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Metabolites Profile of Extracts and Fractions of *Erythroxylum mexicanum* Kunth by UHPLC-QTOF-MS/MS and its Antibacterial, Cytotoxic and Nitric Oxide Inhibitory Activities.

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The present study shows the untargeted metabolite profiling and *in vitro* antibacterial, cytotoxic, and nitric oxide (NO) inhibitory activities of the methanolic leaves extract (MLE) and methanolic stem extract (MSE) of *Erythroxylum mexicanum*, as well as the fractions from MSE. Using ultra-high performance liquid chromatography/quadrupole time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS/MS), a total of 70 metabolites were identified; mainly alkaloids in the MLE, while the MSE showed a high abundance of diterpenoids. The MSE fractions exhibited differential activity against Gram-positive bacteria. Notably, the hexane fraction (HSF) against Streptococcus *pyogenes* ATCC 19615 (MIC=62.5 µg/mL) exhibited a bactericidal effect. The MSE fractions exhibited cytotoxicity against all cancer cell lines tested, with selectivity towards them compared to a noncancerous cell line. Particularly, the HSF and chloroform fraction (CSF) showed the highest cytotoxicity against prostate cancer (PC-3) cells, with IC_{50} values of 19.9 and 18.1 µg/mL and selectivity indexes of 3.8 and 4.2, respectively. Both the HSF and ethyl acetate (EASF) fractions of the MSE inhibited NO production in RAW 264.7 macrophages, with NO production percentages of 50.0% and 51.7%, respectively, at a concentration of 30 µg/mL. These results indicated that *E. mexicanum* can be a source of antibacterial, cytotoxic, and anti-inflammatory metabolites.

Keywords: *Erythroxylum mexicanum* • stem • UHPLC-QTOF-MS/MS analysis • diterpenoids • biological activities

Introduction

For centuries, traditional medicines have depended on plants to treat many diseases. Plants are a source of a wide range of natural products that possess various therapeutic properties and are continuously explored to develop novel drugs.[1] Among various plants, the genus *Erythroxylum* has a wide variety of pharmacological activities, e.g., its crude extracts, fractions or isolated molecules act as antimicrobial,[2, 3] antiproliferative,[2, 4] cytotoxic,[5, 6] proapoptotic,[2] antioxidant[7, 8] and anti-inflammatory.[8] *Erythroxylum* is the only pantropical genus of the four genera constituting the Erythroxylaceae family, being the largest with about 250 species, including trees and shrubs, of about 200 are native to the Neotropics.[9–11] This genus is widely known due to two South American cultivated species, *Erythroxylum coca* Lam. and *Erythroxylum novogranatense* (D.Morris) Hieron,[9, 11] which are natural sources of cocaine. This compound is the main responsible for their biological activities, and is well known as a drug of abuse, but in medicine it is used as a drug for local anesthesia, revolutionizing surgical and dental procedures.[12] In a recent review, Lv *et al.* (2022) classified and summarized the chemical composition of *Erythroxylum* genus: 383 compounds, including diterpenoids, triterpenoids, flavonoids, alkaloids, and other derivates were found in only 67 species.[13] These data show that there is still a lot of information missing on the chemical composition of the genus *Erythroxylum*, therefore, more phytochemical research is needed to better understand about the chemical diversity of this genus.

Erythroxylum mexicanum Kunth is an endemic species of Mexico,[14] and locally it is known as chilillo.[15] Botanically it is

characterized as a deciduous tree or shrub with elliptic leaves, narrowly obovate, up to 7.5 cm in length and 4.5 cm in width, the fruit, an ellipsoid drupe, elongated when immature, 7-10 mm in length (Figure S1).[15] This species has been scarcely explored in terms of its chemical composition, and only two scientific studies has been reported. In a study was analyzed the composition of nortropane alkaloids in forty-six wild and cultivated *Erythroxylum* species and among the results, was reported the presence of the calistegines A3 and B2 in the leaves of *E. mexicanum*.[16] In another study was analyzed the cocaine distribution in 51 *Erythroxylum* species; the cocaine was only detected in 23 species, but not in *E. mexicanum*.[17] Furthermore, to the best of our knowledge, prior to this work, no data existed about the *in vitro* pharmacological activities of crude extracts from *E. mexicanum* or their fractions. So, the aim of this study was to determine the untargeted metabolite profiling of methanolic extracts from the leaves and stem of *E. mexicanum*. Furthermore, the antibacterial, cytotoxic, and nitric oxide (NO) inhibitory activities were evaluated. The extract with the highest biological activity was further fractionated and the fractions were also analyzed.

Results and Discussion

Metabolite Profiling of Extracts and Fractions of E. mexicanum

The result analysis by UHPLC-QTOF-MS/MS displayed the presence of several metabolites in the methanolic leaves extract (MLE) and methanolic stem extract (MSE), as well as in the fractions obtained from MSE, of which 70 were tentatively identified through database matching. Data on retention time (RT min), measured *m/z* value, calculated mass, calculated mass error (ppm), score (DB), and relative abundance of each compound (%) of identified compounds are shown in *Table 1*. The suggested identification by the library allowed the elaboration of the metabolite profiling (*Figure 1*), such as, the MLE showed mainly alkaloids (40.37%) followed by fatty acid derivatives (21.15%) and monoterpenoids (10.36%) (*Figure 2A*). In contrast, MSE showed mainly diterpenoids (42.29%), alkaloids (14.40%), fatty acids (12.97%) and sesquiterpenoids (10.61%) (*Figure 2B*). Regarding the MSE fractions, hexane fraction (HSF) exhibited high abundance of diterpenoids (55.22%) followed by fatty acids and fatty acid derivatives (36.31%) (*Figure 2C*). Chloroform fraction (CSF) showed high abundance of diterpenoids (30.77%), fatty acids and fatty acid derivatives (24.33%), alkaloids (20.22%) and phenolics (11.04%) (*Figure 2D*). Ethyl acetate fraction (EASF) exhibited high abundance of diterpenoids (38.16%), fatty acids and fatty acid derivatives (14.52%) and alkaloids (13.62%) (*Figure 2E*). On the other hand, butanol fraction (BSF) (*Figure 2F*) and aqueous fraction (ASF) (*Figure 2G*) presented high abundance of sesquiterpenoids (45.75 and 44.02%, respectively), diterpenoids (22.15 and 16.24%, respectively) and phenolics (10.05 and 9.35%, respectively). In addition, high abundance of alkaloids (20.28%) was found for ASF.

