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Non-targeted metabolomics characterization of *Annona muricata* leaf extracts with anti-angiogenic activity

Casimiro Cárdenas [a,b], José Antonio Torres-Vargas [a,c], Abel Cárdenas-Valdivia [d], Nuria Jurado [a], Ana R. Quesada [a,c,e], Melissa García-Caballero [a,c], Beatriz Martínez-Poveda [a,c,f], Miguel Ángel Medina [a,c,e,*]

[a] Universidad de Málaga, Andalucía Tech, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, E-29071 Málaga, Spain
[b] Research Support Central Services (SCAI) of the University of Málaga, Spain
[c] IBIMA (Biomedical Research Institute of Málaga), E-29071 Málaga, Spain
[d] Department of Physical Chemistry, Faculty of Sciences, University of Malaga, Malaga, Spain
[e] CIBER de Enfermedades Raras (CIBERER), E-29071 Málaga, Spain
[f] CIBER de Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain

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ABSTRACT

The tropical plant *Annona muricata* has been widely used for traditional ethnobotanic and pharmacologic applications. Extracts from different parts of this plant have been shown to have a wide range of biological activities. In the present study, we carry out a metabolomic study of both aqueous and DMSO extracts from *Annona muricata* leaves that has allowed us to identify 33 bioactive compounds. Furthermore, we have shown that aqueous extracts are able to inhibit endothelial cell migration and both aqueous and DMSO extracts inhibit the formation of tubule-like structures by endothelial cells cultured on Matrigel. We conclude that extracts of *Annona muricata* leaves have great potential as anti-angiogenic natural combinations of bioactive compounds.

1. Introduction

Annona muricata is an evergreen tree with a height of 3–8 m, native to the Caribbean and tropical areas of America, although currently is widely propagated. Its edible fruit is called graviola, guanábana, guyabano or soursop. The seed, the pulp of the fruit and the leaves contain many bioactive compounds related with the traditional ethnopharmacological uses of this plant. In particular, many acetogenins [1–5], megastigmanes [6], as well as isoquinoline and aporphine alkaloids [7,8] have been identified and isolated from *Annona muricata* extracts.

Annona muricata has many traditional and ethnopharmacological uses [9]. Since the identification of the first acetogenins in the nineties, an increasing list of bioactivities is associated to extracts from this plant, including antiparasitic [10–14], antibacterial [15], antiviral [16], molluscocidal [17,18], antidiabetic [19], anti-inflammatory [20,21], antioxidant [16,19,22–25], and antitumoral [25–33] effects. Recently, *Annona muricata* acetogenins have been evaluated as potential anti-SARS-CoV-2 agents [34]. Many of these biological activities of *Annona muricata* extracts have been extensively reviewed elsewhere [9,35–38].

In spite of all this detailed knowledge, few omics studies have been carried out with extracts from this plant. Years ago, a functional proteomics study was published revealing that an ethanol extract of its leaves induced endoplasmic reticulum stress and apoptosis in HepG2 hepatocarcinoma cells [39]. The present study aims to carry out a metabolomic characterization of aqueous and DMSO extracts from *Annona muricata* leaves and to test their potential anti-angiogenic effects using in vitro assays of migration and tube differentiation with endothelial cells.

2. Material and methods

2.1. Plant material

Dried graviola leaf powder was purchased from Tentorium Energy SL

* Corresponding author at: Universidad de Málaga, Andalucía Tech, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, E-29071 Málaga, Spain.
E-mail address: medina@uma.es (M.Á. Medina).

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(Tarragona, Spain). To prepare the aqueous extract, 25 g of graviola powder were weighed and added to 500 mL of MilliQ water, incubated in a water bath at 80 °C for 10 min, centrifuged at 13,000 g and the supernatant was recovered, filtered, and finally, it was frozen at − 80ºC, lyophilized and reconstituted with sterile water to a final concentration of 1 mg/mL. The DMSO extract was prepared by weighing 1 g of graviola powder and resuspending it in 10 mL of DMSO, then it was incubated for 5 min at room temperature with gentle shaking, centrifuged at 13,000 g, and the supernatant was recovered and filtered with PTFE filters. 100 μL of the filtered supernatant were taken, dried under a nitrogen jet, and redissolved in acetonitrile to a final concentration of 1 mg/mL.

2.2. HPLC with diode array detector and charged aerosol detector analyses

HPLC was performed on a Dionex 3000 system (Thermo Scientific) linked to a diode array detector (DAD) and a charged aerosol detector (CAD). Separation was achieved into a C18 reversed phase column (Hypersil Gold, particle size 1.9 μm, 50 ×2.1 mm, Thermo Scientific). The mobile phases consisted of purified water (solvent A) and acetonitrile (solvent B). Compounds were eluted from the analytical column with a 45 min gradient ranging from 2% to 100% solvent B, followed by an isocratic gradient at 100% solvent B for 7 min before returning to initial conditions. The HPLC profiling was performed at 30ºC at a constant flow rate of 400 μL/min. The DAD detector was selected at 280 nm, 320 nm, and 370 nm.

