Edges represent spectral similarities with a cosine score ≥ 0.70 and more than three matched fragment ions



Node size is proportional to the chromatographic peak area of each molecular feature, with larger nodes indicating higher relative abundance.

Table 1 : Putative identification of compounds detected in the ethyl acetate extract of *G. sokotensis*

Code	RT	m/z	Adduct	Formula	Δppm	ΔmDa	MS/MS	Putative identification
1	3.51	223.0751	$[M-H]^-$	$C_{15}H_{12}O_2$	-3.61	-0.8	59.0173	n.i.
2	4.07	265.0908	$[M-H]^-$	$C_{10}H_{18}O_8$	-5.82	-1.54	n.f	n.i.
		311.0920	$[M+FA]^-$					
3	5.47	129.0175	$[M-H]^-$	$C_5H_6O_4$	-9.95	-1.28	n.f	n.i.
4	21.53	549.3065	$[M-H]^-$	$C_{30}H_{46}O_9$	0.26	0.14	87.0091	n.i.
		1099.6024	$[2M-H]^-$					n.i.
5	21.92	463.2615	$[M-H]^-$	$C_{28}H_{36}N_2O_4$	3.92	1.82	371.2508 ; 415.2477 ; 445.2579	n.i.
		927.5474	$[2M-H]^-$					n.i.
6	22.09	549.3076	$[M-H]^-$	$C_{32}H_{42}N_2O_{36}$	4.52	2.03	487.2981 ; 505.3115	n.i.
		1099.6047	$[2M-H]^-$					n.i.
7	22.3	533.3087	$[M-H]^-$	$C_{30}H_{46}O_8$	-5.14	-2.74	87.0092 ; 515.3059	n.i.
8	22.79	533.3068	$[M-H]^-$	$C_{30}H_{46}O_8$	-8.71	-4.64	489.3248	n.i.
9	23.02	591.3116	$[2M-H]^-$	$C_{32}H_{48}O_{10}$	-1.56	-0.92	87.0091	n.i.
		1183.6458	$[2M-H]^-$					n.i.
10	23.34	531.2974	$[M-H]^-$	$C_{30}H_{44}O_8$	3.02	1.61	395.2914 ; 439.2810	n.i.
		1063.5873	$[2M-H]^-$					n.i.
11	23.74	375.0708	$[M-H]^-$	$C_{18}H_{16}O_9$	-2.15	0.81	345.0223 ; 302.0015 ; 175.0014	tetrahydroxy-trimethoxyflavone
		751.1405	$[2M-H]^-$					
12	24.09	515.2931	$[M-H]^-$	$C_{30}H_{44}O_7$	-15.1	-7.78	471.3036 ; 423.2886 ; 379.3020, 453.2901 ; 441.3035 ; 409.3047	triterpenoid derivative I
		1031.5962	$[2M-H]^-$					
13	24.39	531.2973	$[M-H]^-$	$C_{30}H_{44}O_8$	2.84	1.51	485.2888 ; 455.2797 ; 411.2884 ; 437.2664	triterpenoid derivative II
		1063.587	$[2M-H]^-$					
14	24.54	531.2973	$[M-H]^-$	$C_{30}H_{44}O_8$	2.84	1.51	485.2889 ; 455.2798 ; 411.2885 ; 513.2781 ; 397.2582	triterpenoid derivative III
		1063.587	$[2M-H]^-$					
15	24.88	573.2971	$[M-H]^-$	$C_{28}H_{46}O_{12}$	10.46	6	513.3024 ; 407.2887 ; 367.2659 ; 395.2924 ; 469.3014	triterpenoid derivative IV
		1147.6084	$[2M-H]^-$					
16	25.15	345.0647	$[M-H]^-$	$C_{17}H_{14}O_8$	10.6	3.66	300.0220 ; 109.9991 ; 165.9848	tetrahydroxy-dimethoxyflavone
17	25.36	633.3255	$[M-H]^-$	$C_{34}H_{50}O_{11}$	-3.14	-1.99	469.2881 ; 407.2878 ; 439.2812 ; 513.2771 ; 573.3096	n.i.
		1267.6526	$[2M-H]^-$					
18	25.61	515.292	$[M-H]^-$	$C_{26}H_{44}O_{10}$	12.38	6.38	453.3001 ; 471.3014 ; 487.3099 ; 497.2913 ; 391.3025	
		1031.6123	$[2M-H]^-$					