Among the 70 identified metabolites, five diterpenoids namely canavalioside (**22**), 7-*O*-acetylaustroinulin (**53**), kaur-16-en-18-oic acid (**55**), (13*R*,14*R*)-7-labdene-13,14,15-triol (**68**) and manool (**69**) were found very abundant in the MSE extract and its fractions. The diterpenoid glycoside (**22**) was found in BSF (17.37%), ASF (2.36%) and MSE (3.80%), diterpenoid **53** in HSF (8.93%) and MSE (15.01%), diterpenoid **55** in HSF (12.27%), CSF (13.36%), EASF (14.17%) and MSE (6.02%), diterpenoid **68** in HSF (18.94%), CSF (3.36%), EASF (23.37%), BSF (4.97%), ASF (9.09%) and MSE (2.65%) and diterpenoid **69** in HSF (15.08%), CSF (2.58%) and MSE (3.35%). Manool (**69**) with a skeleton of *ent*-labdane type has been isolated from some species of *Erythroxylum*.[18, 19] Contrary, compounds **22**, **53**, **55** and **68** have not been previously reported for this genus, **53** and **68** are *ent*-labdane type, while **22** and **55** are *ent*-kaurane type, and both skeleton-types are biosynthetically related and commonly found in *Erythroxylum*.[13]

Also were identified three compounds of norisoprenoid type, the megastigmane glycosides **23**, **24** and **29**, they were found in EASF (**23**, 2.57%), BSF (**29**, 1.80%), MSE (**23**, 4.29%) and MLE (**23**, 3.67% and **24**, 3.59%). This norisoprenoid compounds found here for *E. mexicanum* are associated with those found previously for *Erythroxylum cambodianum*[20] and *Erythroxylum cuneatum*.[21]

Among identified metabolites that are not associated with the *Erythroxylum* genus and were detected high abundant, are the sesquiterpenoids conchosin A (**6**) and scorpioidin (**12**). In the MSE extract the relative abundance of compounds **6** and **12** was 2.12% and 2.27%, respectively. In its fractions, the content was as follows: CSF (**12**, 1.39%); EASF (**12**, 1.64%); BSF (**6**, 17.37% and **12**, 10.34%); ASF (**6**, 9.34% and **12**, 6.42%). Sesquiterpenes are not representative metabolites of this genus, however, the compounds bisabolene and cadinene were identified from *Erythroxylum monogynum*.[22]

On the other hand, the alkaloids that have been mostly identified from *Erythroxylum* genus are tropane type, which are derived from ornithine.[13, 23] In addition, Macedo Pereira *et al.* (2018),[6] isolated and characterized *N*, *N*-dimethyltryptamine from *Erythroxylum pungens*, an alkaloid derived from tryptophan. In our study for *E. mexicanum*, we identified, by library comparison, pyrrolizidine type alkaloids, namely fulvine (**9**), anacrotine (**11**) and senampeline A (**37**), and no alkaloid of this type has been previously reported for *Erythroxylum* genus to date. In MSE, compounds **9**, **11** and **37** were found in relative abundances of 2.03, 2.27 and 2.71%, respectively, while in its fractions these were found as follows: CSF (**9**, 0.76% and **37**, 12.93%); EASF (**37**, 8.49%); BSF (**9**, 6.08% and **37**, 1.55%); ASF (**9**, 4.22%, **11**, 7.57% and **37**, 8.49%).

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Table 1. Identified compounds of methanolic extracts and fractions of *E. mexicanum* by UHPLC-MS/MS.

							Relative abundance of each compound (%), from the total combined peak area of all identified compounds in each sample							
No.	Identified compound	RT	*m/z*	Molecular Mass	Score (DB)	Error (ppm)	HSF	CSF	EASF	BSF	ASF	MSE	MLE	Class of compound
1	Dolichotheline	0.991	178.1341	195.1374	87.29	-1.07	n.d.	n.d.	n.d.	n.d.	n.d.	5.18	n.d.	Alkaloid
2	15-acetyl-4-deoxynivalenol	1.029	338.1606	338.1374	98.07	-2.36	n.d.	n.d.	n.d.	13.78	12.23	3.93	n.d.	Sesquiterpenoid
3	Allamandin	1.072	313.0687	308.0901	98.94	-1.52	n.d.	n.d.	2.10	n.d.	n.d.	n.d.	n.d.	Sesquiterpenoid
4	Suberic acid	1.086	174.1126	174.0893	99.06	-0.61	n.d.	n.d.	n.d.	n.d.	2.32	10.22	2.27	Fatty acid
5	epi-Tulipinolide diepoxide	1.166	322.1656	322.1424	96.8	-2.28	n.d.	n.d.	n.d.	n.d.	16.03	n.d.	n.d.	Sesquiterpenoid
6	Conchosin A	1.224	278.139	278.1158	86.02	-1.19	n.d.	n.d.	n.d.	17.37	9.34	2.12	n.d.	Sesquiterpenoid
7	3-(1-pyrrolidinyl)-2-butanone	1.278	124.1124	141.1157	97.43	-2.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.42	Alkaloid
8	Myricatomentoside II	1.280	539.1892	534.2106	98.44	-0.86	n.d.	n.d.	4.18	n.d.	n.d.	n.d.	n.d.	Phenolic glycoside
9	Fulvine	1.361	292.155	309.1583	98.51	-2.19	n.d.	0.76	n.d.	6.08	4.22	2.03	n.d.	Alkaloid
10	1-(1-pyrrolidinyl)-2-butanone	1.452	124.1119	141.1152	87.88	1.08	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.70	Alkaloid
11	Anacrotine	1.553	334.164	351.1673	85.6	2.48	n.d.	n.d.	n.d.	n.d.	7.57	2.62	n.d.	Alkaloid
12	Scorpioidin	1.669	276.1592	276.1359	99.19	0.76	n.d.	1.39	1.64	10.34	6.42	2.27	n.d.	Sesquiterpenoid
13	*N*-cyclohexylformamide	1.855	110.0966	127.0999	87.21	-1.57	n.d.	n.d.	n.d.	0.21	0.30	n.d.	0.15	Formamide
14	Coriandrone D	1.857	352.1754	352.1521	99.91	0.26	n.d.	n.d.	n.d.	3.77	3.78	1.28	n.d.	Phenolic
15	Triangularine	1.861	336.1805	335.1732	99.05	0.12	n.d.	4.15	n.d.	n.d.	n.d.	n.d.	n.d.	Alkaloid
16	Ferulic acid	1.939	177.0548	194.0581	99.17	-0.98	n.d.	n.d.	n.d.	0.97	1.03	0.49	n.d.	Phenolic
17	Angustibalin	2.134	304.1544	304.1311	99.31	-0.22	n.d.	0.85	n.d.	n.d.	n.d.	n.d.	n.d.	Sesquiterpenoid
18	Isopentyl gentiobioside	2.237	417.173	412.1944	99.74	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.70	Hemiterpene
19	(10*S*,11*R*)-pterosin C 4-glucoside	2.327	401.1569	396.1782	99.77	0.52	n.d	0.58	2.08	n.d.	n.d.	n.d.	n.d.	Sesquiterpenoid glycoside
20	Phalaenopsine T	2.439	362.1961	361.1889	99.88	0.18	n.d.	1.26	n.d.	n.d.	n.d.	n.d.	n.d.	Alkaloid
21	Hexyl 2-furoate	2.512	179.1065	196.1098	99.39	0.79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.46	Furan derivative
22	Canavalioside	2.630	529.2635	546.2668	96.77	1.53	n.d.	n.d.	n.d.	17.17	2.36	3.30	n.d	Diterpenoid glycoside
23	4-megastigmene-6α,9*R*-diol 9-[apiosyl-(1→6)-glucoside]	2.704	529.2615	506.2723	98.57	0.83	n.d.	n.d.	2.57	n.d.	n.d.	4.29	3.67	Megastigmane glycoside
24	(3β,9*R*)-5-megastigmene-3,9-diol 9-[apiosyl-(1→6)-glucoside]	2.709	529.2629	506.2737	98.15	-1.87	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.59	Megastigmane glycoside
25	Mallotochromene	2.768	442.1867	442.1634	96.61	-1.49	n.d.	6.20	n.d.	n.d.	n.d.	n.d.	n.d.	Phenolic
26	Linalool oxide D 3-[apiosyl-(1→6)-glucoside]	2.815	487.2144	464.2252	98.92	1.2	n.d.	n.d.	n.d.	1.57	n.d.	n.d.	8.93	Monoterpenoid glycoside
27	15-acetoxyscirpene-3,4-diol 4-*O*-α-D-glucopyranoside	2.82	487.2164	486.2091	94.86	2.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.79	Sesquiterpenoid
28	Dihydrozeatin-9-*N*-glucoside-*O*-glucoside	3.008	545.2563	545.2331	99.1	0.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.74	Alkaloid
29	Tsangane L 3-glucoside	3.095	397.2204	374.2312	97.71	-2.06	n.d.	n.d.	n.d.	1.80	n.d.	n.d.	n.d.	Megastigmane glycoside
30	Glaudine	3.145	382.1647	399.168	99.32	0.48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24.20	Alkaloid

