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In vitro antiplasmodial activity and chemical composition of *Combretum aculeatum*, a medicinal plant from Niger

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Malaria remains a significant public health challenge in Niger, accounting for 5.6% of global malaria-related deaths. Local medicinal plants are frequently used as traditional treatments for malaria, although their efficacy and safety are often insufficiently investigated. This study aims to evaluate the antiplasmodial activity and chemical composition of eight medicinal plants from Niger. Plant extracts were tested *in vitro* for their ability to inhibit the uptake of [^{3}H]-hypoxanthine in the *Plasmodium falciparum* NF54 chloroquine-sensitive strain. The most active extract was subjected to chemical analysis using HPLC-PDA-HRMS/MS dereplication, while major compounds were quantified via a validated LC-UV method. The ethyl acetate extract of *Combretum aculeatum* (**2a**) demonstrated important antiplasmodial activity, with an IC_{50} value of 3.1 µg/mL and a selectivity index of 24.5. *C*-glycosyl flavonoids were identified as the primary constituents, present at concentrations ranging from 5.7-9.8 mg of vitexin equivalent per g of extract. However, the low abundance of these compounds suggests that other constituents may contribute to the extract's antiplasmodial effects. Further investigations are required to explore possible synergies among the components of **2a** and to assess its efficacy *in vivo* models.

Keywords: Medicinal plants • malaria • Niger • dereplication • HPLC-UV quantification

Introduction

Malaria remains an important global health burden and is responsible for the death of 608.000 people annually principally in Southeast Asia and Africa, where Niger is located. Most malaria mortality in this country is caused by *Plasmodium falciparum*, the most common species in the highly malaria endemic areas of Africa. In Niger, 5,6% of malaria-related deaths occur, principally because of the emergence of drug-resistant strains of *P. falciparum*, and the limited access to conventional therapy. [1] The WHO recognizes traditional medicine as an important part of healthcare, especially in developing countries, and encourages its integration with modern healthcare systems to address the limited access to primary health care. [2] This must be supported by scientific research to better evaluate the benefits and risks of the traditional preparations.

In Niger, 27 medicinal plants have been reported to treat malaria symptoms. [3] However, few studies dealing with the antiplasmodial activity and the chemical composition of these plants have been yet reported. The aqueous and methanolic extracts of the two main species, *Senna occidentalis* and *Azadirachta indica*, have been reported to show a significant reduction in parasitemia in the *P. berghei* mouse model. The putative antiplasmodial compounds include anthraquinones, flavonoids, and triterpenoids. [4, 5]

To better understand the antimalarial activity of medicinal plants from Niger and with to integrate these extracts into the modern healthcare system, more biological and chemical data must be obtained. Dereplication allows for the quick identification of known compounds within a plant extract, avoiding isolation by comparison of high-resolution MS/MS spectra of an extract to MS/MS spectra of known compounds available in open databases, leading to a reliable chemical fingerprint of plant extracts. By quantifying known compounds that have previously showed antiplasmodial activity using robust HPLC-UV methods, a chemical fingerprint including putative antiplasmodial compounds can be obtained.

In this framework, data on plants traditionally used in the treatment of malaria in Niger were collected through personal contact with local traditional healers during an ethnobotanical survey of medicinal plants, conducted in August 2022. A bibliographic survey permitted to retain eight plant species available for collection, and for which both antiplasmodial activity had not been thoroughly investigated and phytochemical data were lacking. [6]

Aqueous and organic extracts were prepared from the parts of each selected plant indicated as traditionally used in decoction or in maceration by the traditional healers. All extracts were then screened for antiplasmodial activity *in vitro* by assessing its ability to inhibit the uptake of [^{3}H]-hypoxanthine by the parasite. The selectivity of the antiplasmodial activity was evaluated against other protozoan parasites and using L6 mammalian cells. IC_{50} values were determined for the most active extracts. For the most selective extract, the identification of known compounds was performed using an HPLC-PDA-HRMS/MS-based dereplicative approach. When these compounds were previously described for their antiplasmodial activity, their quantification was performed using a validated HPLC-UV methodology, highlighting the significance of accurately measuring their concentration to understand their contributions to the antiplasmodial activity of the extract.

Table 1: Collected medicinal plants.

Scientific names	Families	Some therapeutic indications	Traditional preparation	Administration	Part used	Voucher Number[a]	Reference
Cynanchum boveanum Decne.	Apocynaceae	Malaria convulsion, gonorrhoea	Decoction or maceration	Daily until symptoms disappear	Whole plant	172	6
Combretum aculeatum Vent.	Combretaceae	Malaria, haemorrhoids, stomach aches	Decoction or maceration	Daily until symptoms disappear	Aerial part	16	6
Gardenia sokotensis Hutch.	Rubiaceae	Malaria, diarrhoea, haemorrhoids,	Decoction	Daily until symptoms disappear	Leaves	219	6
Scoparia dulcis L.	Plantaginaceae	Malaria, fever, bronchitis, hepatitis	Decoction	Daily until symptoms disappear	Whole plant	279	6
Tephrosia bracteolata Guill.	Fabaceae	Malaria, diabetes	Decoction	Daily until symptoms disappear	Leaves	754	6
Maerua crassifolia Forssk.	Capparaceae	Malaria, diabetes, sinusitis, intestinal parasitosis	Decoction	Daily until symptoms disappear	Leaves	40	6
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Malaria, cold, bronchitis,	Decoction	Daily until symptoms disappear	Leaves	1033	3
Vitex doniana Sweet	Lamiaceae	Malaria, haemorrhoids, hepatitis	Decoction	Daily until symptoms disappear	Leaves	109	3

[a]Voucher number corresponding to the deposited specimen at the Herbarium of the Biological Department, Abdou Moumouni University.

Results and Discussion

Traditional healers and herbal medicine sellers in the areas of Niamey and Dosso in Niger were consulted directly to gather basic ethnobotanical information about the plants studied. Questions concentrated mainly on plants that are traditionally used against malaria. Based on this information, different databases (Lotus, Reaxys, Dictionary of Natural Products) were consulted to survey the literature concerning these plants. The selected medicinal plants were collected in August 2022, and botanical identification was ensured by Dr. Arzika Tanimoune (Garba Mounkaila Laboratory, Abdou Moumouni University). Vouchers of each specimen were deposited in the Herbarium of the Biological Department, Abdou Moumouni University

(Table1). The parts of the selected plant species traditionally used were extracted to yield 24 extracts, which were tested for antiprotozoal activities. The *in vitro* activity against *P. falciparum* (chloroquine-sensitive NF54 strain, erythrocytic stage) was determined using a [^{3}H]-hypoxanthine incorporation assay, and against *T. brucei rhodesiense* (STIB900 strain, bloodstream form) and *L. donovani* (MHOM/ET/67/L82 strain, axenic amastigotes) using a resazurin reduction assay (Table 2). Finally, the most active extracts were investigated for antiprotozoal IC_{50} values measurement and for their cytotoxic activity towards L6 cells (rat skeletal myoblasts) by CC_{50} measurement (Table 3).