19	25.92	359.0740	$[M-H]^-$	$C_{18}H_{16}O_8$	-7.5	-2.69	329.0306 ; 286.0046	trihydroxy-trimethoxyflavone
20	26.34	375.0709	$[M-H]^-$	$C_{18}H_{16}O_9$	-1.89	-0.71	345.0215 ; 194.9949 ; 302.0108 ; 149.0247	tetrahydroxy-trimethoxyflavone
		751.1407	$[2M-H]^-$					
21	27.36	389.0843	$[M-H]^-$	$C_{19}H_{18}O_9$	-7.6	-2.69	359.0424 ; 344.0115 ; 300.9962 ; 316.0233 ; 328.9901 ; 175.0013 ; 374.0621	trihydroxy-tetramethoxyflavone
		779.1827	$[2M-H]^-$					
22	27.75	517.3103	$[M-H]^-$	$C_{30}H_{46}O_7$	-12.04	-6.23	473.3197 ; 87.0091	triterpenoid derivative V
23	28.18	515.291	$[M-H]^-$	$C_{26}H_{44}O_{10}$	10.44	5.38	423.2867 ; 467.2791 ; 497.2786	n.i
		1031.5919	$[2M-H]^-$					
24	28.65	359.0739	$[M-H]^-$	$C_{18}H_{16}O_8$	-7.78	-2.79	194.9868 ; 329.0311 ; 286.0050 ; 242.0179 ; 179.9711	trihydroxy-trimethoxyflavone
25	28.84	343.077	$[M-H]^-$	$C_{18}H_{16}O_7$	-13.93	-4.78	298.0111 ; 313.0354	dihydroxy-trimethoxyflavone
26	29.11	499.2995	$[M-H]^-$	$C_{30}H_{44}O_6$	-12.95	-6.46	469.2885 ; 481.2947 ; 407.2883 ; 437.3021	triterpenoid derivative VI
		999.6154	$[2M-H]^-$					
27	29.49	499.2998	$[M-H]^-$	$C_{30}H_{44}O_6$	-12.35	-6.16	455.3156	triterpenoid derivative VII
28	29.78	373.0887	$[M-H]^-$	$C_{19}H_{18}O_8$	-9.76	-3.64	328.0246 ; 343.0455 ; 284.9989 ; 200.0231 ; 312.9952	dihydroxy-tetramethoxyflavone
29	30.10	489.351	$[M-H]^-$	$C_{30}H_{50}O_5$	-14.3	-7	245.351	triterpenoid derivative VIII
30	30.24	403.101	$[M-H]^-$	$C_{20}H_{10}O_9$	-4.73	-1.91	373.0541 ; 358.0332 ; 315.0155 ; 343.0024 ; 330.0303	n.i.
31	30.59	489.3481	$[M-H]^-$					n.i.
32	30.74	373.0876	$[M-H]^-$	$C_{19}H_{18}O_8$	-12.71	-4.74	343.0452 ; 300.0228 ; 328.0139	dihydroxy-tetramethoxyflavone
33	31.06	499.302	$[M-H]^-$	$C_{30}H_{44}O_6$	-7.94	-3.96	481.2957 ; 437.3029 ; 407.2543	triterpenoid derivative IX
34	31.20	499.3007	$[M-H]^-$	$C_{30}H_{44}O_6$	-10.54	-5.26	481.2934 ; 482.2871	triterpenoid derivative X
35	31.52	499.3032	$[M-H]^-$	$C_{30}H_{44}O_6$	-5.54	-2.76	471.3047 ; 413.2687	triterpenoid derivative XI
36	32.39	499.3006	$[M-H]^-$	$C_{30}H_{44}O_6$	-10.74	-5.36	471.3016 ; 399.2897	triterpenoid derivative XII
37	32.62	527.2902	$[M-H]^-$				407.2874 ; 483.3073 ; 323.2352 ; 439.3167	n.i.
38	33.60	293.2135	$[M-H]^-$	$C_{18}H_{30}O_3$	6.24	1.83	n.f	lipid derivative I
39	33.88	293.2126	$[M-H]^-$	$C_{18}H_{30}O_3$	3.71	0.93	n.f	Lipid derivative II
40	34.87	481.2949	$[M-H]^-$	$C_{30}H_{42}O_5$	-1.04	-0.5	n.f	triterpenoid derivative XIII
41	35.51	295.2277	$[M-H]^-$	$C_{18}H_{32}O_3$	1.29	0.38	n.f	lipid derivative III
42	35.68	487.3346	$[M-H]^-$	$C_{30}H_{48}O_5$	-15.9	-7.75	303.1981 ; 385.2736	