No.	Identified compound	RT	*m/z*	Molecular Mass	Score (DB)	Error (ppm)	Relative abundance of each compound (%), from the total combined peak area of all identified compounds in each sample							Class of compound
							HSF	CSF	EASF	BSF	ASF	MSE	MLE	
31	Aloesol 7-glucoside	3.400	379.1376	396.1409	95.72	2.89	n.d.	n.d.	n.d.	3.45	n.d.	n.d.	n.d.	Chromone glycoside
32	8-epideoxyloganin	3.404	379.1362	374.1575	99.55	0.5	n.d.	2.53	6.25	4.09	1.14	1.71	n.d.	Monoterpenoid glycoside
33	C1'-C9-glycosylated UWM6	3.595	472.1965	472.1732	99.51	0.24	n.d.	3.60	4.29	n.d.	4.54	1.41	n.d.	Phenolic
34	β-Zearalanol	3.673	322.2021	322.1788	97.63	-2.52	n.d.	n.d.	n.d.	3.77	n.d.	n.d.	n.d.	Phenolic
35	Thesinine 4'-*O*-glucoside	3.673	472.1944	449.2052	97.97	-0.49	n.d.	n.d.	5.14	1.76	n.d.	1.86	n.d.	Alkaloid
36	*N*-Methyl-2,3,7,8-tetramethoxy-5,6-dihydrobenzophenathridine-6-ethanoic acid	3.764	446.1571	423.1679	99.23	0.73	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.31	Alkaloid
37	Senampeline A	3.789	456.2016	473.2049	99.22	0.14	n.d.	12.93	8.49	1.55	8.49	2.71	n.d.	Alkaloid
38	1,3,8-Trihydroxy-4-methyl-2,7-diprenylxanthone	3.868	394.2018	394.1785	99.3	-1.27	n.d.	1.24	n.d.	n.d.	n.d.	n.d.	n.d.	Phenolic
39	Tylophorine	3.870	394.2012	393.1939	99.5	0.24	n.d.	1.12	n.d.	n.d.	n.d.	n.d.	n.d.	Alkaloid
40	Daphnetoxin	3.981	482.217	482.1937	98.96	0.78	n.d.	3.53	n.d.	n.d.	1.95	n.d.	n.d.	Diterpenoid
41	Valerosidatum	4.053	485.1985	462.2093	98.31	1.79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.43	Monoterpenoid
42	Hellicoside	4.246	639.191	656.1943	98.48	1.43	n.d.	n.d.	n.d.	1.55	n.d.	n.d.	0.99	Phenolic glycoside
43	Dimethyl 3-methoxy-4-oxo-5-(8,11,14-pentadecatrienyl)-2-hexenedioate	4.638	403.2462	420.2495	92.27	4.04	n.d.	3.03	n.d.	n.d.	n.d.	0.83	n.d.	Fatty acid derivative
44	17-oxogrindelic acid	4.639	317.2113	334.2146	85.13	-0.47	n.d.	1.61	n.d.	n.d.	n.d.	n.d.	n.d.	Sesquiterpenoid
45	Methylgingerol	4.744	313.1769	308.1982	98.33	1.75	n.d.	6.67	5.48	1.53	1.32	2.38	n.d.	Methoxybenzene
46	Momilactone B	4.748	313.1787	330.182	93.10	3.40	n.d.	n.d.	n.d.	n.d.	n.d.	1.99	n.d.	Diterpenoid
47	5-*O*-methylembelin	4.749	313.1784	308.1997	95.19	-3.18	1.75	n.d.	5.10	n.d.	1.55	2.11	n.d.	Benzoquinone
48	9,10-dihydroxy-12,13-epoxyoctadecanoate	5.214	353.23	330.2408	99.56	-0.56	n.d.	n.d.	4.15	n.d.	n.d.	n.d.	n.d.	Fatty acid derivative
49	Annosquamosin A	5.904	345.2415	362.2448	92.41	2.56	n.d.	n.d.	n.d.	n.d.	2.84	2.47	n.d.	Diterpenoid
50	Tussilagone	6.096	373.2364	390.2397	96.1	2.32	n.d.	n.d.	n.d.	n.d.	n.d.	2.30	n.d.	Sesquiterpenoid
51	Compactin	6.100	373.2365	390.2398	96.46	2.03	6.72	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Cyclic poliketide
52	9,10-epoxyoctadecatrienoic acid	6.172	275.2009	292.2041	85.88	-1.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.30	Fatty acid derivative
53	7-*O*-acetylaustroinulin	6.289	347.2571	364.2604	95.27	2.68	8.93	n.d.	n.d.	n.d.	n.d.	15.01	n.d.	Diterpenoid
54	Guggulsterone	6.371	325.2155	342.2188	96.31	1.98	n.d.	n.d.	n.d.	n.d.	3.48	3.22	n.d.	Steroid
55	Kaur-16-en-18-oic acid	6.372	285.2211	302.2244	85.86	0.48	12.27	13.36	14.79	n.d.	n.d.	6.02	n.d.	Diterpenoid
56	Methyl dihydrophaseate	6.563	301.141	296.1624	99.42	0.04	n.d.	n.d.	n.d.	4.25	n.d.	n.d.	n.d.	Sesquiterpenoid
57	Eicosapentaenoic Acid	6.567	325.2137	302.2245	92.28	0.33	11.65	5.58	n.d.	n.d.	n.d.	n.d.	n.d.	Fatty acid
58	10-oxo-11-octadecen-13-olide	6.669	277.2168	294.22	85.21	-1.86	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.85	Fatty acid derivative
59	Pheophytin a	6.750	871.5743	870.5671	98.62	-1.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.52	Chlorophyll derivative

No.	Identified compound	RT	*m/z*	Molecular Mass	Score (DB)	Error (ppm)	Relative abundance of each compound (%), from the total combined peak area of all identified compounds in each sample							Class of compound
							HSF	CSF	EASF	BSF	ASF	MSE	MLE	
60	18-oxooleate	6.754	279.2326	296.2358	94.59	-2.35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.07	Fatty acid derivative
61	Dihydrovaltrate	6.756	425.2168	424.2095	99.45	0.48	n.d.	n.d.	n.d.	n.d.	n.d.	2.01	n.d.	Monoterpenoid
62	6-*O*-acetylaustroinulin	6.867	347.2575	364.2608	97.18	1.66	n.d.	n.d.	n.d.	n.d.	n.d.	2.32	n.d.	Diterpenoid
63	(*Z*)-15-oxo-11-eicosenoic acid	6.871	347.2554	324.2662	99.68	0.77	9.24	3.67	5.04	n.d.	n.d.	2.75	n.d.	Fatty acid
64	Levopimaradienal	7.145	287.237	286.2298	85.67	-0.32	n.d.	7.95	n.d.	n.d.	n.d.	n.d.	n.d.	Diterpenoid
65	1-palmitoyl lysophosphatidylcholine	7.328	496.3405	496.3411	90.63	-1.56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.82	Fatty acid derivative
66	Sagittariol	7.334	289.2541	306.2574	92.01	-4.99	n.d.	n.d.	n.d.	n.d.	n.d.	4.68	n.d.	Diterpenoid
67	MG(0:0/16:0/0:0)	7.521	353.2668	330.2776	98.18	-1.71	15.42	3.06	5.32	n.d.	n.d.	n.d.	2.72	Fatty acid derivative
68	(13*R*,14*R*)-7-labdene-13,14,15-triol	7.530	329.2449	324.2662	97.99	0.69	18.94	3.36	23.37	4.97	9.09	2.65	n.d.	Diterpenoid
69	Manool	7.722	273.258	290.2612	99.15	-0.97	15.08	2.58	n.d.	n.d.	n.d.	3.35	n.d.	Diterpenoid
70	Atemoyacin B	8.88	599.4289	594.4503	99.23	-1.2	n.d.	8.99	n.d.	n.d.	n.d.	n.d.	8.39	Fatty acid derivative