A range of extraction yield was obtained between 1.4% (decoction of *G. sokotensis*) and 21.4% (methanolic extraction of *C. aculeatum*). Methanol and decoction extracts from all plants showed no important antiparasitic activity against the three tested parasites and only the ethyl acetate extract showed high levels of activity (≥ 50% at 10 µg/mL) against *P. falciparum*. The ethyl acetate extract of *C. aculeatum* (**2a**), *G. sokotensis* (**3a**), *S. dulcis* (**4a**), and *E. camaldulensis* (**7a**), showed the strongest antiplasmodial activity with a percentage of inhibition ranging from 93.4% to 69.8% at 10 µg/mL and with IC_{50} values of 3.1-7.8 µg/mL. Among the tested extracts, only **2a** showed selectivity when compared to the other protozoan evaluated species. As reported previously in the literature against *P. falciparum* strains, the most active isolated compounds from medicinal plants are usually lipophilic compounds, which could explain that only the lipophilic extracts showed high levels of antiplasmodial activity *in vitro* in comparison with methanolic and decoction extracts. [7]

Table 2: *In vitro* percentage of growth inhibition of extracts against *P. falciparum*, *T. b. rhodesiense* and *L. donovani* at 10 and 2 µg/mL.

Scientific names	Extracts	Code	Extraction yield (%)[a]	Parasite growth inhibition (%)[b]					
				P. falciparum		*T. b. rhodesiense*		*L. donovani*	
				10 µg/mL	2 µg/mL	10 µg/mL	2 µg/mL	10 µg/mL	2 µg/mL
C. boveanum	AcOEt[c]	1a	2.7	3.2 ± 4.8	5.4 ± 3.3	6.9 ± 2.6	0 ± 0.0	13.6 ± 10.4	0 ± 0.0
	MeOH[d]	1m	11.2	7.0 ± 4.7	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
	H_2O[e]	1d	9.5	8.1 ± 0.6	2.6 ± 1.6	5.8 ± 3.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
C. aculeatum	AcOEt[c]	2a	3.0	**69.8 ± 7.5**	**7.5 ± 6.9**	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
	MeOH[d]	2m	21.4	11.2 ± 4.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
	H_2O[e]	2d	9.5	6.3 ± 2.8	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
G. sokotensis	AcOEt[c]	3a	12.7	**88.2 ± 1.5**	**10.4 ± 2.4**	6.7 ± 0.2	0 ± 0.0	**81.1 ± 20.4**	**29.6 ± 0.0**
	MeOH[d]	3m	12.0	0 ± 0.0	0 ± 0.0	9.8 ± 2.1	0 ± 0.0	20.6 ± 5.2	0 ± 0.0
	H_2O[e]	3d	1.4	0 ± 0.0	0 ± 0.0	0.0 ± 0.0	0 ± 0.0	19.9 ± 6.0	0 ± 0.0
S. dulcis	AcOEt[c]	4a	4.2	**93.4 ± 2.9**	**0 ± 0.0**	**86.7 ± 19.4**	**0 ± 0.0**	23.0 ± 0.8	0 ± 0.0
	MeOH[d]	4d	4.7	25.2 ± 7.1	2.4 ± 0.5	3.2 ± 2.1	0 ± 0.0	10.9 ± 5.9	0 ± 0.0
	H_2O[e]	4m	2.1	21.4 ± 10.6	8.5 ± 3.9	0 ± 0.0	3.8 ± 1.5	10.5 ± 7.1	0 ± 0.0
T. bracteolata	AcOEt[c]	5a	2.5	17.1 ± 5.1	0 ± 0.0	0 ± 0.0	0 ± 0.0	12.4 ± 8.9	7.5 ± 0.0
	MeOH[d]	5m	6.7	15.0 ± 3.9	0 ± 0.0	0 ± 0.0	0 ± 0.0	11.8 ± 3.1	0 ± 0.0
	H_2O[e]	5d	1.9	4.3 ± 1.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	15.9 ± 2.9	0 ± 0.0
M. crassifolia	AcOEt[c]	6a	1.7	26.6 ± 6.1	2.8 ± 1.8	5.6 ± 3.2	0 ± 0.0	24.6 ± 1.9	0 ± 0.0
	MeOH[d]	6m	11.2	0 ± 0.0	0 ± 0.0	0 ± 0.0	5.1 ± 3.7	22.1 ± 2.2	0 ± 0.0
	H_2O[e]	6d	7.9	0 ± 0.0	0 ± 0.0	0 ± 0.0	2.6 ± 0.4	23.8 ± 5.8	0 ± 0.0
E. camaldulensis	AcOEt[c]	7a	16.3	**80.7 ± 6.4**	**0 ± 0.0**	0 ± 0.0	5.8 ± 3.0	**94.0 ± 9.6**	**29.5 ± 0.0**
	MeOH[d]	7m	16.2	8.8 ± 0.3	0 ± 0.0	5.2 ± 2.3	5.6 ± 3.1	9.9 ± 3.3	0 ± 0.0
	H_2O[e]	7d	4.7	30.2 ± 0.6	11.2 ± 2.9	0 ± 0.0	4.1 ± 2.0	13.3 ± 0.2	0 ± 0.0
V. doniana	AcOEt[c]	8a	3.2	26.6 ± 5.7	13.3 ± 8.8	8.2 ± 1.8	0 ± 0.0	32.4 ± 4.2	0 ± 0.0
	MeOH[d]	8m	12.1	9.8 ± 4.7	0 ± 0.0	2.6 ± 0.3	12.4 ± 3.6	18.8 ± 3.6	0 ± 0.0
	H_2O[e]	8d	2.8	20.1 ± 9.0	8.2 ± 5.8	6.6 ± 2.0	6.4 ± 1.7	18.4 ± 2.0	0 ± 0.0
	Chloroquine			100 ± 0.0	100 ± 0.0	-	-	-	-
	Melarsoprol			-	-	100 ± 0.0	100 ± 0.0	-	-

Miltefosine	-	-	-	-	100 ± 0.0	100 ± 0.0

[a]Extraction yield calculated in rapport of the initial plant mass (n=1). [b]The results are expressed as means ± SD of triplicates measurements (n=3). [c]Ethyl acetate extract maceration. [d]Mehtanolic maceration extract. [e]Decoction extract. -: No tested.