2.3. UHPLC-HRMS analysis

Samples were injected into an Easy nLC 1200 UHPLC system coupled to a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). Data was acquired using Tune 2.9 and Xcalibur 4.1.31.9 (Thermo Scientific). Separation was achieved by automatically loading samples into a 25 cm analytical column (PepMap RSLC C18, 2 μm, 100 A, 50 μm x 15 cm, Thermo Scientific). The binary gradient mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 80% acetonitrile (solvent B). Compounds were eluted from the analytical column with a 60 min gradient ranging from 2% to 70% solvent B, followed by a 10 min gradient from 70% to 98% solvent B and finally, to 98% solvent B for 10 min before re-equilibration to 2% solvent B at a constant flow rate of 300 nL/min.

Data acquisition was performed in the electrospray ionization positive mode. MS1 scans were acquired from *m/z* 100–1300 at a resolution of 120,000. For target-Selected Ion Monitoring data-dependent MS/MS acquisition method (t-SIM/ddMS2), precursor ions were isolated within a 4 *m/z* window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher energy collisional dissociation cell (HCD) with a collision energy of 35 eV and detected in the Orbitrap mass analyzer at a resolution of 60,000.

Raw data was analyzed using Xcalibur Qual Browser 4.2 software (Thermo Fisher).

2.4. CFM-ID peak assignment

Prediction of MS/MS fragmentation and search of the unknown spectrum against predicted candidate spectra were based on the use of the freely available software Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) version 3.0 [40]. To get the assignment of a putative fragment annotation to the peaks in each spectrum, a single input spectrum (for high energy level) with the top 20 peaks and their most likely chemical structures (in InChI format) based on their formula and the available data in *A. muricata* literature were used. For CFM-ID peak assignment, chemical structures were obtained from PubChem database, positive ionization mode was selected, and 10 ppm mass tolerance was applied to matching peaks of unknown and candidates.

2.5. GNPS platform

Briefly, Thermo RAW files from UHPLC-HR targeted SIM data dependent MS/MS data were submitted to the Global Natural Products Social (GNPS) Molecular Networking web-platform [41] and a molecular network was created using the online workflow on the GNPS website (http://gnps.ucsd.edu). The data were filtered with default parameters and the network was then created with edges filtered to have a cosine score above 0.70 and more than 6 matched peaks. The MS/MS spectra in the network were then searched against GNPS' spectral libraries filtered in the same manner as the input data.

2.6. Cell culture

Bovine aortic endothelial cells (BAECs) were isolated from bovine aortic arches as previously described [42] and maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% FBS.

2.7. MTT cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical, St. Louis, MO) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, which can be measured spectrophotometrically. BAEC were incubated for 3 days in each well with serial dilutions of aqueous or DMSO *A. muricata* extracts (37 ºC, 5% CO2 in a humid atmosphere), 10 μL of MTT (5 mg/mL in PBS) was added to each well and the plate was incubated for a further 4 h (37 ºC). The resulting formazan was dissolved in 150 μL of 0.04 N HCl-2 propanol and read at 550 nm. Four samples for every tested concentration were included in each of three independent experiments. IC_{50} values were calculated as those concentrations of *A. muricata* extract yielding a 50% of cell survival, taking the values obtained for control as 100%.

2.8. Wound healing assay

The wound healing assay was carried out as described by us elsewhere [43]. Briefly, confluent monolayers of BAEC in 6-well plates were wounded and wells were supplied with complete medium in the absence or presence of *A. muricata* extracts. Wounded areas were observed and photographed at 0 and 7 h of incubation with a microscope camera Nikon DS-Ri2 coupled to a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). The amount of migration was determined by image analysis and was first normalized to their respective values at time zero, and then normalized against the control using Image J software to determine the difference of treatment versus control.

2.9. Tube formation on Matrigel by endothelial cells

The formation of tubule-like structures by endothelial cells on Matrigel is frequently used. We carried out this assay as previously described by us elsewhere [44]. Briefly, Matrigel (50 μL of 10.3 mg/mL) at 4 ºC was used to coat each well of a 96-well plate and allowed to polymerize at 37 ºC for a minimum of 30 min 5×10^4 BAECs were added with 200 μL DMEM. Finally, the indicated concentrations of the compounds to be tested were added and incubated at 37 ºC in a humidified chamber with 5% CO2. After incubation for 5 h, cultures were photographed with camera Nikon DS-Ri2 coupled to a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). Each concentration was tested in duplicate with three independent experiment, and staurosporine 2 μM was used as a routine positive assay control. Two different observers evaluated the inhibition of tube formation.