Methanolic leaves extract (MLE); methanolic stem extract (MSE); hexane stem fraction (HSF); chloroform stem fraction (CSF); ethyl acetate stem fraction (EASF); butanol stem fraction (BSF); aqueous stem fraction (ASF). n.d. = no detected.

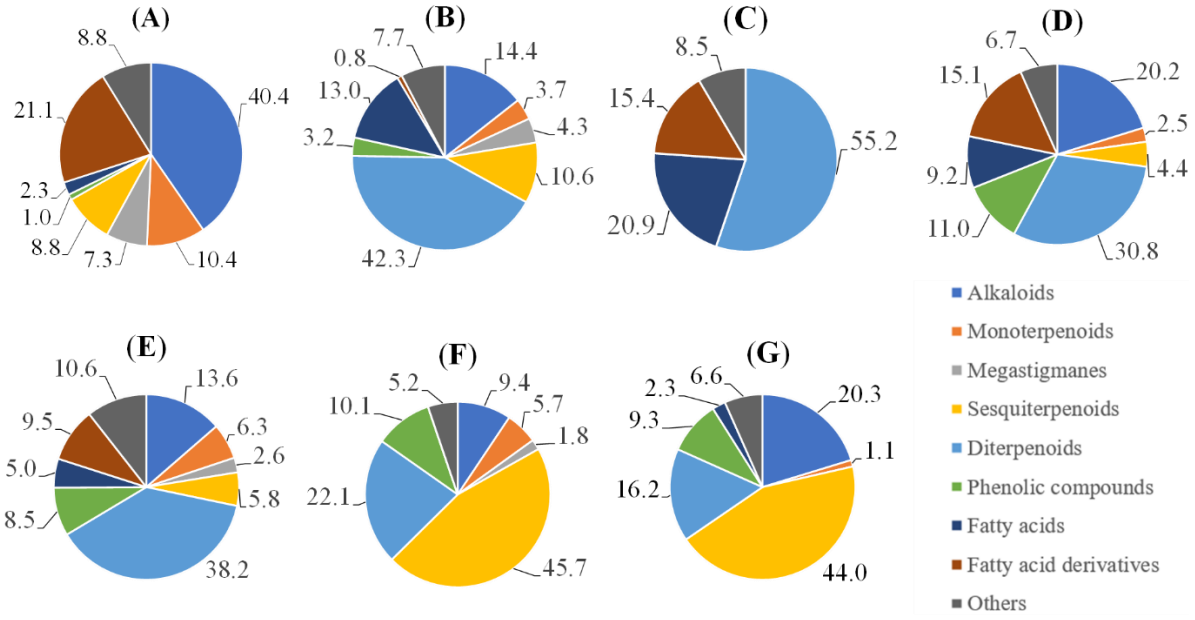

Figure 1. Metabolite Profiling by compound classes of methanolic extracts and fractions of *E. mexicanum*. Each compound class is the sum of relative abundance (%) of the same type of compound in a sample. Methanolic leaves extract (MLE) (A); methanolic stem extract (MSE) (B); hexane stem fraction (HSF) (C); chloroform stem fraction (CSF) (D); ethyl acetate stem fraction (EASF) (E); butanol stem fraction (BSF) (F); aqueous stem fraction (ASF) (G).

Pyrrolizidine alkaloids are toxic alkaloids present in several families including Apocynaceae, Asteraceae and Boraginaceae. The core pyrrolizidine moiety is derived from homospermidine, which is formed from putrescine and spermidine by a reaction catalyzed by homospermidine synthase. In plants, putrescine can be derived from arginine or ornithine. Ornithine decarboxylation is a typical pathway in eukaryotic cells and ornithine decarboxylases have been identified and characterized in plants that produce nicotine and tropane alkaloids.[24]

In Vitro Antibacterial Activity

The antibacterial activity of methanolic leaves extract (MLE) and methanolic stem extract (MSE) of *E. mexicanum* was investigated against six pathogenic strains, three Gram-positive (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus*-MRSA ATCC 43300 and *Streptococcus pyogenes* ATCCC 19615) and three Gram-negative strains (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* clinical isolate, and *Salmonella typhimurium* ATCC 14028). *Table 2* shows the minimum inhibitory concentrations (MICs) of extracts evaluated against the Gram-positive strains with gentamicin (GEN) as the positive control. MSE presented strong antibacterial activity against *S. aureus*, *S. aureus*-MRSA and *S. pyogenes*, with MIC values of 62.5 µg/mL against each strain. The other methanolic extract (MLE) was ineffective against Gram-positive strains up to 500 µg/mL. According to these results, the MSE was subjected to a fractionation with solvents of polarity different, and the five fractions obtained were evaluated against the three Gram-positive strains (*Table 2*). Gram-positive bacteria were susceptible to the fractions, i.e., HSF fraction exhibited strong activity against the three strains (MIC = 62.5 µg/mL against each one). CSF fraction showed moderate activity (MIC = 125 µg/mL) against *S. aureus* and *S. pyogenes* but was ineffective against *S. aureus*-MRSA. EASF fraction showed strong activity (MIC = 62.5 µg/mL) against *S. aureus* and *S. aureus*-MRSA and moderate activity against *S. pyogenes* (MIC = 250 µg/mL). BSF fraction exhibited strong activity (MIC = 62.5 µg/ml) against *S. aureus* and *S. aureus*-MRSA and was ineffective against *S. pyogenes* and ASF fraction showed strong activity against *S. aureus*-MRSA (MIC = 62.5 µg/ml) but was ineffective against *S. aureus* and *S. pyogenes*.

With respect to evaluations against Gram-negative bacteria, both methanolic extracts and fractions obtained from MSE were ineffective, even when applying 500 µg/mL, while the gentamicin positive control showed MIC values of ≤ 0.62 µg/mL against *S. typhimurium* and 1.2 µg/mL against both *E. coli* and *P. aeruginosa*.

On the other hand, the bacteriostatic or bactericidal activity of the five fractions was investigated using MTT assay on the susceptible bacterial strains according to the microdilution assays. Our results showed that CSF, EASF, BSF and ASF presented bacteriostatic activity against susceptible strains. HSF fraction also showed bacteriostatic activity against *S. aureus* and S. aureus-MRSA but showed bactericidal activity against S. pyogenes (*Table 2*). All fractions were evaluated at the corresponding MIC.

Table 2. Antibacterial activity (MIC in µg/mL) of methanolic extracts and fractions of *E. mexicanum* against three Gram-positive strains.