Table 3: Antiplasmodial, antitrypanosomal and antileishmanial IC_{50}, CC_{50} values (µg/mL ± SD) and SI of the most active extracts.

Code	IC_{50} (µg/mL)[a]			CC_{50} (µg/mL)[a]	SI*
	P.falciparum	*T. b.rhodesiense*	*L.donovani*	L6	
2a	3.1 ± 0.2	-	-	76.0 ± 19.2	24.5
3a	4.3 ± 0.5	-	4.3 ± 2.6	8.2 ± 3.4	1.9
4a	6.7 ± 0.3	21.5 ± 1.9	-	45.9 ± 4.5	6.8
7a	7.8 ± 1.4	-	3.0 ± 1.6	22.8 ± 2.5	2.9
Chloroquine	0.003±0.001	-	-	-	/
Melarsoprol	-	0.005 ± 0.001	-	-	/
Miltefosine	-	-	0.29 ± 0.08	-	/
Podophyllotoxine	-	-	-	0.008±0.0	/

[a]The results are expressed as means ± SD of triplicates measurements (n=3). * Selectivity index between CC_{50} value and IC_{50} antiplasmodial values (SI = CC_{50}/IC_{50}); -: No tested; /: No calculated.

Previous studies have investigated the *in vivo* antiplasmodial activity of two extracts with different polarities from the leaves of *E. camaldulensis*. A chloroform extract of *E. camaldulensis*, sourced from Nigeria, showed a significant reduction in the average parasitemia of *P. berghei*-infected mice when administered at daily doses of 25 and 300 mg/kg of weight (66% compared with the untreated group on day 5). [8] In another study, the aqueous extract of *E. camaldulensis* leaves displayed 60% efficacy in reducing parasitemia on the same model at daily doses of 200 and 100 mg/kg of weight. [9] In the case of *S. dulcis*, the ethyl acetate extract (**4a**) showed an IC_{50} value of 6.7 µg/mL, comparable to the activity reported in the literature for an ethyl acetate extract and a hydroethanolic extract against the chloroquine-resistant FCR-3 strain (IC_{50} = 19.5 and 6.6 respectively). Moreover, the hydroethanolic extract showed no effect on inhibiting *in vitro* haemozoin formation, suggesting that other mechanisms may be responsible for its antiplasmodial activity. These *in vitro* and *in vivo* antiplasmodial activities of *S. dulcis* and *E. camaldulensis* have been partially attributed to the presence of bioactive compounds such as polymethoxyflavonoids, glycosylflavonoids, triterpenoids, and bicyclic diterpenoids and of mono- and triterpenoids, respectively. [10, 11]

For *G. sokotensis* extracts, **3a** showed antiplasmodial activity with an IC_{50} value of 4.3 µg/mL, similar to the activity reported for the dichloromethane extract of the leaves against the *P. falciparum* 3D7 chloroquine-resistant strain (IC_{50} = 14 µg/mL). [12] In another study, the *in vivo* antiplasmodial activity in *P. berghei*-infected mice was reported for both dichloromethane and dichloromethane/methanol extracts of *G. sokotensis* leaves (72% and 87% reduction of parasitemia) at a daily dose of 500 mg/kg of weight. [13] However, no phytochemical reports were found for *G. sokotensis*.

The active extracts, which were also active against *T. brucei rhodesiense* and *L. donovani*, of *S. dulcis* (**4a**), *E. camaldulensis* (**7a**) and *G. sokotensis* (**3a**) showed poor selectivity indices (1.9-6.8) against the mammal cell line L6, indicating that the antiplasmodial compounds could also exert cytotoxic effects on eukaryote cells. These results must be taken in consideration for further biological assays regarding the safe administration of these medicinal plants.

For *C. aculeatum*, no previous reports have documented antiparasitic activities. In this study, extract **2a** (ethyl acetate extract) was found active against *P. falciparum* (IC_{50} = 3.1 µg/mL) and showed the highest selectivity, with a SI of 24.5. To identify the bioactive compounds responsible for the antiplasmodial activity of **2a**, a dereplicative analysis was carried out by HPLC-PDA-HRMS/MS analysis (Figure 1) and visualized through a molecular network representation (Figure 2). Additionally, the detected compounds were quantified by a validated HPLC-UV method.

The molecular network of **2a** organizes the fragmented compounds detected (Table 4) in the HPLC-PDA-HRMS/MS analysis in several clusters, corresponding principally *C*-glycosyl flavonoids (Figure 2). After some sugars derivatives and small organic compounds, the first eluted phenolic compound was putatively identified as *C*-glycosyl-flavonoid (**4**), which gave a pseudo-molecular ion $[M-H]^-$ at *m/z* 447 and fragmentated on the glycosidic moiety, at *m/z* 327 $[M-C_4H_8O_4-H]^-$ and at *m/z* 357 $[M-C_3H_6O_3-H]^-$, characteristic of fragmentation loss of *C*-glycosyl-flavonoids.[14] The same fragmentation pattern was

observed for the phenolic compounds **5**, **7** and **8**. Their structure was confirmed by co-elution with pure standards and identified as isoorientin (**4**), orientin (**5**), vitexin (**7**) and isovitexin (**8**). In the case of isoorientin **4**, its node was not connected with the rest of *C*-glycosyl flavonoids, based on the important intensity difference of the main fragment ions when compared with the respectively fragmentation spectra of **5**, **7** and **8**. *O*-galloyl-*C*-glycosyl-flavonoids derivatives were also detected with a pseudo-molecular ion $[M-H]^-$ at *m/z* 599 for *O*-galloyl-orientin (**9**) and at *m/z* 583 for *O*-galloyl-vitexin (**12**) derivatives, respectively. *O*-cinnamoyl-vitexin (**13**) was also putative detected with a pseudo-molecular ion $[M-H]^-$ at *m/z* 577 with a fragmentation signal at *m/z* 431, corresponding to the vitexin ion. *O*-glycosyl flavonoids were also detected, principally kaempferol and quercetin derivatives. Rutin (**6**, $[M-H]^-$ at *m/z* 609, $[M\text{-hexosyl-hexosyl-H}]^-$ at *m/z* 300/301) and quercetin-hexoside (**10**, $[M-H]^-$ at *m/z* 463, $[M\text{-hexosyl-H}]^-$ at *m/z* 300/301), presented the characteristic fragment signal corresponding to the quercetin aglycon with the loss of one or two H. [15] Kaempferol-*O*-hexoside (**11**, $[M-H]^-$ at *m/z* 447) was identified as kaempferol derivative by the important presence of the fragmented signal at *m/z* 285, corresponding to the aglycone. In addition, eight triterpenoids were putatively detected, corresponding to isomers of quinovic acid (**17**, $[M-H]^-$ at *m/z* 485), of asiatic acid (**18**, **19** and **20**, $[M-H]^-$ at *m/z* 487) and of maslinic acid (**23**, **24**, **27** and **28**, $[M-H]^-$ at *m/z* 471). The identity of compound **18** was confirmed as asiatic acid by co-elution with the respective commercial standard. Lipids derivatives were also detected at *m/z* 327 (**15**), *m/z* 329 (**16**) and *m/z* 295 (**25**). The presence of these class of compounds in **2a** is supported by previous studies of leaves of *C. aculeatum* collected in Sudan. [16] This is the first evidence of the presence of **4**, **5** and **18** *in C. aculeatum*.