		975.6801	$[2M-H]^-$					n.i
43	36.3	487.3339	$[M-H]^-$	$C_{30}H_{48}O_5$	-17.34	-8.45	303.1963 ; 385.2713	triterpenoid pentacyclic derivative I
		975.6767	$[2M-H]^-$					
44	36.72	483.3081	$[M-H]^-$	$C_{30}H_{43}O_5$	-6.1	-2.95	n.f	triterpenoid pentacyclic derivative II
45	36.86	471.3395	$[M-H]^-$	$C_{30}H_{48}O_4$	-16.83	-7.93	n.f	triterpenoid pentacyclic derivative III
		943.6931	$[2M-H]^-$					
46	37.04	487.3376	$[M-H]^-$	$C_{30}H_{48}O_5$	-9.75	-4.75	455.3167	triterpenoid pentacyclic derivative IV
		975.6859	$[2M-H]^-$					
47	37.80	483.3082	$[M-H]^-$	$C_{30}H_{44}O_5$	-5.9	-2.85	439.3175 ; 421.3084	triterpenoid pentacyclic derivative V
48	38.35	469.326	$[M-H]^-$	$C_{30}H_{46}O_4$	-12.33	-5.78	407.3243	triterpenoid pentacyclic derivative VI
		939.6659	$[2M-H]^-$					
49	40.88	377.1374	$[M-H]^-$	$C_{23}H_{22}O_5$	-3.97	-1.5	118.9266 ; 99.9211 ; 84.9366	n.i.
50	49.1	455.3508	$[M-H]^-$	$C_{30}H_{48}O_3$	-3.78	-1.72		triterpenoid pentacyclic derivative VII

Note: ‘n.f.’ means no fragmentation signal; ‘n.i.’ means no compound identified.

Antiplasmodial and antileishmanial activities of extract fractions

The ethyl acetate extract was fractionated by open column chromatography using a dichloromethane-methanol gradient of increasing polarity, yielding eighteen fractions (**3af1**–**3af18**). Their biological activities were evaluated at 10 and 2 µg/mL against *P. falciparum* and *L. donovani*, the two parasites against which the crude extract was previously found to be active (Damé et al., 2025) (Table 2). Three fractions (**3af8**, **3af9**, and **3af11**) were selected for preparative HPLC purification; these were among the fractions displaying antiplasmodial and/or antileishmanial activity.

Table 2 : Parasite growth inhibition (%) by the ethyl acetate extract fractions of *G. sokotensis* against *P. falciparum* and *L. donovani*