Sample	Strain		
	S. aureus ATCC 6538	*S. aureus*-MRSA ATCC 43300	*S. pyogenes* ATCC 19615
MLE	n.a.	n.a.	n.a.
MSE	62.5	62.5	62.5
HSF	62.5†	62.5†	62.5††
CSF	125†	n.a.	125†
EASF	62.5†	62.5†	250†
BSF	62.5†	62.5†	n.a.
ASF	n.a.	62.5†	n.a.
GEN	≤0.62	20	5

Methanol leaves extract (MLE); methanol stem extract (MSE); hexane stem fraction (HSF); chloroform stem fraction (CSF); ethyl acetate stem fraction (EASF); butanol stem fraction (BSF); Aqueous stem fraction (ASF). Gentamicin positive control (GEN). n.a.: No activity at concentrations evaluated. † Bacteriostatic effect. †† Bactericidal effect.

In this study, the antibacterial inactivity of MLE contrasted with the promising antibacterial activity possessing MSE and its fractions against Gram-positive bacteria tested. These results can be explained from the chemical composition de each sample. According to the results of the UHPLC-QTOF-MS/MS analysis, the chemical profiles of stem extract (MSE) and some its fractions stand out for their high content of diterpenoids. Some of these diterpenoids have been shown to have antibacterial properties, as manool (**69**) and kaur-16-en-18-oic acid (**55**) and probably the presence of such compounds in MSE and its HSF and CSF fractions is related to the activity shown against Gram-positive bacteria. In a study conducted by Ulubelen *et al*., they obtained the manool compound from *Salvia sclarea* and it was found be active against *S. aureus* ATCC 6538 (MIC = 13.75 µg/mL).[25] In another study, manool was obtained from *Salvia tingitana* and it was active against different *Enterococcus* strains, reaching MIC values ranging from 4 to 32 µg/mL.[26] Kaur-16-en-18-oic acid compound also showed antibacterial activity against *S. aureus* ATCC 6538 (MIC = 250 µg/mL) but showed negligible activity against clinical isolates strains (*S. aureus* and *E. coli*) and *E. coli* ATCC 25999.[27] Others compounds detected in high abundance in the fractions obtained from MSE are, the kaurene diterpenoid canavalioside (**22**), the labdane diterpenoids 7-*O*-acetylaustroinulin (**53**) and (13*R*,14*R*)-7-labdene-13,14,15-triol (**68**), as well as the sesquiterpenoids conchosin A (**6**) and scorpioidin (**12**), and the alkaloid senampeline A (**37**); however, to our knowledge, there is not reports on their antibacterial activity.

Our findings show that the MSE of *E. mexicanum* contain important metabolites that can be isolated in further research to investigate their activity against human pathogenic bacteria, including antibiotic-resistant strains.

In Vitro Cytotoxic Activity

Extracts and fractions of *E. mexicanum* were assayed for their cytotoxicity against five human cancer cell lines (HeLa, CaSki, PC3, BT549 and A549) with paclitaxel (PTX) as the positive control. A human noncancerous cell line (HFF) was also used to determinate the selectivity of treatments. Extracts and fractions were evaluated at various concentrations and their effect on cell growth was assessed by MTS cell viability assay. Dose-response curves (Figure S2) were generated for each treatment from which the IC_{50} values were obtained (*Table 3*). MSE exhibited moderate cytotoxicity against PC3 (IC_{50} = 71.7 µg/mL), CaSki (IC_{50}= 93.9 µg/mL), HeLa (IC_{50} = 95.3 µg/mL), A549 (IC_{50} = 98.7 µg/mL) and BT549 (51.6% of cell viability at 100 µg/mL) cell lines and was more potent than MLE against all cancer cell lines tested. Therefore, only MSE fractions were evaluated. When cells were exposed to various concentrations of fractions, cytotoxicity was observed in a concentration-dependent manner (Figure S2). HSF and CSF exhibited the strongest cytotoxic activity against all cancer cell lines with IC_{50} values ranging from 18.1 to 30.8 µg/mL, followed by BSF and ASF with IC_{50} values from 40.2 to 52.0 µg/mL, and EASF showed the lowest cytotoxic activity with IC_{50} values from 80.0 to 94.5 µg/mL. The cell lines more sensible for HSF and CSF fractions were HeLa (IC_{50} =23.6 µg/mL and IC_{50} =20.3 µg/mL, respectively) and PC3 (IC_{50} =19.9 µg/mL and IC_{50} =18.1 µg/mL).

The preferential of an extract, fraction or isolated molecule for kill malignant cells compared to noncancerous cells is essential for therapeutic treatments, this selectivity is identified with the selectivity index (SI), the higher the SI, the greater the toxicity towards cancerous cells than towards noncancerous cells. In this study, all fractions showed 2.1 to 4.2-fold higher cytotoxicity towards cancer cell lines than noncancerous fibroblast (HFF) cells. It is worth mentioning the SI values showed for HSF and CSF fractions against PC3 cells of 3.8 and 4.2, respectively, and of CSF fraction against HeLa cells of 3.8 (*Table 3*).

In this study, manool compound (**69**) was found in the HSF and CSF fractions. This compound has been shown to have cytotoxic and antitumor activities. In a study manool was isolated from *Salvia officinalis* and evaluated against different cancer cell lines exhibiting high cytotoxic activity against HeLa (IC_{50} = 6.7 µg/mL), MO59J (IC_{50} = 9.6 µg/mL) and U343 (IC_{50} = 6.7 µg/mL). In addition, manool was significantly more cytotoxic for tumor cell lines than V79 normal cell line (IC_{50} = 49.3 µg/mL).[28] In another study, manool (also isolated from *S. officinalis* L.) showed a selective cytotoxic effect against murine melanoma cells, and using an *in vivo* model in mice, a significantly decrease of the tumor mass was observed by reduction percentages of 62.4% (oral), 48.5% (intraperitoneal) and 38.8% (subcutaneous) at dose of 20 mg/kg, without toxic effects.[29]

Table 3. Cytotoxic activity (IC_{50} in µg/mL) of methanolic extracts and fractions of *E. mexicanum* against five human cancer cell lines and one noncancerous cell line and selectivity index (SI).

Sample	Cell lines										
	HFF†	HeLa		CaSki		PC3		BT549		A549	
	IC_{50}	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
MLE	> 100	> 100	n.d.	> 100	n.d.	> 100	n.d.	> 100	n.d.	> 100	n.d.
MSE	> 100	95.3 ± 2.3^{d}	n.d.	93.9 ± 2.2^{c}	n.d.	71.7 ± 1.3^{c}	n.d.	> 100	n.d.	98.7 ± 3.6^{c}	n.d.
HSF	75.0 ± 1.6^{a}	23.6 ± 1.4^{a}	3.2	24.1 ± 1.2^{a}	3.1	19.9 ± 1.3^{a}	3.8	25.3 ± 1.2^{a}	3.0	28.2 ± 1.2^{a}	2.6
CSF	76.9 ± 1.8^{a}	20.3 ± 1.4^{a}	3.8	26.6 ± 1.5^{a}	2.9	18.1 ± 1.4^{a}	4.2	28.0 ± 1.3^{a}	2.7	30.8 ± 1.4^{a}	2.5
EASF	240.0 ± 1.4^{d}	80.0 ± 2.3^{c}	3.0	86.7 ± 2.8^{c}	2.8	82.9 ± 2.1^{d}	2.9	87.1 ± 1.9^{c}	2.8	94.5 ± 2.7^{c}	2.5
BSF	129.9 ± 1.3^{c}	40.2 ± 1.3^{b}	3.2	43.4 ± 1.7^{b}	3.0	46.4 ± 1.3^{b}	2.8	52.0 ± 1.3^{b}	2.5	49.2 ± 1.3^{b}	2.6
ASF	106.0 ± 1.2^{b}	41.4 ± 1.5^{b}	2.6	44.1 ± 1.5^{b}	2.4	40.9 ± 1.3^{b}	2.6	47.2 ± 1.4^{b}	2.2	50.0 ± 1.6^{b}	2.1
PTX*	n.d.	7.0×10^{-3}	n.d.	12.0×10^{-3}	n.d.	13.2×10^{-3}	n.d.	17.0×10^{-3}	n.d.	18.0×10^{-3}	n.d.