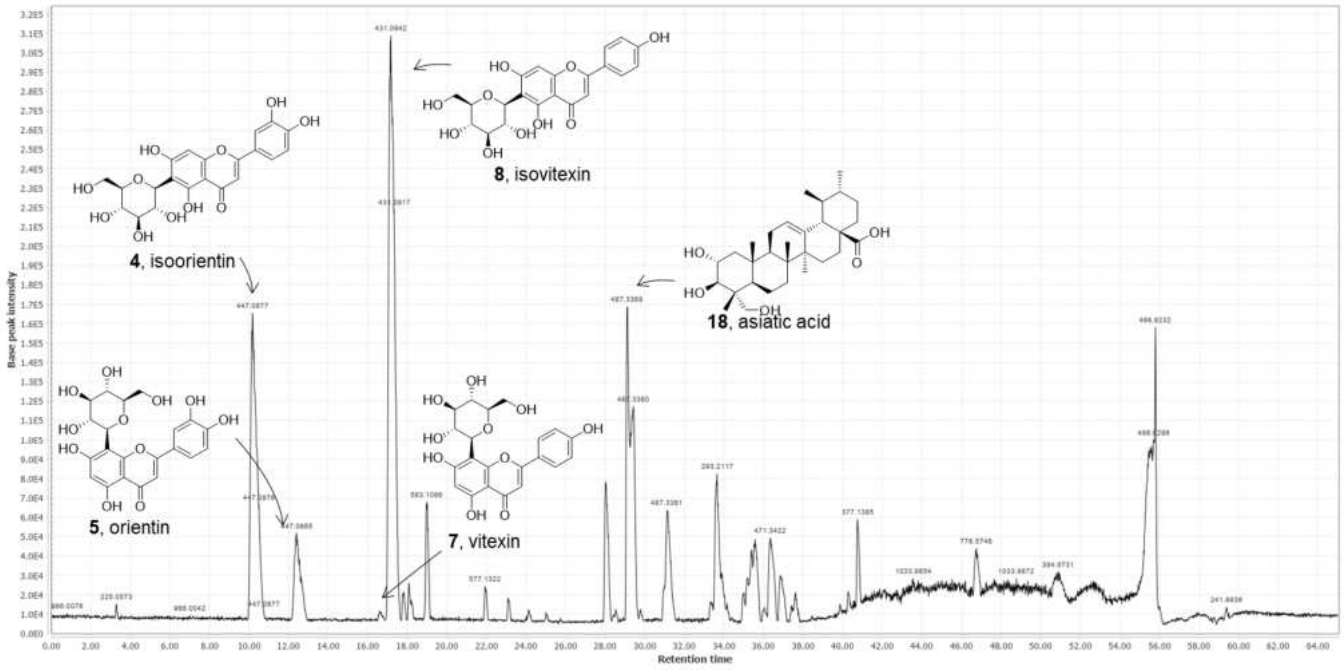

Figure 1: LC-MS/MS chromatogram of **2a** in negative mode showing the main compounds identified. **4** Isoorientin, **5** orientin, **7** vitexin, **8** isovitexin and **18** asiatic acid.

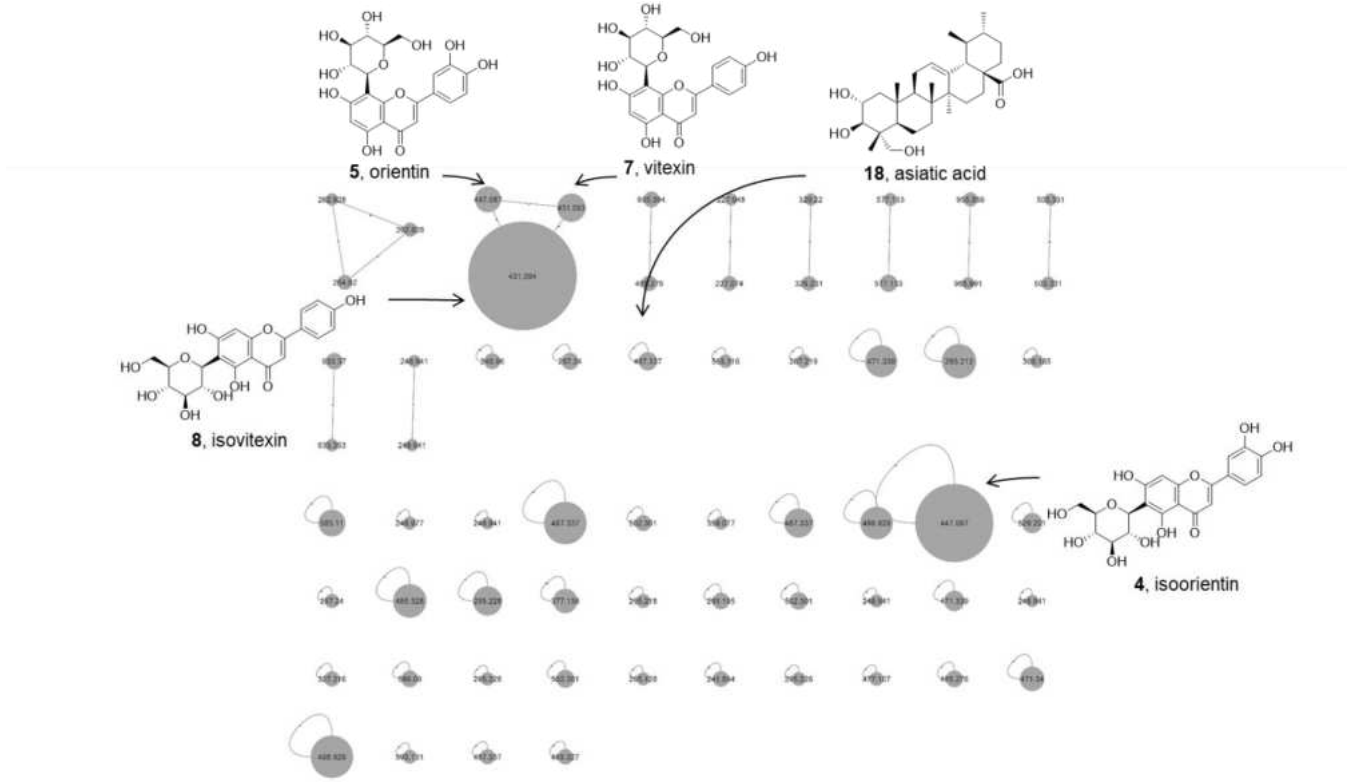

Figure 2: Molecular network of **2a**. Node size is proportional to peak area detected in negative mode.

