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Identification of Bioactive Compounds in Polar and Nonpolar Extracts of *Araujia sericifera*

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Details on extraction and properties of the extracts as **Supporting information** is available online at http://www.thieme-connect.de/products

ABSTRACT

Araujia sericifera is a native perennial, climbing laticiferous shrub from South America that is currently naturalized in many other countries. Previous data describe promising properties for *A. sericifera*, but no systematic study of its bioactive compounds and possible medicinal applications has been conducted to date. In the present study, aerial parts of *A. sericifera* (leaves, stems, and fruits) were explored by combining GC-MS and NMR spectroscopy analysis for both nonpolar (hexane) and polar (methanol) extracts. The hexanic extracts contained high amounts of pentacyclic triterpenes including two new metabolites, 3-tigloyl germanicol (**18**) and 3-tigloyl lupeol (**19**). The methanolic extracts revealed the presence of luteolin-7-glucoside (**24**), trigonelline (**22**), and conduritol F (**23**) as the main constituents. A multivariate study of a meaningful number of extracts allowed us to determine the distribution of compounds inside the plant. A cytotoxic evaluation in vitro showed that both leaf and fruit hexanic extracts presented a moderate activity against human breast carcinoma cell lines (MDA-MB-453 and MCF-7) and human colon carcinoma cell line (HCT-116) by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay.

Introduction

Araujia sericifera Brot. (Asclepiadaceae), also called the bladder flower, is a native perennial, climbing laticiferous shrub from South America that was introduced to other countries as an exotic ornamental plant. Currently, it is naturalized in Europe, South Africa, North America, Australia, and New Zealand. In the Mediterranean coastline, it competes with different crops, such as citrus trees, for water and nutrients [1, 2]. *A. sericifera* was reported to possess emetic, analgesic, antihistaminic, and anti-inflammatory properties [3–5]. Its seeds were described to exert toxicity on the central nervous system [3]. Its fruits contain luteolin-7-glucoside, serotonin [3], lupeol-3-cinnamate, and germanicol-3-acetate [4]. Nevertheless, despite the promising properties of *A. sericifera*, there is no systematic study of its chemical composition.

In a plant, different classes of secondary metabolites exert individual biological functions. They accumulate in specific tissues playing different roles in physiological processes or ecological interactions [6]. Metabolic profiling of different plant organs (root, shoot,

leaves, etc.) has been a tool to achieve better authentication and chemotaxonomic analyses of plants [7]. In line with this, the metabolic composition of the different organs of *A. sericifera*, with a broad tolerance for environmental conditions, has attracted our attention. Several methods exist to detect and quantify plant metabolites, such as NMR spectroscopy- and MS-based techniques, the major analytical tools used for metabolite profile studies. Both analytical techniques are considered complementary to obtain optimal results, each with its own particular advantages [8]. The aim of this study was to analyze the composition and cytotoxic properties of both nonpolar (hexane) and polar (methanol) extracts of the three aerial parts of *A. sericifera* (leaves, fruits, and stems) using GC-MS and NMR spectroscopy methods in conjunction with multivariate statistical analyses in order to explore this poorly studied plant.