Results are expressed as means ± SE. Methanol leaves extract (MLE); methanol stem extract (MSE); hexane stem fraction (HSF); chloroform stem fraction (CSF); ethyl acetate stem fraction (EASF); butanol stem fraction (BSF); Aqueous stem fraction (ASF). † Noncancerous cell line. * IC_{50} in µM; n.d.: not determined. Different lowercase letters within a column indicate statistically significant differences between the means ($p < 0.05$).

The studies described above indicate that the manool diterpenoid might be involved partly to the selective cytotoxicity induced by the HSF and CSF fractions towards cancer cells. Furthermore, as we previously mentioned, this same compound may be involved in the antibacterial activity of the same fractions. However, due to manool compound was found greater abundance in HSF than in CSF, probably another metabolite contained in CSF may be participating as a cytotoxic agent.

Pyrrolizidine alkaloids fulvine (**9**), anacrotine (**11**) and senampeline A (**37**) were found in our study for *E. mexicanum*, these metabolites also could be involved in the cytotoxicity of the fractions that contain them. Pyrrolizidine alkaloids are known to have acute hepatotoxicity, genotoxicity and neurological damage in both humans and animals. In last decades, the application of their biological activities to cure disease have increased the interest of researchers. However, most results of pharmacological activities of pyrrolizidine alkaloids are derived from laboratory studies and *in vitro* tests, but clinical studies are needed.[30] For instance, in a study conducted by Culvenor,[31] he evaluated eighteen pyrrolizidine alkaloids and several derivatives against cancer cells. The fulvine alkaloid showed strong activity against adenocarcinoma 755 and Walker 256 (subcutaneous) system. However, activity of fulvine and other metabolites was confined to the solid tumors, being inactive against leukemia 1210 and KB cells.[31]

Our results of cytotoxic activity of extracts and fractions of *E. mexicanum* suggest that important metabolites could be isolated of HSF and CSF to be used in further studies on various human cancer types.

In Vitro Nitric Oxide Inhibitory Activity

In RAW 264.7 murine macrophage cells, lipopolysaccharide (LPS) induces NOS-2, and then NO production. Therefore, this cell line provides an excellent model for drug screening and for evaluation of potential inhibitors on the pathway leading to the induction of NOS-2. The reactive free radical NO synthesized by NOS-2 is a major macrophage-derived inflammatory mediator and has been reported to be involved in the development of inflammatory diseases.[32] Here, the anti-inflammatory activity of extracts and fractions of *E. mexicanum* was evaluated as the ability to inhibit NO production in LPS-stimulated RAW 264.7 cells. To rule out activity due to cytotoxic effects, the effect of extracts and fractions on RAW 264.7 cells growth were evaluated using the MTS cell viability assay. The results showed that the leaves extract (MLE) at concentrations ranging from 1 to 100 µg/mL did not have significantly effect on RAW 264.7 cells growth (*Figure 2A*); neither significantly decreased on NO production at the same concentrations (*Figure 2B*). On the other hand, stem extract (MSE) was significantly cytotoxic at 25 and 50 µg/mL, showing viability rates of 49.2 and 31.2%, respectively; however, at concentrations ≤ 15 µg/mL, it had no negative effect on macrophage viability (*Figure 2A*). Then, the pre-treatment with MSE at 15 µg/mL induced a significant reduction on NO production (56.1%) compared to cells treated only with LPS (*Figure 2B*). This encouraged to continue with the evaluation of the fractions obtained from MSE. Fractions at concentrations of 1 to 30 µg/mL did not affect the cell viability, excluding CSF which significantly affected cell viability at 25 µg/mL (82.6%) and 30 µg/mL (39.0%) (*Figure 2C*). On the other hand, HSF and EASF fractions induced a significant decreased in NO production with a concentration-dependent effect, showing NO production percentages of 50.0 and 51.7%, both at 30 µg/mL. This effect was close to that of the indomethacin (Indo) positive control (NO production 47.9% at 25 µg/mL). CSF also induced a significantly reduction on NO production (69.3%) at the non-cytotoxic concentration of 15 µg/mL. While the

most polar fractions (BSF and ASF) did not show a significantly inhibitory effect on NO production (*Figure 3D*).