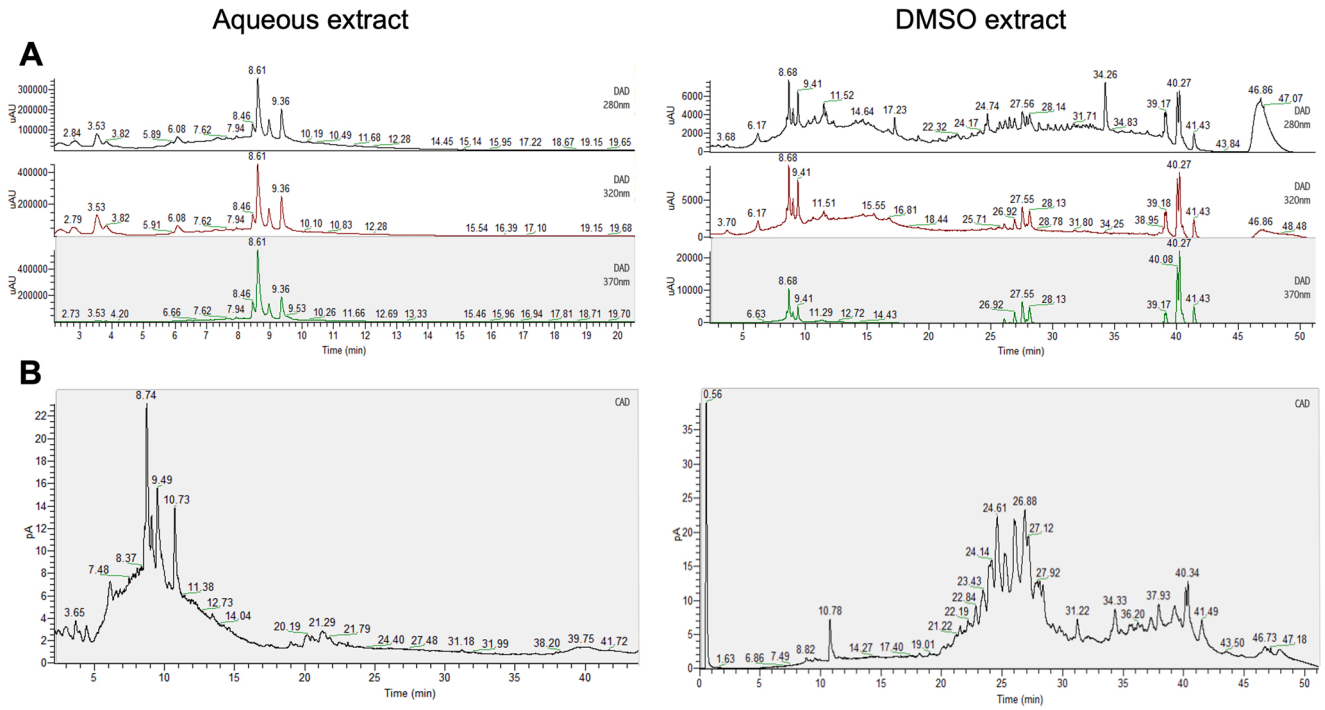


Fig. 1. High performance liquid chromatography diode array detector (HPLC-DAD) (A) and charged aerosol detector (-CAD) (B) profiling of aqueous (left) an DMSO (right) *Annona muricata* extracts. A C18 reversed phase column was used to achieve separation and the DAD detector was selected to display ultraviolet absorbance patterns at 280 nm, 320 nm and 370 nm.