Table 4: Identification of chemical constituents present in *C. aculeatum* extract **2a** by comparison of the MS/MS data.

Code	RT	*m/z*	Adduct	Formula	Δppm	ΔmDa	MS/MS	Putative identification
1	3.30	179.0577 225.0573	$[M-H]^-$ $[M+FA]^-$	$C_6H_{12}O_6$	11.93	2.14	59.0132; 71.0143; 85.0321	hexose isomer
2	3.54	221.0691	$[M-H]^-$	$C_8H_{14}O_7$	13.45	2.97	59.0127; 85.0367; 108.1255	acetyl hexose isomer
3	4.77	169.0111	$[M-H]^-$	$C_7H_6O_5$	-15.40	-2.6	n.f.	gallic acid
4	10.17	447.0877 895.1821	$[M-H]^-$ $[2M-H]^-$	$C_{21}H_{20}O_{11}$	-11.30	-5.04	327.0494; 357.0583; 297.0424; 299.0508; 285.0374	isoorientin[a]
5	12.40	447.0865 895.1797	$[M-H]^-$ $[2M-H]^-$	$C_{21}H_{20}O_{11}$	-14.00	-6.24	327.0480; 357.0568; 297.0412; 299.0496; 285.0362	orientin[a]
6	16.61	609.1447	$[M-H]^-$	$C_{27}H_{30}O_{16}$	-1.41	-0.86	300.0222; 301.0256	rutin[a]
7	17.05	431.0932	$[M-H]^-$	$C_{21}H_{20}O_{10}$	-10.72	-4.62	311.0507; 283.0568; 341.0625	vitexin[a]
8	17.16	431.0942 863.2064	$[M-H]^-$ $[2M-H]^-$	$C_{21}H_{20}O_{10}$	-8.40	-3.62	311.0517; 283.0577; 341.0636; 312.0531	isovitexin[a]
9	17.81	599.1042	$[M-H]^-$	$C_{28}H_{24}O_{15}$	0.84	0.50	309.0439; 327.0500; 429.0854	galloyl-orientin isomer
10	18.08	463.0913	$[M-H]^-$	$C_{21}H_{20}O_{12}$	7.88	3.65	300.0237; 301.0271	quercetin hexose
11	18.22	447.0894	$[M-H]^-$	$C_{21}H_{20}O_{11}$	-7.46	-3.34	285.0385	kaempferol hexose
12	18.96	583.1086 1167.2	$[M-H]^-$ $[2M-H]^-$	$C_{28}H_{24}O_{14}$	-0.31	-0.18	431.0932; 169.0114; 311.0514; 293.0514	galloyl-vitexin isomer
13	21.92	577.1322	$[M-H]^-$	$C_{30}H_{26}O_{12}$	-4.16	-2.4	293.0461; 311.0515; 431.1052	cinnamoyl-vitexin isomer
14	23.12	695.3937	$[M-H]^-$	$C_{37}H_{60}O_{12}$	-10.00	-6.95	487.338	
15	24.13	327.2157	$[M-H]^-$	$C_{18}H_{32}O_5$	-4.43	-1.45	203.0965; 183.1454; 155.1025; 57.0314	trihydroxyoctadecadienoic acid isomer

16	25.00	329.2304	$[M-H]^-$	$C_{18}H_{34}O_5$	-7.29	-2.40	229.1431; 127.1172	trihydroxyoctadecaenoic acid isomer
17	28.02	485.3275 971.6612	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{46}O_5$	1.65	0.80	n.f.	quinovic acid isomer
18	29.10	487.3369 975.6845	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_5$	-11.20	-5.45	n.f.	asiatic acid[a]
19	29.39	487.338 975.687	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_5$	-8.93	-4.35	n.f.	asiatic acid isomer I
20	31.12	487.3381 975.6871	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_5$	-8.72	-4.25	n.f.	asiatic acid isomer II
21	33.31	633.3709 1267.8	$[M-H]^-$ $[2M-H]^-$	$C_{35}H_{54}O_{10}$	11.09	7.03	145.0299; 469.3273	n.i.
22	33.65	293.2117 587.4321	$[M-H]^-$ $[2M-H]^-$	$C_{18}H_{30}O_3$	0.10	0.03	n.f.	n.i.
23	35.00	485.2759	$[M-H]^-$	$C_{25}H_{42}O_9$	1.74	0.84	227.2166	n.i.
24	35.17	471.3391 943.6923	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_4$	-17.7	-8.33	n.f.	maslinic acid isomer I
25	35.38	471.3392 943.6926	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_4$	-17.5	-8.23	n.f.	maslinic acid isomer II
26	35.58	295.2285	$[M-H]^-$	$C_{18}H_{32}O_3$	4.00	1.18	229.1434; 127.1170	hydroxyoctadecadienoic acid isomer
27	36.03	485.2749	$[M-H]^-$	$C_{25}H_{42}O_9$	-0.33	-0.16	277.2157	n.i.
28	36.34	471.3422 943.6985	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_4$	-11.10	-5.23	n.f.	maslinic acid isomer III
29	36.86	471.3391 943.6924	$[M-H]^-$ $[2M-H]^-$	$C_{30}H_{48}O_4$	-17.70	-8.33	n.f.	maslinic acid isomer IV
30	37.40	297.24	$[M-H]^-$	$C_{18}H_{34}O_3$	-9.99	-2.97	n.f.	n.i.
31	37.60	297.2406	$[M-H]^-$	$C_{18}H_{34}O_3$	-7.97	-2.37	n.f.	n.i.
32	40.75	377.1385	$[M-H]^-$	$C_{23}H_{22}O_5$	-1.06	-0.40	116.9267; 100.9320; 84.9366	n.i.