	***In vitro* parasite growth inhibition (%)**			
	P. falciparum		***L. donovani***	
	10 µg/mL	**2 µg/mL**	**10 µg/mL**	**2 µg/mL**
3af1	42.1 ± 4.5	8.3 ± 3.1	11.7 ± 1.5	8.7 ± 4.6
3af2	26.1 ± 8.5	15.6 ± 4.7	20.9 ± 9.9	17.9 ± 7.8
3af3	82.1 ± 1.3	20.1 ± 1.9	32.1 ± 10.4	14.7 ± 0.8
3af4	18.7 ± 5.3	19.4 ± 2.2	26.2 ± 3.4	12.7 ± 3.8
3af5	98.2 ± 0.6	34.4 ± 1.2	74.9 ± 2.5	22.6 ± 5.9
3af6	54.5 ± 3.3	9.6 ± 2.6	38.3 ± 8.8	14.4 ± 0.0
3af7	95.2 ± 0.6	20.5 ± 1.8	78.1 ± 1.8	25.7 ± 0.0
3af8	100.1 ± 0.2	18.9 ± 4.6	90.2 ± 0.0	27.7 ± 5.1
3af9	79.1 ± 1.8	11.4 ± 9.2	100.9 ± 0.1	64.8 ± 13.4
3af10	98.5 ± 0.1	49.4 ± 2.0	100.1 ± 1.0	58.2 ± 15.3
3af11	73.0 ± 3.2	10.5 ± 0.1	50.9 ± 2.1	15.9 ± 6.7
3af12	98.6 ± 1.2	86.8 ± 0.6	99.7 ± 0.9	91.7 ± 1.2
3af13	102.2 ± 1.8	80.3 ± 0.8	97.6 ± 0.7	46.0 ± 1.5
3af14	101.9 ± 1.2	43.5 ± 5.5	87.4 ± 1.9	28.3 ± 0.7
3af15	102.6 ± 1.7	65.8 ± 1.9	66.8 ± 1.5	15.3 ± 4.3
3af16	100.3 ± 1.5	47.4 ± 8.5	60.8 ± 5.5	14.1 ± 10.7
3af17	98.2 ± 1.7	23.7 ± 10.4	60.9 ± 12.5	26.2 ± 17.2
3af18	101.3 ± 1.7	33.2 ± 13.6	69.3 ± 9.5	21.6 ± 14.5

Structural identification of the isolated polymethoxyflavonoids

Building on the bioactivity screening described above, fractions **3af8**, **3af9** and **3af11** were subjected to preparative HPLC purification. This process led to the isolation of three compounds (Figure 2), identified through the combined interpretation of mass spectrometric data, ^{1}H NMR, HSQC and HMBC spectra, together with comparison with published spectroscopic data.

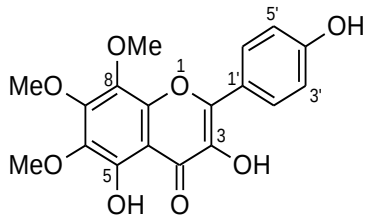
The first of the three isolated compounds, corresponding to one of the two trihydroxy-trimethoxyflavones proposed from LC-MS/MS data, was isolated (2 mg) from fraction **3af9** and identified as 3,5,4′-trihydroxy-6,7,8-trimethoxyflavone (**19**). Its mass spectrum (Figure S1) showed a $[M+H]^+$ ion at *m/z* 361.0926, consistent with the molecular formula $C_{18}H_{16}O_8$. The ^{1}H NMR spectrum (Figure S2) showed a singlet at δ 12.36 ppm, characteristic of the hydroxyl proton at C-5 (hydrogen-bonded to the carbonyl group), two

doublets (J = 9.1 Hz, 2H each) characteristic of an AB system, attributed to H-2′/H-6′ and H-3′/H-5′ in ring B of the flavonoid skeleton, and three singlets (3H each) around δ 4 ppm corresponding to the OCH_3 groups. The HSQC spectrum (Figure S3) showed three correlations between carbons at δ 61.28–59.49 ppm and protons at δ 3.95–3.80 ppm, corresponding to the OCH_3 methyl groups. In the HMBC spectrum (Figure S4), no correlation was observed between the methyl protons and the aromatic carbons at C-2′/C-5′ (δ 130.60 ppm), C-3′/C-6′ (δ 115.77 ppm), or the carbonyl carbon (δ 179.42 ppm). The only possible positions for the three OCH_3 groups were therefore C-6, C-7, and C-8, consistent with the correlations observed between the methyl protons (δ 3.95–3.80 ppm) and the C-6, C-7, and C-8 carbons (δ 138.22–127.77 ppm). Based on these data, compound **19** was identified as 3,5,4′-trihydroxy-6,7,8-trimethoxyflavone, previously isolated from the aerial parts of *Helichrysum stoechas* (Lavault & Richomme, 2004).