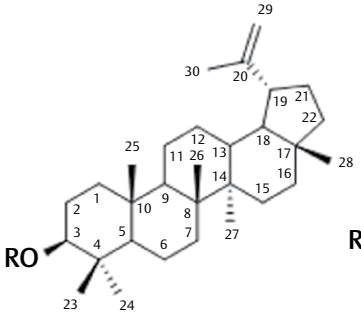
Results and Discussion

Polar and nonpolar metabolites were extracted by an automated Soxhlet extraction (Soxtec) of each aerial plant part with n-hexane and, subsequently, methanol to give the hexanic and methanolic extract, respectively. The leaves, stems, and fruits extracted with n-hexane gave extract yields (w/w), which were 5.6 % for both leaves and fruits, and 2.2 % for stems. The subsequent extraction with methanol gave higher extract yields for leaves and stems (21.0 % and 16.4 %, respectively) than for fruits (11.0 %). In the hexanic extracts, 20 different metabolites were identified by a combination of GC-MS (after trimethylsilyl derivatization) and 1D and 2D NMR spectroscopy. These nonpolar metabolites consisted of fatty esters (FEs), fatty acids [FAs: palmitic (**1**), linoleic acid (**2**), oleic acid (**3**), stearic acid (**4**)], squalene (**5**), n-alkanes (**6**-**8**), β-sitosterol (**9**), β-amyrin-3-acetate (**12**, oleanane type), and chiefly triterpen-3-ols and/or their esters, such as lupeol (lupane type) and germanicol esters (oleanane type), and compounds **10**, **11**, and **13**-**19**. For an unequivocal characterization of the lupeol and germanicol esters, an aliquot of the fruit hexanic extract was subjected to silica gel column chromatography and semipreparative reversed-phase HPLC purification. Several pentacyclic triterpenes could be isolated, including to new compounds, 3-O-tigloyl germanicol (**18**, oleanane type) and 3-O-tigloyl lupeol (**19**, lupane type) (▶ **Fig. 1**). The structure elucidation of **18** and **19** was carried out by GC-MS, 1D and 2D NMR spectroscopy, and UPLC-Q-TOF analysis. The ^{1}H and ^{13}C NMR spectra of compound **18** and **19** (▶ **Table 1** and **Fig. 3S**, **4S**, and **5S**, **Supporting Information**) exhibited the typical signals of an (E)-tigloyl group; e.g., for compound **19** at δ_H (ppm) 6.83 (q, J = 7.1 Hz, H-33), 1.83 (s, H_3-35) and 1.78 (d, J = 7.1 Hz, H_3-34) and δ_C (ppm) 167.9 (CO), 136.5 (CH-33), 129.3 (C-32), 14.3 (CH_3-34) and 12.1 (CH_3-35). The HMBC spectrum of these compounds also showed a correlation from H-3 (δ_H 4.51 ppm) to the carbonyl carbon (CO) of the tigloyl group at δ_C 167.9 ppm. The GC-MS data also displayed a characteristic MS fragmentation of oleanane and lupane skeletons for compounds **18** and **19**, respectively. Intense fragment ions were observed at m/z 204, 189, and 177 for compound **18** (Δ^{18}-oleanene with a methyl group at C-17). However, compound **19** with a C-20-29 double bond and a methyl group at C-17 presented a main peak at m/z 207 ($[C_{14}H_{23}O]^+$, $[A+B]^+$ rings) and at m/z 189 ($[C_{14}H_{21}]^+$, $[A+B]^+$ rings-H_2O). Fragmentation peaks at m/z 508, 493, and 408 corresponded to the molecular ion peaks $[M]^+$, $[M\text{-}CH_3]^+$, and [M-tiglic acid]$^+$, respectively. The HRESIMS analysis for compounds **18** and **19** displayed an ion peak at m/z 409.3832 or 409.3829 [(M-C_5H_7O-H_2O) + H]$^+$, respectively, which suggests the molecular formula $C_{30}H_{48}$ (calcd. 409.3829) for both compounds corresponding to the loss of the tigloyl group and one water molecule.

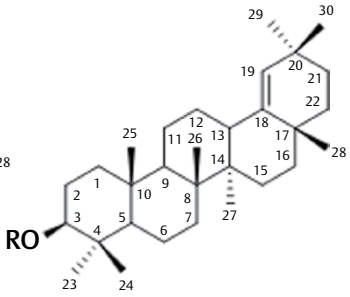
Known triterpenes were also identified by making a direct comparison of their NMR spectroscopic and GC-MS data with those reported in the literature, including germanicol (**10**) [9], lupeol (**11**) [10], β-amyrin-3-acetate (**12**) [11], germanicol-3-acetate (**13**) [12], lupeol-3-acetate (**14**) [9], lupeol-3-cinnamate (**15**) [13], germanicol-3-propionate (**16**), and germanicol-3-butyrate (**17**) [14] (▶ **Fig. 1**). Among them, lupeol-3-acetate (**14**), germanicol-3-propionate (**16**), and germinacol-3-butyrate (**17**) were identified in *A. sericifera* for the first time. A representative GC-MS chromatogram section of the three plant aerial parts containing principally triterpen-3-ols and/or their esters is drawn in ▶ **Fig. 2a**. In ▶ **Fig. 2b**, the ^{13}C NMR spectra of the hexanic extracts of leaves, stems, and fruits are depicted. The acquisition of ^{13}C spectra seemed an optimal choice for these nonpolar fractions since the ^{1}H NMR spectra were highly overlapped due to the high amounts of alkanes and enough sample amount was available to obtain a good signal/noise ratio in the less sensitive ^{13}C spectroscopy. It is worth mentioning that β-amyrin-3-acetate (**12**), present in very small amounts in leaves and stems, was only detected by the most sensitive technique GC-MS but not in the NMR spectra. In addition, only general signals of alkanes and FAs were assigned in the NMR spectra due to overlapping. On the other hand, NMR spectroscopy allowed for the quantification of several nonvolatile compounds such as long-chain FEs that could not be analyzed by GC-MS. Similarly, the quantification of lupeol-3-cinnamate (**15**) was only feasible by NMR spectroscopy since its peak overlapped with germanicol-3-propionate (**16**) in the GC-MS chromatogram (t_R = 26.09 min). Relative percentages of metabolites from the hexanic extracts determined by GC-MS and ^{13}C NMR in leaves, stems, and fruits are shown in **Fig. 6S** and **7S**, **Supporting Information**. Thus, we found that the combination of NMR and GC-MS is optimal for the detection of nonpolar metabolites in *A. sericifera*.