[a] Compound identified by standard comparison. n.f, no fragmentation signal; n.i., no identified compound.

To determine the concentrations of *C*-glycosyl derivatives previously identify by co-elution with standards (isoorientin, orientin, vitexin and isovitexin) in **2a**, an HPLC-PDA quantification methodology was developed, performed and validated according to EMA guidelines, assessing trueness, precision, accuracy and linearity parameters (Supplementary Information, Figure S.6, S.7 and S.8) at 280 nm (Figure 3, Table 6). [17] Optimized separation was obtained with isocratic conditions (15% of acetonitrile and 85% of water). The methodology allowed us to determine a concentration of 5.7 mg of vitexin (**7**) per g of dry extract (corresponding to a concentration of 1.32 x 10^{-4} M at the tested extract solution). Relative quantification of isoorientin (**4**), orientin (**5**) and isovitexin (**8**) revealed concentrations of 9.8, 6.0 and 9.3 mg of vitexin equivalent per g of dry extract (2.20 x 10^{-4} M, 1.33 x 10^{-4} M and 2.18 x 10^{-4} M at the tested extract solution), respectively.

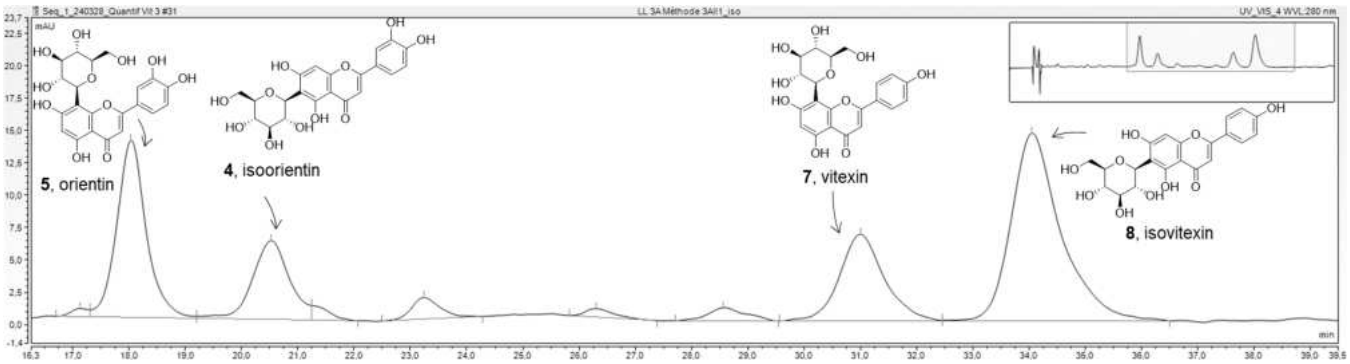

Figure 3: Chromatographic profile of **2a** at 280 nm obtained in isocratic conditions.

Table 6: Validation results obtained for the quantification method of vitexin.

Validation criteria		Concentration levels of vitexin (mg/mL)				
		0.05	0.1	0.2	0.4	0.6
Response function	Linear regression					
	Calibration Range			4 points		
	µg/mL			0.05 - 0.6		
Trueness	Relative bias (%)	-4.84	1.65	0.88	-0.25	1.38
Precision	Repeatability (RSD %)	3.44	2.31	0.91	0.75	0.35
	Intermediate precision (RSD %)	3.45	2.23	1.60	0.67	3.31
Accuracy	Lower and upper tolerance limits (%)	-13.23 3.55	-3.73 7.02	-4.83 6.60	-1.84 1.34	-15.03 17.79
Linearity	Slope			95.269		
	Int.			0.2169		
	R^2			0.9998		

RSD: Relative Standard Deviation

In the literature, vitexin showed an IC_{50} value of 4.4 µM (1.9 µg/mL) against the *P. falciparum* K1 chloroquine-resistant strain, while isovitexin was not active. [18] Isoorientin, on the other hand, was found to be active against the chloroquine-resistant RKL-9 and sensitive 3D7 strains with IC_{50} values of 14.4 µM (6.4 µg/mL) and 13.2 µM (5.9 µg/mL), respectively. [19] Regarding the IC_{50} value of **2a** (3.1 µg/mL), the proportion in the extract of vitexin or isoorientin should be very important to be the only responsible of the *in vitro* activity (1.9-6.4 µg/mL in the literature against *P. falciparum*). However, vitexin and isoorientin are present in very low concentration (5.7-9.8 mg of vitexin equivalent per g of extract) in the extract, to justify by themselves the activity of **2a**. These findings indicate that other compounds could play a role in the antiplasmodial activity of ethyl acetate extract of *C. aculeatum*.

In the case of asiatic acid, another compound identified in **2a**, an administration of 10 mg/kg of weight per oral in a pre-infection *P. berghei in vivo* model, resulted in a prolongation of the pre-patent period, reduction the percentage of parasitaemia at day 7 in comparison to the untreated group, reduction of white cell count and an augmentation of haemoglobin levels at days 9, 12 and 21 of infection.[20] Other asiatic acid isomers, such as arjunolic and arjunic acids showed an antiplasmodial *in vitro* activity of 9.9 and 9.7 µM as IC_{50} values against *P. falciparum* K1 chloroquine-reisistant strain.[21] In addition, 2´´-*O*-galloylvitexin, also demonstrated a moderate *in vitro* activity with an IC_{50} value of 38 µM.[22]

These findings indicate that the antiplasmodial activity of **2a** could be explained not only by the presence of a single or various bioactive compounds, but by a potential synergistic or additive effect of their chemical constituents. In fact, flavonoids extracted from *Artemisia annua* (artemetin, casticin, chrysoplenetin, chrysosplenol-D and circilineol) with moderate *in vitro* antiplasmodial activity exhibited a synergistic effect at 5 µM in combination with artemisinin. Triterpenes, such as 2,3,19-trihydroxyursolic acid, isolated from *Kligelia africana* (Bignoniaceae), showed synergistic effect with a combination of artemether and also with a combination with atranorin, a polyphenol compound isolated from the same plant, on the W2mef *P. falciparum*

strain with a combination index of 0.26 and 0.78, respectively.[23] This evidence highlights the complexity of natural compound interactions and the need to consider synergistic effects when assessing the antiplasmodial activity of medicinal plants. It suggests that the therapeutic potential of natural products against malaria may lie not just in isolated compounds, but in the intricate interplay of various constituents within these natural matrices.