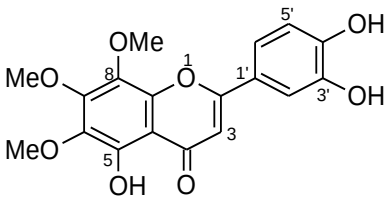
The second trihydroxy-trimethoxyflavone, compound **24**, was isolated (7.8 mg) from fraction **3af8**. Its mass spectrum (Figure S5) showed a pseudomolecular ion at *m/z* 361.0930 $[M+H]^+$, also corresponding to the molecular formula $C_{18}H_{16}O_8$. The ^1H NMR spectrum (Figure S6) showed a signal at δ 12.33 ppm attributed to 5-OH, a vinyl proton signal at δ 6.19 ppm (H-3), and three aromatic proton signals at δ 5.22, 6.93, and 7.97 ppm, assigned to H-2′, H-5′, and H-6′, respectively, together with three methoxy singlets around δ 4.0 ppm. The HSQC spectrum (Figure S7) showed three correlations between the methyl protons (δ 3.83–3.75 ppm) and their corresponding carbons (δ 60.96–55.54 ppm), a correlation between the proton at δ 6.93 ppm and C-2′/C-5′ (δ 115.89 ppm), and a correlation between the proton at δ 7.97 ppm and C-6′ (δ 130.66 ppm). In the HMBC spectrum (Figure S8), no correlation was observed between the methyl protons and C-3 (δ 104.32 ppm), C-2′/C-5′ (δ 115.89 ppm), or C-6′ (δ 123.28 ppm). Several correlations were instead observed between the methyl protons (δ 3.81-3.75 ppm) and C-6 (δ 131.95 ppm), C-7 (δ 138.35 ppm), and C-8 (δ 127.81 ppm), confirming the positions of the three methoxy groups. Additional correlations were observed between the hydroxyl proton at C-5 and C-6, and between the vinyl proton at C-3 and C-4. These data matched those of 3′,4′,5-trihydroxy-6,7,8-trimethoxyflavone, previously isolated from *Loranthus acutifolius* leaves (Apaza Ticona et al., 2021).

The third isolated compound, **25**, was obtained (2.5 mg) from fraction **3af11**. Unlike compounds **19** and **24**, the structure of compound **25** was assigned on the basis of its ^1H NMR chemical shifts compared with literature data, as mass spectrometric data and 2D correlation experiments were not acquired for this compound. Its ^1H NMR spectrum (Figure S9) showed three methoxy singlets around δ 4 ppm, a vinyl proton signal, and

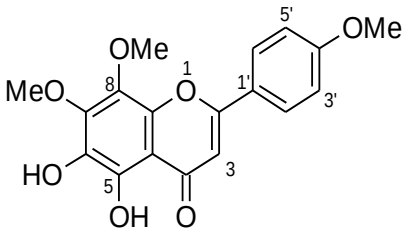
two aromatic proton doublets, consistent with the chemical shifts reported for 5,6-dihydroxy-7,8,4′-trimethoxyflavone (Kumar Rana et al., 2018).



3,5,4′-trihydroxy-6,7,8-trimethoxyflavone (19)



3′,4′,5-trihydroxy-6,7,8-trimethoxyflavone (24)



5,6-dihydroxy-7,8,4′-trimethoxyflavone (25)

Figure 2 : Chemical structures of compounds **19**, **24**, and **25**, isolated from *G. sokotensis*

Discussion

The limited annotation capacity of LC-MS/MS profiling and molecular networking reflects the lack of detailed phytochemical information available for *G. sokotensis*. While molecular networking provided an overview of the chemical families present (polymethoxyflavonoids, triterpenoids derivatives) in the extract, the analysis alone did not give access to individual molecular structures, nor, consequently, to information regarding their biological activity, motivating the complementary bio-guided fractionation step. The detection of these tow families is consistent with earlier phytochemical screenings of the species, which reported triterpenes and flavonoids together with tannins, saponins and alkaloids (Sama et al., 2022; Ouedraogo et al., 2025).