Concerning the polar fraction, 25 metabolites were identified in the methanolic extracts using GC-MS (after methoximation and trimethylsilyl derivatization) and NMR spectroscopy. In order to unambiguously identify several polar metabolites, an aliquot of the fruit methanolic extract was subjected to a solid-phase extraction (SPE) C_{18} cartridge and was eluted firstly with 100 % H_2O, followed by 100 % MeOH. The methanolic fraction (F-2) was further purified by reversed-phase HPLC to give viburnitol (**21**, SF_{meth}2-1) [15], trigonelline (**22**, SF_{meth}2-2) [16], and conduritol F (**23**, SF_{meth}2-3, also known as L-leuchanthemitol) [17] (▶ **Fig. 1**). The GC-MS analysis of the whole extracts also detected viburnitol (**21**), malate (**37**), L-asparagine (**31**), sucrose (**38**), glucose (**40**), fructose, and myo-inositol. A representative GC-MS chromatogram of the fruit methanolic extract is shown in **Fig. 8S**, **Supporting Information**. NMR spectroscopy confirmed the presence of metabolites detected by MS and further allowed for the identification of luteolin-7-glucoside (**24**), serotonin (**25**), allantoin (**26**), choline (**28**), and malate (**37**). In fact, luteolin-7-glucoside (**24**) and serotonin (**25**) were pre-

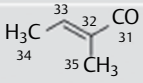
Germanicol and lupane derivatives from hexanic extracts



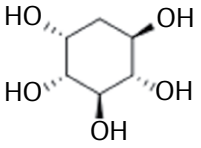
lupeol derivatives
(11,14,15,19)



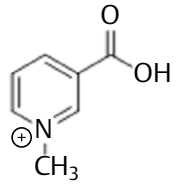
germanicol derivatives
(10,13,16,17,18)

Compound	R
Germanicol (**10**)	H
Lupeol (**11**)	
Germanicol-3-acetate (**13**)	CH_3CO
Lupeol-3-acetate (**14**)	
Lupeol-3-cinnamate (**15**)	PhCH = CHCO
Germanicol-3-propionate (**16**)	CH_3CH_2CO
Germanicol-3-butyrate (**17**)	$CH_3(CH_2)_2CO$
3-O-Tigloyl-germanicol (**18**)	
3-O-Tigloyl-lupeol (**19**)	

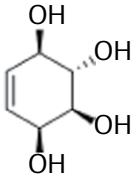
Metabolites from methanolic extracts



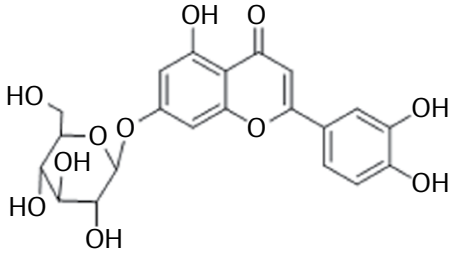
Viburnitol (**21**)



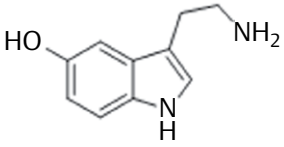
Trigonelline (**22**)



Conduritol F (**23**)



Luteolin-7-glucoside (**24**)



Serotonin (**25**)

▶ **Fig. 1** Nonpolar secondary metabolites identified in hexanic extracts, germanicol type and lupeol type, and polar metabolites from methanolic extracts of *A. sericifera*.