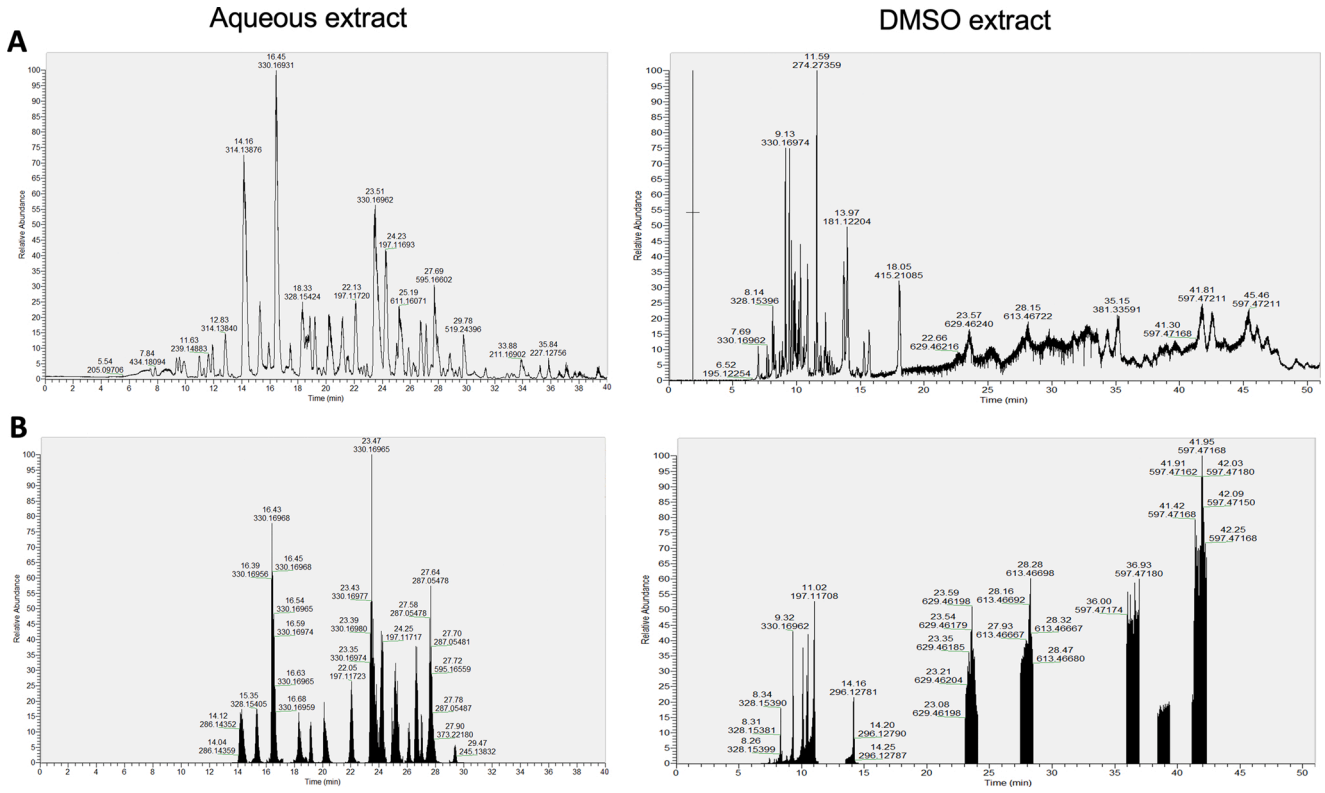


Fig. 2. High-resolution mass spectrometry analysis performed to identify bioactive compounds associated with the biological activities evaluated in this study. Images show UHPLC-HRMS full scan base peak (A) and targeted SIM-MS/MS total ion chromatograms (B) of aqueous (left) an DMSO (right) *Annona muricata* extracts.

Table 1
Compounds identified from *Annona muricata* leaf extracts by UHPLC-HRMS using the CFM-ID.

Compound identified	Formula	Retention time (min)	*m/z* $[M+H]^+$	Error (ppm)	Top 20 assigned fragments	MassBank similhinarity	Class[a]
Aqueous extract							
Loliolide	C11H16O3	22.12	197.1172	0.1015	11	947	MG
Vomifoliol	C13H20O3	18.84	225.1484	0.5330	15	–	MG
Coclaurine	C17H19NO3	14.10	286.1435	0.9436	12	980	ALK
Kaempferol	C15H10O6	26.75	287.0547	1.0799	4	986	MG
Catechin	C15H14O6	19.20	291.0861	0.7214	13	605	FG
Argentinine	C19H21NO2	27.49	296.1643	0.6753	8	–	FG
Stepharine	C18H19NO3	14.10	298.1434	1.2410	6	–	ALK
Quercetin	C15H10O7	26.31	303.0498	0.3960	7	999	FG
Norcorydine	C19H21NO4	18.43	328.1541	2.4318	5	–	ALK
Reticuline	C19H23NO4	16.45	330.1697	0.8480	11	997	ALK
Chlorogenic acid	C16H18O9	16.69	355.1021	0.7266	12	999	FG
Citroside A	C19H30O8	18.84	387.2010	0.8781	17	–	MG
Annoionoside	C19H34O9	16.41	407.2273	0.6139	6	–	MG
Isoquercitin	C21H20O12	26.31	465.1028	0.1075	8	936	FG
Kaempferol 3-O-rutinoside	C27H30O15	27.69	595.1655	0.4032	5	999	FG
Rutin	C27H30O16	25.19	611.1602	0.7527	4	976	FG
DMSO extract							
Loliolide	C11H16O3	10.84	197.1172	0.1015	13	970	MG
Anonaine	C17H15NO2	9.51	266.1173	0.9394	5	–	ALK
Coclaurine	C17H19NO3	7.29	286.1434	1.2931	10	878	ALK
Kaempferol	C15H10O6	10.31	287.0547	1.0799	9	997	FG
Xylopine	C18H17NO3	14.03	296.1279	0.7092	13	–	ALK
Quercetin	C15H10O7	9.88	303.0496	1.0559	15	997	FG
Isolaureline	C19H19NO3	9.56	310.1433	1.5154	7	–	ALK
Norcorydine	C19H21NO4	8.16	328.1539	1.3104	7	–	ALK
Reticuline	C19H23NO4	9.15	330.1696	1.1509	6	–	ALK
Chlorogenic acid	C16H18O9	8.69	355.1019	1.2898	4	–	FG
Isoquercitin	C21H20O12	10.20	465.1024	0.7525	14	997	FG
Corossolone	C35H62O6	38.90	579.4612	1.2425	16	–	AGE
Rutin	C27H30O16	9.88	611.1601	0.9163	9	–	FG

[a] ALK: alkaloid; AGE: acetogenin; MG: megastigmane; FG: flavonol glycoside.

Table 2
Compounds identified from *Annona muricata* leaf extracts using GNPS.

Compound identified	Retention time (s)	*m/z* $[M+H]^+$	Shared Peaks	MQScore
Kaempferol 3-O-rutinoside	1584.64	595.166	9	0.963
Chlorogenic acid	973.70	355.102	10	0.948
Rutin	1484.79	611.161	10	0.944
Isoquercitin	1549.85	465.103	9	0.939
Reticuline	958.45	330.170	7	0.887
Coclaurine	845.81	286.144	9	0.853
Loliolide	1298.21	197.117	6	0.833
Blumenol C glucoside	1599.96	373.222	6	0.759
Quercetin	1494.26	303.051	7	0.751
Datiscetin	1588.88	287.055	6	0.701

Compounds potentially identified by MS/MS spectral comparison; Retention time (min); *m/z* of the precursors for detecting MS/MS fragments; shared peaks indicate the number of MS/MS fragments shared between the experimental spectra and the reference spectra; MQScore suggests the chemical similarity, the MQScore value ranges from 0 to 1.

2.10. Statistical analysis

Quantitative data are given as means±S.D. for at least three independent experiments. Statistical significance was determined using the two-sided unpaired Student's *t*-test. Differences were considered to be significant when $p < 0.05$.