Both families detected by molecular networking have been associated with antiparasitic activity. Pentacyclic triterpenoids are among the most extensively studied natural antiparasitic scaffolds, with betulinic, ursolic, oleanolic and asiatic acids as representative examples. *In vitro*, betulinic, ursolic and oleanolic acids showed modest activity against *P. falciparum* (IC_{50} 19.6–88.8 µg/mL), yet betulinic acid did not reduce parasitaemia in *P. berghei*-infected mice at up to 250 mg/kg/day and showed some toxicity (Steele et al., 1999). By contrast, asiatic acid suppressed parasitaemia in *P.*

berghei-infected rats (Mavondo et al., 2016), and ursolic and oleanolic acids were active against intracellular *L. amazonensis* amastigotes (Torres-Santos et al., 2004). Flavonoids, the other family detected, have also shown antiparasitic activity, including methoxylated flavones. Yenjai et al. (2004) isolated nine flavonoids from *Kaempferia parviflora*, among which 5,7,4′-trimethoxyflavone and 5,7,3′,4′-tetramethoxyflavone were active against *P. falciparum* (IC_{50} 3.70 and 4.06 µg/mL, respectively). In a screening of a large flavonoid library against *L. donovani* axenic amastigotes, most compounds were active *in vitro*, the most potent being hydroxylated flavonoids (fisetin, luteolin and quercetin; IC_{50} 0.6–1.0 µg/mL), although only general structure-activity trends emerged (Tasdemir et al., 2006). As for possible mechanisms, polymethoxylated flavones form adducts with heme *in vitro* and pentamethoxyquercetin inhibited hemozoin formation in *P. falciparum*; however, heme-binding affinity did not correlate with antiplasmodial activity across the series (Ortiz et al., 2020). Both classes may therefore contribute to the antiparasitic activity of the extract, but this activity cannot be attributed directly to the putatively annotated triterpenoids or to methoxylated flavones.

The bioactivity data for the fractions indicate that the antiplasmodial and antileishmanial activities of the crude extract are distributed across several fractions obtained by silica gel chromatography. At 10 µg/mL, most fractions inhibited *P. falciparum* growth by more than 70%, and several also showed substantial activity against *L. donovani*. Antiplasmodial activity is not always concentrated in a single fraction. For the stem bark of *Lepidobotrys staudtii*, a 70% ethanolic extract and all six of its liquid-liquid partition fractions were active against *P. falciparum* (Magoudjou Pekam et al., 2025). *For G. sokotensis*, Jansen (2013) reported that the activity of the dichloromethane extract was not attributable to a single major compound but resulted from a combined action of several constituents. In the present study, the distribution of activity across adjacent fractions may reflect several active constituents with additive or synergistic effects, or the same constituents eluting across successive fractions. Fraction-level data alone cannot distinguish between these possibilities, and individual testing of the isolated compounds is required.

To our knowledge, this is the first report of these three polymethoxylated flavones from *G. sokotensis*, and their isolation provides confirmation of the polymethoxyflavonoid derivatives previously suggested by the LC-MS/MS profiling and molecular networking, illustrating the complementarity of chemical profiling and purification in this study. Compounds **19**, **24**, and **25** were isolated from fractions that displayed antiplasmodial and/or antileishmanial activity at 10 µg/mL (Table 2). Such complex fractions may also contain other constituents, including the

triterpenoid derivatives putatively annotated by molecular networking. Therefore, the individual contribution of compounds **19**, **24** and **25** to the observed activity cannot be established from the present data, and their mechanisms of action remain to be determined.

Conclusions

LC-MS/MS profiling and molecular networking of the ethyl acetate leaf extract of *Gardenia sokotensis* revealed a complex profile dominated by pentacyclic and tetracyclic triterpenoid, and polymethoxyflavonoid derivatives. Bio-guided fractionation identified several fractions with marked antiplasmodial activity, while the purification of selected fractions led to the isolation and structural identification of three polymethoxyflavonoids, namely 3,5,4′-trihydroxy-6,7,8-trimethoxyflavone (**19**), 3′,4′,5-trihydroxy-6,7,8-trimethoxyflavone (**24**), and 5,6-dihydroxy-7,8,4′-trimethoxyflavone (**25**), reported here for the first time in this species. These findings provide new insights into the chemical composition of *G. sokotensis*. The individual contribution of the isolated compounds to the observed antiplasmodial activity remains under investigation.

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