In addition to identifying the compounds responsible for the antiplasmodial activity of *C. aculeatum*, optimizing the extraction procedures could also be considered. Parameters such as solvent type, number of cycles, and temperature should be optimized to obtain and standardize a potentially more active extract.

Conclusions

Ethyl acetate extracts of four medicinal plants showed important inhibition of parasite growth at 10 µg/mL against *P. falciparum*. The best IC_{50} value of 3.1 ± 0.2 µg/mL was obtained for ethyl acetate extract of *C. aculeatum*, which presented also the best selectivity index (24.5). Dereplication by molecular networking of this extract allowed the identification of *C*-glycosyl flavonoids, triterpenoids and lipids derivatives. Quantification suggests that *C*-glycosyl flavonoids are not the responsible by themselves of the antiplasmodial activity of the extract. Further research should focus on uncovering potential synergies among the chemical constituents of *C. aculeatum* to enhance our understanding of its antiplasmodial activity.

Experimental Section

Materials, chemicals and reagents

RPMI 1640 medium, MEM medium, SM medium, dimethylsulfoxide (DMSO), chloroquine, hypoxantine, HEPES, $NaHCO_3$, neomycin, AlbumexR, MEM-non essential amino acids solution, 2-mercaptoethanol, sodium pyruvate, horse serum, foetal bovine serum, resazurin, L-glutamine, miltefosine and podophyllotoxin were purchased to Sigma-Aldrich (St. Louis, USA). Melarsorpol was purchased to Sanofi-Aventis (Paris, France). Ethyl acetate, methanol, trifluoroacetic acid (TFA) and acetonitrile (ACN) were purchased to Carlo Erba (Emmendingen, Germany). Ethyl acetate was distilled before maceration.

Plant material and extraction

Eight plants from Niger were collected in August 2022: at Botanical Garden, Science and Techniques Faculty of Niamey University (13°30'05.4"N 2°05'28.6"E) for *Combretum aculeatum* and *Eucalyptus camaldulensis*; at Harobanda market, Niamey (13°29'31.0"N 2°05'51.2"E) for *Cynanchum boveanum* and *Gardenia sokotensis*; in Bolbol, Dosso Region (12°57'20.6"N 3°34'55.8"E) for *Scoparia dulcis* and *Tephrosia bracteolate*; and in Doutchi, Dosso Region (13°42'26.7"N 3°59'27.9"E) for *Maerua crassifolia* and *Vitex doniana*. Botanical identification of plants was done by Dr Arzika Tanimoune (Garba Mounkaila Laboratory, Abdou Moumouni University). Voucher of each collected plant was deposited at the Herbarium of the Biological Department, Abdou Moumouni University (Table 1). Plants were then dried at room temperature and powdered.

Preparation of extracts

For each plant, three types of extracts were prepared. Ethyl acetate and methanolic extracts were obtained through sequential maceration—first using ethyl acetate, then methanol—at 10% w/v, over two 24-hour cycles. The aqueous extract was made by boiling 10 g of dried plant powder in 100 mL of distilled water at 105°C for 15 minutes. Extracts were filtered through a Whatmann® filter system (11 µm pore size). The filtrate was concentrated in a rotary evaporator (Vaccubrand®) under reduced pressure at 35°C. Decoction extracts were then frozen at -80°C and then freeze dried (BenchTop Pro Lyophilizer) until complete solvent evaporation. The extraction yield was calculated according the following equation:

$$Extraction\ yield = \frac{Dry\ extract\ mass\ (g)}{Plant\ material\ mass\ (g)} * 100$$

Extract solutions were prepared at 10 mg/mL in DMSO until biological evaluation.

In vitro antiprotozoal assays

The effectiveness of the extracts in inhibiting the growth of *Plasmodium falciparum*, *Trypanosoma brucei rhodesiense*, and *Leishmania donovani* were first tested at concentrations of 10 and 2 µg/mL. Extracts that showed over 50% growth inhibition at 10 µg/mL against any of the parasites were selected for IC_{50} value determination by serial dilution. These values were calculated from the inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA).

Antiplasmodial assay

In vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]-hypoxanthine incorporation assay using the drug sensitive NF54 strain (Swiss Tropical and Public Health Institute) and the standard drugs chloroquine. [24] Continuous *in vitro* cultures of asexual erythrocytic stages of *P. falciparum* were maintained in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 mg/mL), $NaHCO_3$ (2.1 mg/mL), neomycin (100 U/mL), AlbumaxR (5 mg/mL) and washed human red cells A+. Initial concentration of each

plant extract was 30 µg/mL diluted with two-fold dilutions to make 7 concentrations, the lowest being 0.47 µg/mL. After 48 hours of incubation at 37 °C, [^{3}H]-hypoxanthine was added to each well and the incubation was continued for another 24 hours at the same temperature. The plates were then harvested with a Betaplate™ cell harvester (Wallac, Zurich, Switzerland), and the red blood cells transferred on to a glass fibre filter then washed with distilled water. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid, and counted in a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). Chloroquine was used as control.

Antitrypanosomal assay

In vitro culture of *T. b rhodesiense* STIB900 strain (Swiss Tropical and Public Health Institute) of bloodstream form [25] were maintained in axenic culture conditions (MEM medium supplemented with 25 mM HEPES, 1mg/mL additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM sodium pyruvate and 15% heat inactivated horse serum). Initial concentration of each plant extract was 30 µg/mL diluted with two-fold dilutions to make 7 concentrations, the lowest being 0.47 µg/mL. Then 4x10^3 bloodstream forms of *T. b. rhodesiense* STIB900 in 50 µL on modified medium was added to each well and the plate incubated at 37 °C under a 5 % CO_2 atmosphere for 70 hours. Then, 10 µL of resazurin solution (0.125 mg/mL) was then added to each well and incubation continued for a further 2 hours.[25] Then, the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Melarsoprol was used as control.

Antileishmanial assay

Amastigotes of *L. donovani* strain MHOM/ET/67/L82 (Swiss Tropical and Public Health Institute) are grown in axenic culture at 37 °C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. Initial concentration of each plant extract was 30 µg/mL diluted with two-fold dilutions to make 7 concentrations, the lowest being 0.47 µg/mL. One hundred microliters of culture medium with 1x10^5 amastigotes from axenic culture with or without a serial drug dilution are seeded in 96-well microtitre plates. After 70 hours of incubation the plates are inspected under an inverted microscope to assure growth of the controls and sterile conditions. Then, 10 µL of resazurin (0.125 mg/mL) are then added to each well and the plates incubated for another 2 h. Then the plates are read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm.[26] Miltefosine was used as positive control.