3. Results and discussion

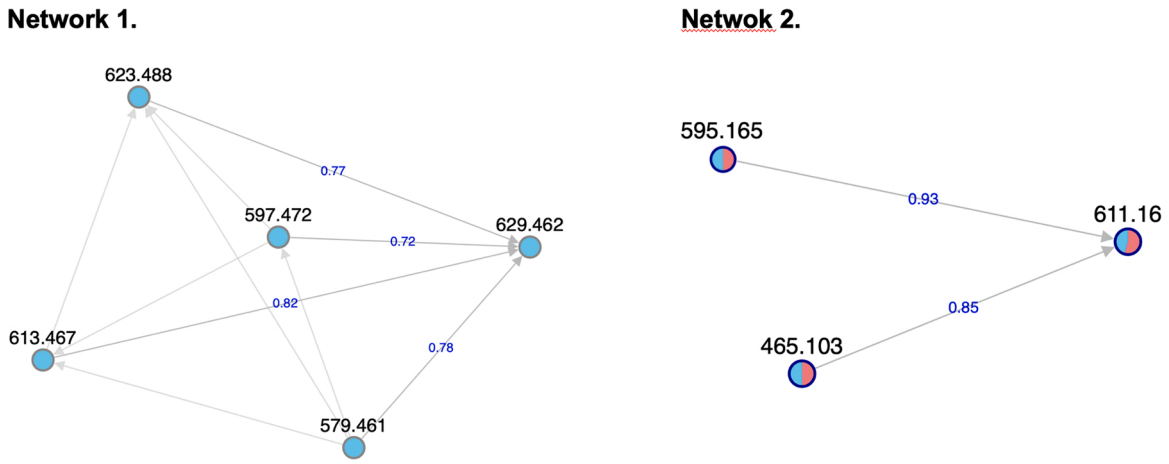
3.1. Extraction and general analysis of Annona muricata extracts

It is well known that *Annona muricata* is a rich source of acetogenins, megastigmanes and other bioactive compounds with different polarity [1–8]. In order to better characterize this plant as a source of bioactive compounds with pharmacological interest, we carried out both an aqueous and a DMSO extract of powder leaves. Both extracts were then submitted to high performance liquid chromatography (HPLC) using a C18 reversed phase column to achieve a proper separation and two different detectors, a diode array detector (DAD) selected to display ultraviolet absorbance patterns at 280, 320 and 370 nm, and a charged aerosol detector (CAD). The obtained profiles are depicted in Fig. 1.

Table 3
Absorption peaks of 10 compounds identified from *Annona muricata* leaf extracts.

	Wavelengths (nm)	Reference
Kaempferol 3-O-rutinoside	266, 348	[45]
Chlorogenic acid	240, 298, 326	[45]
Rutin	256, 266, 352	[45]
Isoquercitin	256, 266, 352	[45]
Quercetin	256, 372	[45]
Datiscetin	348	[46]
Reticuline	280	[47]
Coclaurine	304	[48]
Loliolide	230	[49]
Blumenol C glucoside	245	[50]

As expected, chromatograms from aqueous extracts were very different to those obtained from DMSO extracts. The ultraviolet absorption pattern of the DMSO extract is significantly more complex than the aqueous extract (Fig. 1A). This suggests that a greater diversity of compounds is isolated by DMSO extraction than by aqueous extraction. Most of the compounds in the aqueous extract detected by ionization with CAC eluted on the reversed phase column at a very short retention time (RT), concentrating from 5 to 20 min (Fig. 1B). Since the mobile phase gradient ranges from 2% to 100% acetonitrile in water, it suggests that most of the compounds obtained by aqueous extraction are polar in nature, as expected. However, when the extraction was done with DMSO



Spectral Families

Cluster ID	Number of spectra	Precursor m/z $[M+H]^+$	Group(s)	Library ID
Network 1				
28	12	579.461	G2	-
30	12	597.472	G2	-
32	6	613.467	G2	-
33	6	623.488	G2	-
34	6	629.462	G2	-
Network 2				
29	9	595.165	G1,G2	Kaempferol 3-O-rutinoside
27	10	465.103	G1,G2	Isoquercitin
31	11	611.160	G1,G2	Rutin

Fig. 3. Spectral families molecular network created using the web-based mass spectrometry ecosystem Global Natural Products Social (GNPS) workflow. Each circle (node) in the network panel represents a consensus spectrum and edges represent related fragmentation patterns showing the cosine score. Blue nodes correspond to compounds present in the DMSO extract (G2 group) and red nodes correspond to compounds present in aqueous extract (G1 group). The table shows the two distinct spectral families that comprise the molecular network, the number of consensus spectra, the precursors for detecting MS/MS fragments, the group they belong to and the compound that were identified by library search.

the elution profile practically concentrated at a RT range of 20–45 min (Fig. 1B), suggesting compounds of a more lipophilic nature.

3.2. *Identification of compounds from Annona muricata extracts by non-targeted metabolomics methods*

To further proceed with the characterization of the extracts and the identification of bioactive compounds present in them, we used non-targeted metabolomics methods. Firstly, a full scan was performed, and the peaks were manually filtered searching, on the one hand, for the mass peaks coinciding with known compounds from a bibliographic searching on *A. muricata*, and on the other, for the unknown peaks whose isotopic profiles looked like organic compounds. Secondly, from the data of these masses and their corresponding retention times an inclusion list was created and a targeted SIM-MS/MS was performed. 59 SIMs traces were set in total. Full scan base peak and targeted SIM-MS/MS total ion chromatograms are depicted in Fig. 2A and B, respectively.