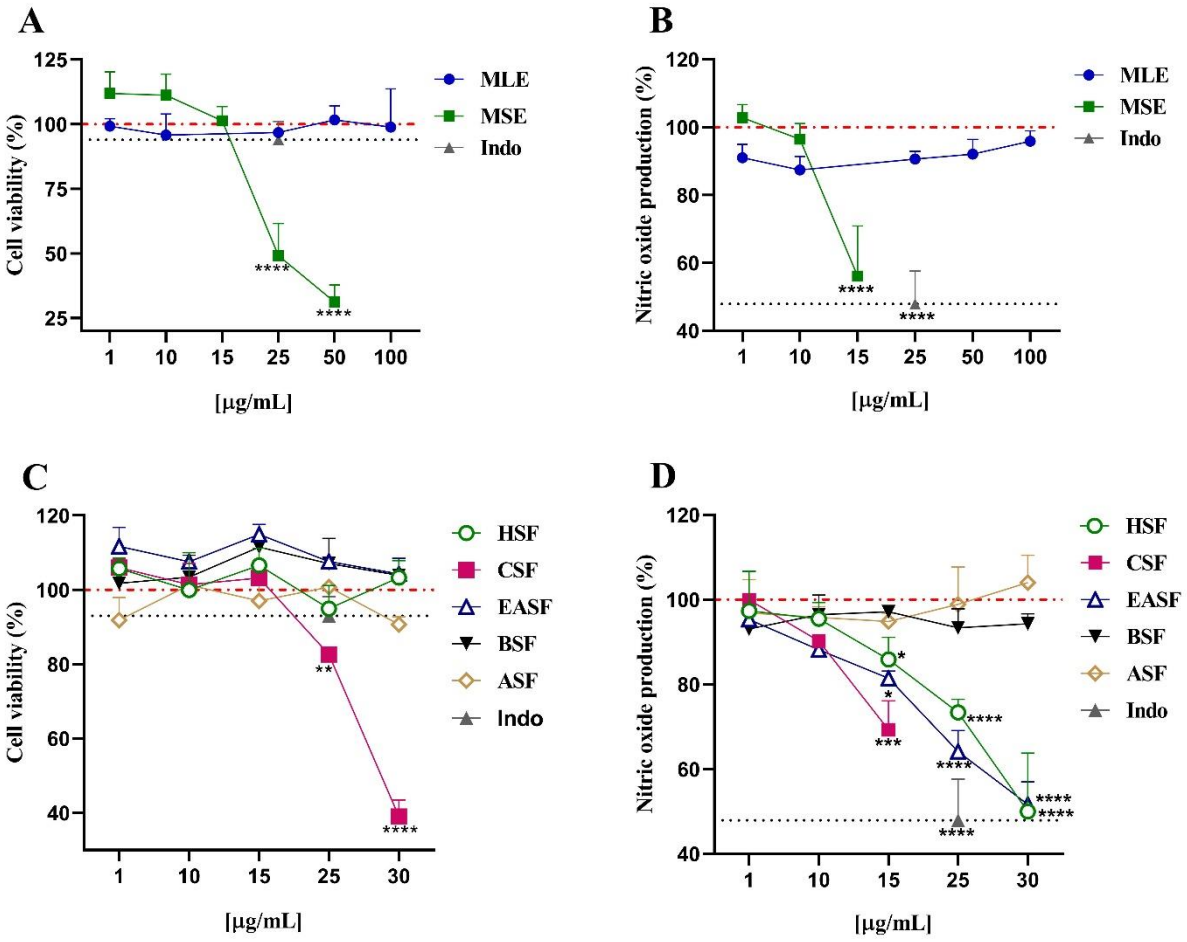

Figure 2. Effect of extracts (A and B) and fractions (C and D) of *E. mexicanum* on cell viability (A and C) and inhibition NO production (B and D) in LPS-stimulated RAW 264.7 cells. Methanol leaves extract (MLE); methanol stem extract (MSE); HSF: hexane stem fraction; CSF: chloroform stem fraction; EASF: ethyl acetate stem fraction; BSF: butanol stem fraction; ASF: aqueous stem fraction. The black horizontal dashed line indicates the effect of indomethacin (Indo) at 25 µg/mL. Values are means ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ represent significance compared to control cells, treated only with LPS (red horizontal dashed line).

The anti-inflammatory activity of HSF, CSF and EASF could be attributed to their high abundance in diterpenoids of *ent*-labdane and *ent*-kaurane type. These metabolites type have been reported with anti-inflammatory activity. For instance, Jang and Yang[33] report that manool (8,14-labdadien-13-ol) from *Phellinus pini* inhibited NO production in LPS-activated RAW 264.7 cells (IC_{50} = 59.9 µM) and the inhibition was not due to cytotoxicity, as determined by MTT assay. In other study in LPS-activated RAW 264.7 cells conducted by Díaz-Viciedo *et al.*,[32] was reports that pre-treating cells with three diterpene acids (1–20 µM), among them kaurenoic acid, an epimer of kaur-16-en-18-oic acid, reduced dose dependently production NO and PGE2. Besides, all diterpenoids significantly inhibited the release of TNF-α in a dose dependent manner. Díaz-Viciedo *et al.*[32] also studied whether the inhibitory effects on the pro-inflammatory mediators are related to NOS-2 and COX-2 modulation, using Western blot. Diterpenoids assayed at 20 µM potently inhibited the expression of NOS-2 and were less efficient regarding the effects on COX-2. Moreover, analysis of NOS-2 and COX-2 mRNA by quantitative PCR revealed that these compounds inhibited LPS-induced expression, once again showing greater activity against NOS-2 induction at the concentrations assayed (1–20 µM). Noteworthy, the diterpene acids did not exert any cytotoxic effects as indicated by MTT reduction assay.[32]

Our results reported here provide new information regarding the anti-inflammatory activity found in fractions obtained from MSE of *E. mexicanum*. In addition, they support that the HSF, CSF and EASF

fractions represent a significant source of diterpenoids that can be considered as a therapeutic alternative of anti-inflammatory drugs.

Conclusions

In this work we determined the chemical profile of extracts and fractions from *Erythroxylum mexicanum* plant using UHPLC-QTOF-MS/MS, in which a great abundance of diterpenoid compounds were found in stem extract and some its fractions, and a great abundance of alkaloids in leaves extract. Our first results demonstrated a strong antibacterial activity of stem extract (MSE) and their fractions against Gram-positive bacterial strains including an antibiotic-resistant strain. Regarding cytotoxic activity, HSF and CSF fractions stood out for having greater selectivity against all cancer cell lines tested. In addition, HSF, CSF, and EASF suppressed NO production in LPS-activated RAW 264.7 cells. Our findings for this species are novel, supporting that *E. mexicanum* represents an important source of compounds such as diterpenoids, alkaloids, and other metabolites. It also provides opportunities to conduct future research focused on the isolation of those metabolites that may be related to antibacterial, cytotoxic and anti-inflammatory effects, as well as to comprehend their molecular mechanism in these biological activities.

Experimental Section

Chemicals

Extracts and fractions were prepared using ACS grade solvents (Fermont®, Monterrey, México). For UHPLC-QTOF-MS/MS analysis, mass spectrometry (MS) grade methanol (J.T. Baker; Phillipsburg, NJ, USA) were used. Dimethyl sulfoxide (DMSO), Indo, LPS from *Escherichia coli* (0111:B4), MTT [3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide], *N*-(1-naphtyl) ethylenediamine dihydrochloride, PTX, sodium nitrite and sulphanilamide were purchased from Sigma–Aldrich (St. Louis, MO, USA). GEN (Garamycin®) was purchased from Schering-Plough (Kenilworth, NJ, USA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI, USA). MEM, high glucose DMEM, DMEM/F12, Advanced DMEM/F12 and RPMI-1640 culture mediums were purchased from GIBCO (Waltham, MA, USA).

Plant Material

Stem, leaves, and fruits of *E. mexicanum* species were collected in August 2018 in the locality of La Gorupa, proximity to the community of Llano Grande de Ipala, El Tuito, Cabo Corrientes, Jalisco, México. The geographical coordinates are 20° 19' 44" North Latitude and 105° 26' 37" West Longitude at 406 meters above sea level. The botanical identification was done by Dr. Daniel Sánchez Carbajal and Dr. Pablo Carrillo Reyes of the Luz María Villarreal de Puga Herbarium of Botany Institute of the University of Guadalajara (IBUG) and a voucher specimen was registered and deposited with number 209657.

Preparation of Crude extracts and Fractions

Stem and leaves of *E. mexicanum* were processed separately to obtain MSE and MLE. In brief, both stem and leaves were dried at 40 °C immediately after collection and then powdered. Then, samples of stem (2.0 kg) and leaves (0.87 kg) was macerated with methanol at room temperature in the dark, three extraction cycles were performed on the same sample for 48 h each. Extracts were filtered and concentrated using an EL-131 Rotavapor (Büchi, Flawil, Switzerland) at 40 °C using reduced pressure. Yields of each crude extract was 4.85% for MSE and 10.31% for MLE. To obtain the fractions, the MSE was subjected to a liquid-liquid fractionation using hexane, chloroform, ethyl acetate, butanol, and water to give five fractions, i.e., 1.89% HSF, 0.70% CSF, 0.36% EASF, 0.84% BSF and 1.04% ASF. All dry extracts and fractions were maintained in the dark at 4°C until used.

UHPLC-QTOF-MS/MS Analysis

Extracts and fractions were dissolved in MS grade methanol (0.5 mg/mL) and filtered through 0.22 µm PTFE membranes before proceeding to UHPLC-QTOF-MS/MS analysis. An Agilent 1290 Infitniy II chromatograph coupled to an AJS ESI source, and an Agilent 6545 QTOF mass spectrometer was used. A sample injection volume of 5 µL was used for chromatographic separation in an Agilent Zorbax C18 reversed phase column (2.1 x 100 mm, 1.7 µm) at 25 °C. The mobile phase consisted of water (A) and methanol (B), both with 0.1% of formic acid at a flow rate of 0.25 mL/min. The gradient used was 35 – 100% B from 0 – 8 min, kept constant for 2 min and initial conditions were recovered in 1 min and keep for 1 additional min. For detection, positive scan and MS/MS was used with range from 100 to 3000 *m/z*, collision energies were fixed at 20, 30 and 40 CE, with VCap and fragmentor values of 3500 V and 175 V, respectively. Results were obtained with MassHunter software for auto MS/MS allowing all adducts for positive mode. Peaks were identified by comparison with Metline_Metabolites_AD library with a mass tolerance of 5 ppm, and a minimum general score of 85.