Cytotoxicity assay

Assay was performed in 96-well microtiter plates, each well containing 100 µL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4000 L-6 cells (ATCC CRL-1458, bought from LGC standards, France), a primary cell line derived from rat skeletal myoblasts. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. After 70 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Ten microliters of resazurin (0.125 mg/mL) was then added to each well and the plates incubated for another 2 hours. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. [27] Podophyllotoxin is used as control. The selectivity index (SI) corresponds to the ratio of the IC_{50} value of the cytotoxic activity to the IC_{50} value of antiplasmodial activity.

Statistical analysis

For biological evaluations, data were normalized as percentage of inhibition in relation with the corresponding parasite control (0% absence de parasites and 100% normal growth of parasites). All experiments were performed independently at least three times. Means and standard deviation (± SD) of the means were calculated with the program Softmax Pro 7 (Molecular Devices Cooperation, Sunnyvale, CA, USA).

HPLC-PDA-HRMS/MS analysis

HPLC-PDA-HRMS/MS analyses were performed on an HPLC-PDA Agilent 1200 system coupled with an Agilent Accurate Mass QToF 6520 mass spectrometer (Agilent, USA), controlled with a Mass Hunter software. The chromatographic separation was done on a 5 µm particles Uptisphere C18, 250 x 4.6 mm column. Stock solutions of crude extract and standards were prepared in methanol at 10 and 1 mg/mL concentration respectively and the injection volume was 10 µL. The mobile phase consisted of 0.1% of formic acid in water (solvent A) and 0.1% of formic acid in acetonitrile (solvent B). Elution of the mobile phase was performed with a flow rate of 0.8 mL/min in gradient mode: 0-10 min (15%, v/v, B); 10-40 min (15-65%, v/v, B); 40-45 min (65-100%, v/v, B); 45-55 (100%, v/v, B); 55-60 (100-15%, v/v, B); and 60-65 (15%, v/v, B). Detection wavelengths for chromatograms were set between 190 and 500 nm. HRMS analyses were performed in ESI positive and

negative modes with the following inlet conditions for both modes: ESI gas temperature of 340 °C, nebulizer of 30 psig, MS TOF fragmentor of 120 V, skimmet of 68 V and a collision energy of 30 eV. The MS/MS events were performed on the most abundant ions detected in full MS scans.

MS/MS Data treatment

HRMS data (.d) files obtained in negative mode (more informative set), were converted to .mzML files with MSConvert 3.0 software and then treated using an open source software MZmine 2.53. Noise level was set to 1.5×10^2 and 10 for mass detection at MS1 and MS2 levels, respectively. The ADAP chromatogram builder was used by setting to a minimum group size of 5 scans, group intensity threshold of 1.0×10^3, minimum highest intensity of 1.0×10^3, and m/z tolerance of 10 ppm. The intensity window S/N as an S/N estimator was used to deconvolute the chromatogram by ADAP wavelets 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 isotope pattern forming peaks and to remove additional minor isotopic peaks using an *m/z* tolerance of 10 ppm, RT tolerance of 0.5 min (absolute), maximum charge set at 1. The most intense isotope was selected as the representative isotope. The resulting mass list was filtered using "duplicate peak filter" and "feature list rows filter" to remove all duplicates and features and keep only MS/MS scans.

Mass spectral organization and dereplication

MZmine, after MS data treatment, gave a .mgf file which was exported to the GNPS website to construct a molecular network. The precursor ions mass tolerance was set to 2.0 Da with MS/MS fragmentation tolerance of 0.5 Da. A network was created by setting the edges to have a cosine value of 0.70 and more than 3 common peaks. The spectra in the network were then searched against GNPS's spectral libraries, under the same conditions. Additional putative identification of unmatched peaks was carried out by comparing available MS/MS fragmentation patterns in the literature (https://lotus.naturalproducts.net/). Data were visualized using Cytoscape 3.8.0 software. Data of peak area was added to the network from.csv file, obtained from MZmine. Size of nodes was set proportionally to the total area of each peak, detected in the analyzed extract.

HPLC-PDA quantification of *C*-glycosyl flavonoids

HPLC separations were conducted as described previously in HPLC-PDA-HRMS/MS analysis with the same analytical column and with an isocratic method of solvent with 15% (v/v) of ACN with 0.1% of TFA and 85% (v/v) of H_2O with 0.1% of TFA and a flow rate of 0.8 mL/min for 45 minutes. Stock solution of vitexin standard was prepared at 1 mg/mL concentration in HPLC grade methanol and then serially diluted to achieve five different concentrations in the range of 0.05 to 0.6 mg/mL (1.16×10^{-10} M to 1.39×10^{-9} M, Supplementary Information, Figure S.1, S.2, S.3, S.4 and S.5). The relative quantification of isovitexin, orientin, and isoorientin was performed using vitexin solutions as surrogate standards. The results were expressed in milligrams of vitexin equivalents per gram of extract. All analysis were carried out with 10 µL injection volume and with a flow rate of 0.8 mL/min. Chromatograms were integrated at 280 nm. Extract solution was freshly prepared at 10 mg/mL in HPLC grade methanol and analyzed under the same conditions as the vitexin standard. Validation criteria such as response function, trueness, precision, accuracy and linearity using a calibration range of 0.05 – 0.6 mg/mL (1.16×10^{-10} M to 1.39×10^{-9} M) of vitexin in methanol are presented in Table 5. Linear regression was selected as the most adequate regression model, with 95% expectation tolerance intervals included inside the ± 20 % acceptance limits for each concentration level of the validation standards. The calculated bias was less than 1.65%, showing the good trueness of the method. Precision was evaluated inter-day (intermediate precision) and intra-day (repeatability) and expressed as relative standard deviations (RSD) with 3.45% and 3.44% as maximum deviations respectively. All the trueness and precision results follow EMA guidelines criteria (≤15%). [17] Accuracy profiles, evaluating the sum of systematic and random errors (total error), indicated the relative upper and lower 95% β-expectation tolerance limits were inside the acceptance limits set at ± 20%. LOD and LOQ were calculated from the residual standard deviation (σ) of the regression curves and the slopes (S), according to the following equation: LOD = 3.3σ/S (0.020 mg/mL) and LOQ = 10σ/S (0.061 mg/mL).

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Author Contribution Statement

Conceptualization, S.M.D., A.T.I., S.O; methodology, S.M.D., S.O; software, S.O; formal analysis, S.M.D, L.L.; resources, S.M.D, C.V.S.; data curation, C.V.S., S.M.D, S.O; writing-original draft preparation, S.M.D, S.O; writing-review and editing, S.M.D, C.V.S., A.T.I., S.O.; project administration, A.T.I., S.O. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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