Using the freely available software Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) version 3.0 [40] as described in the Material and methods section, it was possible to identify 16 bioactive compounds in the aqueous extract and 13 in the DMSO extract, as listed in Table 1. All these compounds are included in four major classes of bioactive compounds, namely, alkaloids, acetogenins, megastigmanes, and flavonol glycosides.

Additionally, Thermo RAW files from UHPLC-HR targeted SIM data dependent MS/MS data were submitted to the Global Natural Products Social (GNPS) Molecular Networking web-platform [41] as described in Material and methods section. Table 2 shows all the 10 library matches found between the consensus MS/MS spectra and the GNPS MS/MS Spectral Libraries. Two of them are compounds that had not been previously identified with CFM-ID, namely, blumenol C and datiscetin.

Figs. S1 to S10 (Supplementary information) show all the library matches between the consensus MS/MS spectra and the GNPS MS/MS Spectral Libraries. In connection with the peaks detected after the initial HPLC analysis (Fig. 1), a bibliographic search [45–50] has allowed to assign peaks to each of the 10 compounds listed in Table 2 (see Table 3).

All the 33 consensus spectra in the molecular networking analysis are listed in Table S1 (Supplementary information).

The use of the web-based mass spectrometry ecosystem Global Natural Products Social (GNPS) workflow allowed us to obtain two molecular networks of spectral families (Fig. 3).

Network 1 includes spectra from acetogenins present in DMSO extracts. They were included in five clusters, from which only cluster 28 could be identified by CFM-ID as corossolone, an annonaceous acetogenin. For the rest of clusters in this network 1, from their exact molecular mass, their formulae can be deduced and, taking into account the available literature, 1–7 different acetogenins can be suggested for each cluster (Table 4).

Network 2 includes only three clusters (29, 27 and 31), corresponding to 3 well characterized flavonol glycosides present in both

Table 4
Compounds identified (only corossolone) or suggested for the five clusters of network 1.

	Compound	Formula	m/z $[M+H]^+$
AGE	Corossolone	C35H62O6	579.46192
AGE	Isoannonacin	C35H64O7	597.47248
AGE	Annocatalin	C35H64O7	597.47248
AGE	Annocatacin	C35H64O7	597.47248
AGE	Annonacin	C35H64O7	597.47248
AGE	Goniothalamicin	C35H64O7	597.47248
AGE	Gigantetrocin A	C35H64O7	597.47248
AGE	Muricatetrocin A	C35H64O7	597.47248
AGE	Annomuricin A	C35H64O8	613.46739
AGE	Muricapentocin	C35H64O8	613.46739
AGE	Muricatocin	C35H64O8	613.46739
AGE	Annopentocin	C35H64O8	613.46739
AGE	Gigantetronenin	C37H66O7	623.48813
AGE	Annohexocin	C35H64O9	629.46231
AGE	Murihexocin	C35H64O9	629.46231
AGE	Muricoreacin	C35H64O9	629.46231

All the compounds are acetogenins (AGE). Corossolone is the AGE identified corresponding to cluster 28. Suggested AGEs for clusters 30, 32, 33 and 34 are highlighted on green, blue, pink and orange backgrounds.

aqueous and DMSO extracts, namely, kaempferol-3-O-rutinoside, isoquercitin and rutin, respectively (see Fig. 3).

Taking all the so far presented data, our metabolomics approach has allowed for the deepest molecular characterization of *Annona muricata* extracts described so far.

3.3. Evaluation of the anti-angiogenic potential of Annona muricata extracts

Extracts of *Annona muricata* have been related with different biological effects, some of them sharing features with anti-angiogenic agents, as is the case for several anti-inflammatory [20,21] and anti-tumor [25–33] effects previously described. Furthermore, several of the identified compounds have been previously shown to have anti-angiogenic effects [51–54]. However, the anti-angiogenic potential of *Annona muricata* extracts has not been previously studied. To fulfill this aim, in the present study we used BAECs cultured in the presence or absence of either the aqueous or the DMSO extract of *Annona muricata* leaves.

Fig. 4 shows the survival curves of BAEC incubated for 3 days in the presence of different concentrations of both aqueous and DMSO extracts of *Annona muricata* leaves, as determined with the MTT assay according to the instructions provided in Material and methods section. From these curves, IC_{50} values of 962 ± 38 μg/mL and 983 ± 345 ng/mL were determined for the aqueous and the DMSO extract, respectively. It is noteworthy that DMSO extracts yielded IC_{50} values three orders of magnitude lower than aqueous extracts. In any case, the IC_{50} values for aqueous extracts were similar to those we obtained using HT-1080 human fibrosarcoma cells as internal controls (results not shown). This seems to indicate that the long-term cytotoxic effects of *Annona muricata* leaf extracts were not cell specific.