Antibacterial Activity

Bacterial Strains

Antibacterial activity of extracts and fractions of *E. mexicanum* were evaluated against *Escherichia coli* ATCC 8739, *Streptococcus pyogenes* ATCC 19615, *Pseudomonas aeruginosa* (clinical isolate), *Staphylococcus*

aureus ATCC 6538, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus*-MRSA ATCC 43300.

Broth Microdilution Method

The antibacterial activity was conducted by the microdilution method according to the Clinical Laboratory Standard Institute for microdilution testing for bacteria in broth.[34, 35] The bacteria were incubated under aerobic conditions at 37 °C. Extracts and fractions were solubilized in DMSO and Tween 80, then diluted in Mueller Hinton broth (Difco, Detroit, MI, USA). DMSO and Tween 80 were used as negative controls. Bacterial inoculums of each strain were prepared in 0.85% saline solution adjusted to 0.5 McFarland Nephelometer to assure 1.5×10^8 CFU/mL, with further dilution to a final concentration of 5×10^5 CFU/mL. 96-well plates were used, and a row of wells was considered as a growth control (100 mL of broth plus 100 mL of inoculum) and another row was used as a sterility control (200 mL broth). To test the antibacterial activity, was mixed 100 ml of the bacterial inoculum plus 100 ml of the extract or fraction solution resulting in concentrations ranging from 3.9 to 500 µg/mL. The plates were covered and incubated for 24 h at 37 °C. The absorbances at 600 nm were obtained using Glomax Multidetection system (Promega, Madison, WI, USA). GEN was used as a reference antibiotic using concentrations from 0.15 to 40 µg/mL. The MIC values were defined as the lowest concentrations at which no bacterial growth was observed after incubation.

MTT Bacterial Viability Assay

The MTT assay was performed according to the microplate protocol of Grela *et al.*[36] The bacterial inoculum plus fractions at MIC concentrations were added in 96-well plates as the method described above. After 24 h of bacterial incubation with the treatments at 37 °C, 10 µL of reconstituted MTT (0.4 mg/mL) were added to each well and incubated for 4 h. MTT is a yellow tetrazolium salt, which is reduced to purple formazan by dehydrogenases in a living cell. The amount of formazan produced is directly proportional to the number of living cells; then, we can determine of bacteriostatic or bactericidal activity.

In Vitro Cytotoxic Activity

Cell Lines

The cytotoxic activity of extracts and fractions were evaluated in five human cancer cell lines: A549 (lung), BT-549 (breast), PC-3 (colon), HeLa and CaSki (cervical). In addition, a human fibroblast cell line (HFF) was included as a control of noncancerous cells. All cell lines were obtained from ATCC (Manassas, VA, USA). PC3, CaSki and BT-549 cells were cultured in RPMI-1640 medium, HeLa cells in high glucose DMEM medium, A549 cells in DMEM/F12 medium and HFF cells in MEM medium. All mediums were supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. All cultures were incubated at 37 °C in 5% CO_2 atmosphere.

Cytotoxicity Assay

For cytotoxicity assay, 8000 cells per well were seeded in 96-well plates. Cancer cells were treated with extracts or fractions at the concentrations of 0.01, 0.1, 1, 10 and 100 µg/mL, while for HFF cells the concentration of 1000 µg/mL was also included. PTX drug was used as a positive control. Samples were dissolved in DMSO, ensuring that the final concentration in the wells did not exceed 0.5%. Additionally, DMSO (0.5%) (vehicle) was used as a negative control, in which no cell growth inhibition was observed. Cells treated were incubated for 48 h at 37 °C in 5% CO_2. Then, the cytotoxicity was determined by the MTS assay according to manufacturer's instructions. The absorbances were measured at 450 nm using an automatic microplate reader (Promega, Madison, WI, USA). The IC_{50} values were determined by nonlinear regression analysis. To determine degree of selectivity of the treatments, SI was established by following equation (Eq. 1).[37]

SI = IC_{50} value of test sample on HFF normal cells / IC_{50} value of test sample on cancer cells corresponding (Eq. 1)

Inhibition NO Production in RAW 264.7 Cells

Cell Culture

RAW 264.7 cells (ATCC TIB-71: murine macrophages) were cultured in Advanced DMEM/F12 medium supplemented with 1% glutamax, 3% FBS and 1% antibiotic/antimycotic at 37 °C in a humidified incubator containing 5% CO_2.

Stimulation with LPS and Cell Viability Assessment

The effect of extracts and fractions on cell viability of LPS-stimulated RAW 264.7 cells was determined by using the MTS assay. Briefly, RAW 264.7 cells (2×10^4 cells/well in 200 µL of medium) were seeded into 96-well plates and incubated at 37 °C for 24 h under 5% CO_2. Then, cells were treated with different concentrations of extracts or fractions (1–30 µg/mL) or drug control Indo (25 µg/mL). DMSO (0.42%) was considered as a negative control to exclude the deleterious effect of the vehicle. The cells treated were incubated for 2 h, followed stimulation with LPS (1 µg/mL) for 21 h. Then, cell-free supernatants were collected, and a fresh portion was used for nitrite quantification (see the next section). After supernatants were collected, 200 µL of serum-free fresh medium and 20 µL of MTS was added to each well, and cells were incubated for another 4 h. Then, absorbance at 490 nm

was measured on a microplate reader. The mean absorbance at 490 nm of cells treated only with LPS was considered as the control of 100% cell viability. Cell viability percentage induced by treatments was estimated by following equation (Eq. 2).

Cell viability (%) = [Mean A of cells with treatment / Mean A of cells treated only with LPS] x 100 (Eq. 2)

Where A means absorbance.

Determination of Nitrite

Nitrite is a stable metabolite of NO, and its concentration in the supernatants was calculated with Griess reagent as in a previous report.[38] Briefly, cell-free supernatants (50 µL) were mixed with Griess reagent solution [50 µL of 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride and 50 µL of 1% sulphanilamide both in 2.5% phosphoric acid)] and the mixture incubated at room temperature for 10 min. Then, the absorbance at 540 nm was measured with a microplate reader and nitrite concentration was calculated using a standard curve of $NaNO_2$. The mean nitrite concentration of supernatants of cells control treated only with LPS was taken as 100% NO production. A batch of cells maintained only with culture medium was considered as a control of unstimulated cells.

Statistical Analyses

All experiments were conducted in three independent experiments, each in triplicate. Statistical analyses were performed using GraphPad Prism 8.0 software. One-way analysis of variance (ANOVA) was used to detect statistically significant differences in cytotoxicity and NO inhibition assays, followed by Tukey's and Dunnett's post hoc tests, respectively. A p value < 0.05 was considered statistically significant.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

I.H.-D. designed the study and writing original draft. M.A.R.-C., L.A., J.N.S.-C., M.C.C.-P. and A.B.-A. contributed to the study design. M.A.R.-C. performed the experiments by UHPLC-QTOF-MS/MS and analyzed the data obtained. I.H.-D., J.N.S.-C. and M.C.C.-P. performed the *in vitro* experiments and acquired and analyzed the data. L.A, J.A.S.-G. and A.B.-A provided resourcing. A.B.-A supervised the study. All authors reviewed draft and participated in the paper final version.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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