The rest of experiments with BAECs described in the present study were carried out after short-term treatments, under conditions where there were no relevant cytotoxic effects. Migration of activated endothelial cells is a key step in the angiogenic process [55]. The effects of *Annona muricata* on the migratory potential of BAEC could be easily determined by using the wound healing assay as described in Material and methods. Fig. 5 shows that tested concentrations of aqueous extracts

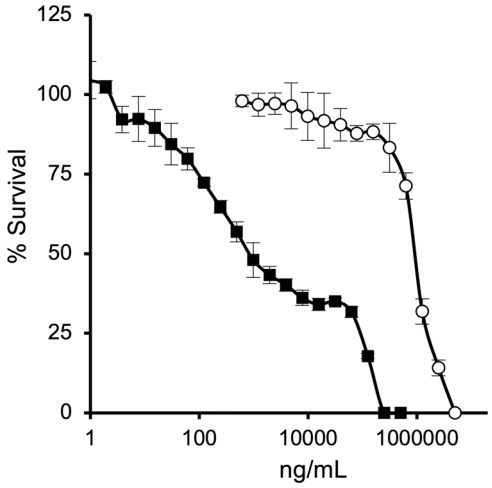


Fig. 4. Survival curves of BAEC, bovine aortic endothelial cells, treated with aqueous (white circles) and DMSO (black squares) *Annona muricata* leaf extracts. Concentrations are represented in logarithmic scale. Depicted data are means of values of three independent experiments, each one with quadruplicate samples.

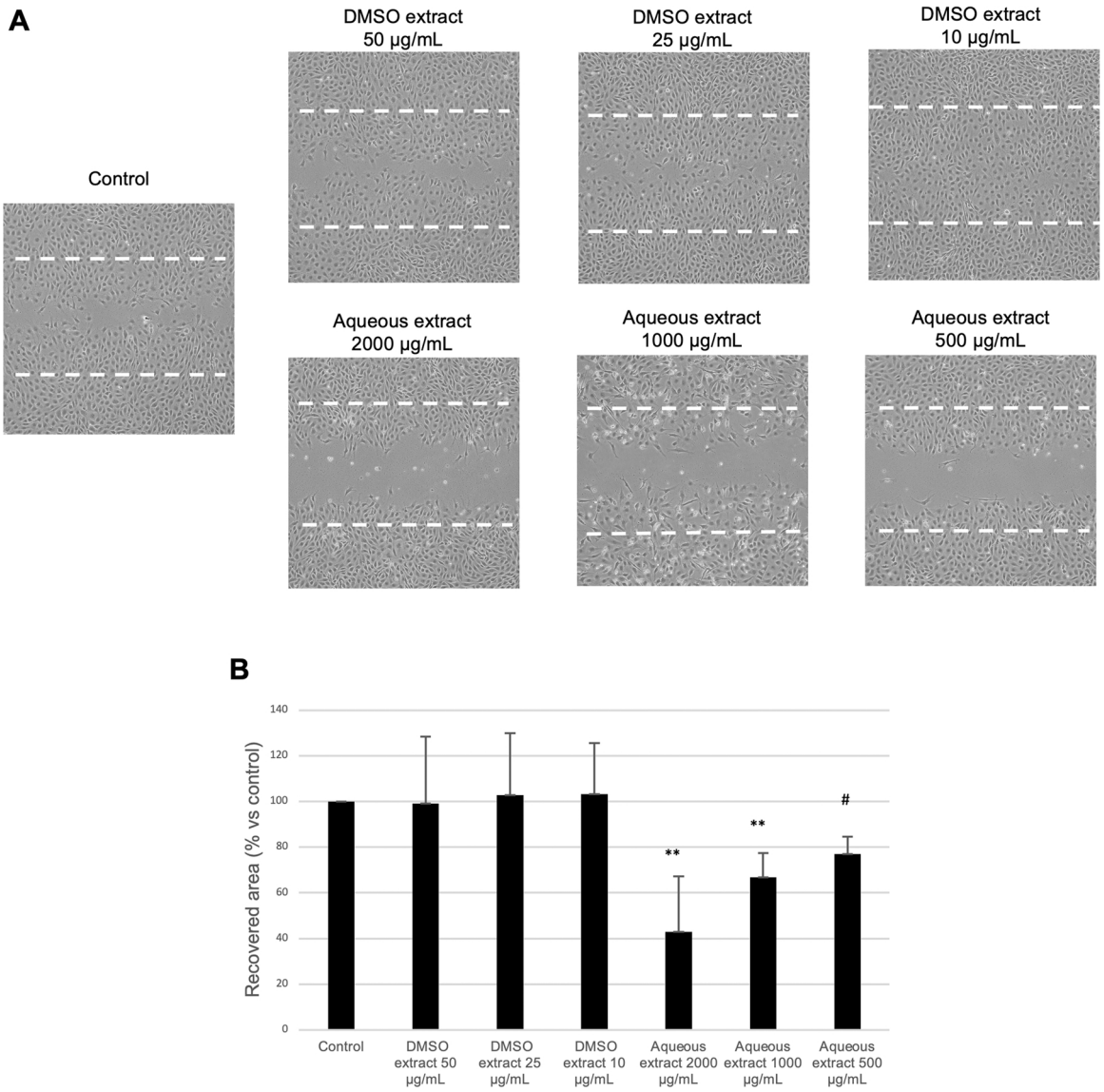


Fig. 5. Effect of aqueous and DMSO extracts on the migratory capabilities of endothelial cell. (A) Confluent BAEC monolayers were wound and fresh culture medium was added either in the absence or presence of the indicated concentrations of aqueous and DMSO extracts. Photographs were taken at the beginning of the assay and after 7 h of incubation (shown in the pictures). Broken lines indicate the initial (time 0) wound edges. (B) Quantitative analysis of data for recovered area of BAEC in the wound healing assay after 7 h at the indicated treatments. For wound-healing, data are means ± SD of at least three independent experiments (**$p < 0.01$, #$p < 0.001$).

inhibited in a dose dependent manner migration of BAECs for 7 h of incubation. In contrast, DMSO extracts had no inhibitory effect on the migratory potential of BAECs.

It had been previously shown that *Annona muricata* leaves can accelerate wound healing in rats and that this effect was mediated with the involvement of Hsp70 and antioxidant defense [56]. This is in contrast with our results of the wound healing assay with BAECs. On the other hand, our wound healing results with BAECs are comparable to the previously reported inhibitory effects of *Annona muricata* extracts on motility of cancer cells [30,57,58].

The differentiation of endothelial cells in new vessels and their stabilization constitutes the last and critical stage of the complex angiogenic process. To test the potential effects of *Annona muricata* leaf extracts on this last step of angiogenesis, we made use of the assay evaluating the formation of bidimensional "tubule-like" structures when endothelial cells are grown on Matrigel. In fact, we use this "tubule-like" formation on Matrigel assay as a key discriminant assay when we screen new potential antiangiogenic compounds [43,44,59,60]. Fig. 6 shows that all the tested concentrations of both extracts inhibited endothelial cell tube formation on Matrigel and that this inhibition was complete from 1 mg/mL for aqueous extracts and from 25 µg/mL for DMSO extracts.

4. Conclusion

In the present study we have carried out a first metabolomics characterization of relevant bioactive components present in both aqueous and DMSO extracts from *Annona muricata* leaves. We have tentatively identified 16 bioactive compounds in aqueous extracts and 13 in DMSO extracts. Ten of these compounds exhibited very good library matches between the consensus MS/MS spectra and the GNPS MS/MS Spectral Libraries. With regards to the bioactivities tested for these extracts, aqueous extracts (but not DMSO extracts) showed a dose-dependent inhibitory effect in the wound healing assay. The actual combination of bioactive compounds present in both extracts demonstrated to have clear and potent inhibitory effects in the assay of formation of

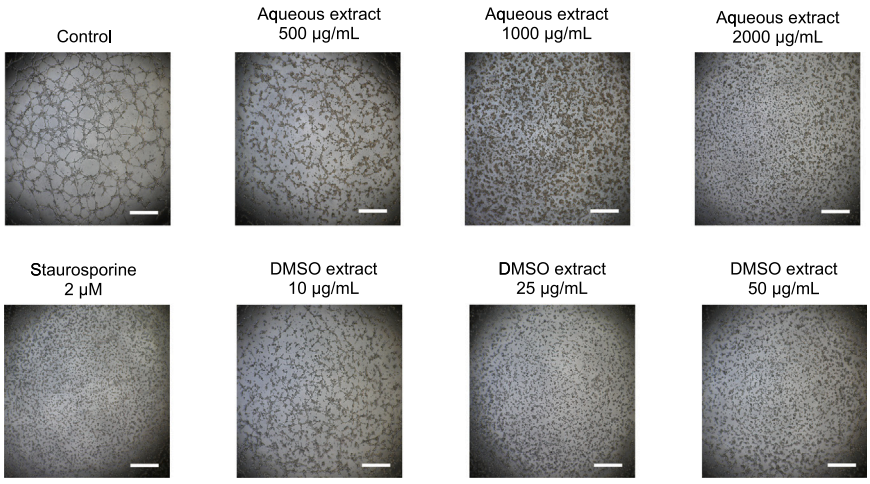


Fig. 6. Aqueous and DMSO *A. muricata* extract inhibits endothelial cell tube formation in vitro in a dose-dependent manner. BAECs seeded on Matrigel formed tubes like those shown in control. Aqueous and DMSO extract at 1000 µg/mL and 25 µg/mL respectively completely inhibited BAEC cord formation, with partial inhibition observed at 500 µg/mL (Aqueous extract) and 10 µg/mL (DMSO extract). 2 µM staurosporine was used as a positive control of inhibition. Cells were photographed 5 h after seeding under an inverted microscope (bar = 500 µm).

bidimensional "tubule-like" structures when endothelial cells are grown on Matrigel. Therefore, both aqueous and DMSO extracts of *Annona muricata* leaves have great potential as anti-angiogenic natural combinations of bioactive compounds. Future in vivo and translational studies should be warranted.

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CRediT authorship contribution statement

Casimiro Cárdenas: Conceptualization, Methodology, Investigation, Writing – Original draft. **José Antonio Torres-Vargas:** Investigation. **Abel Cárdenas-Valdivia:** Investigation. **Nuria Jurado:** Investigation. **Ana R. Quesada:** Writing - review & editing, Funding acquisition. **Melissa García-Caballero:** Writing – review & editing, Funding acquisition. **Beatriz Martínez-Poveda:** Writing – review & editing, Funding acquisition. **Miguel Ángel Medina:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

No potential conflicts of interest were disclosed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112263.

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