▶ **Table 1** ^{1}H and ^{13}C NMR data for **18** and **19** in $CDCl_3$ (δ in ppm, J in Hz).

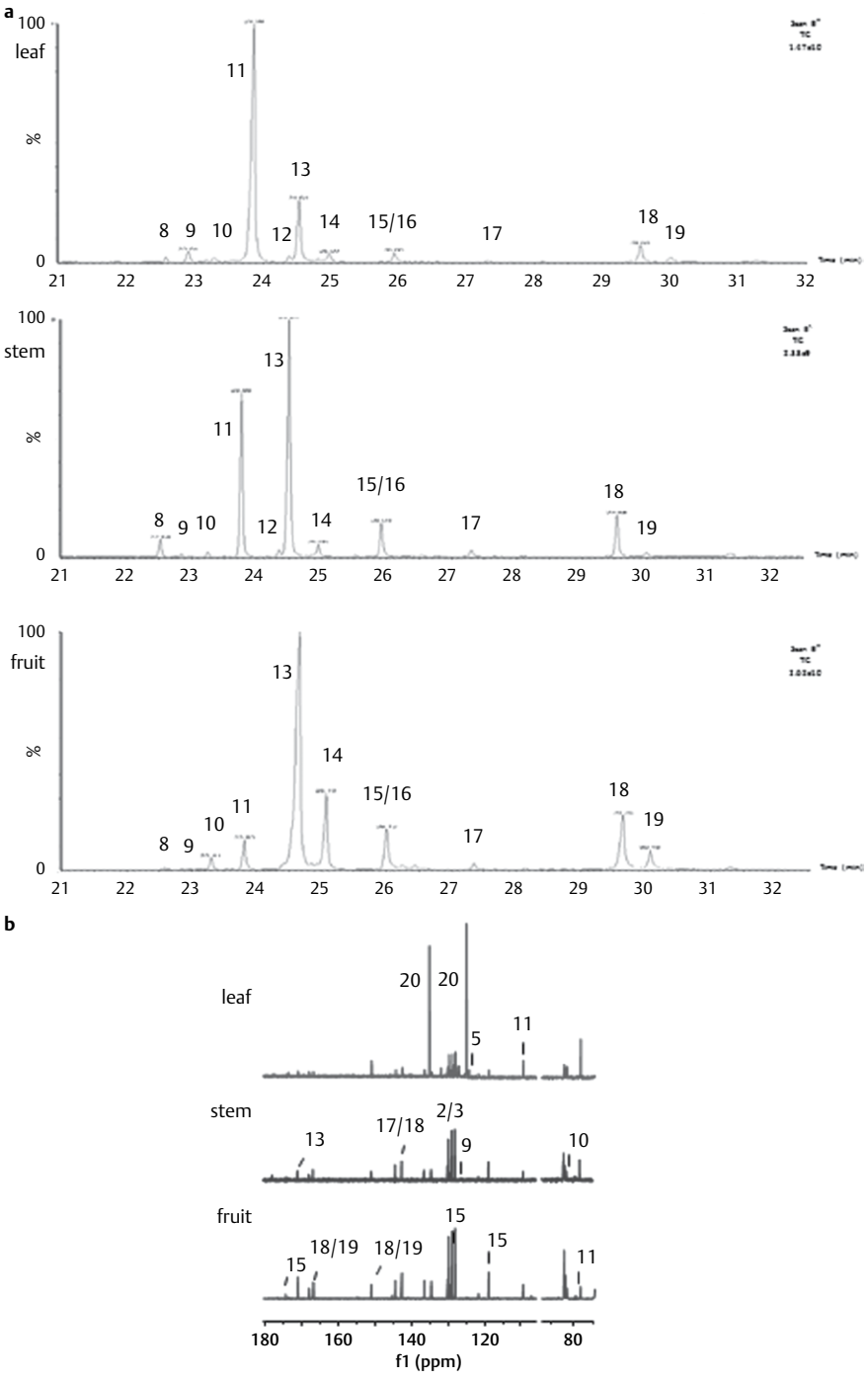
	18		19	
Position	**δ_C (mult.)**	**δ_H (J in Hz)**	**δ_C (mult.)[a]**	**δ_H (J in Hz)**
1a	37.3 (CH_2)	1.50, m	38.4 (CH_2)	1.69, m
1b				1.00, m
2a	23.4 (CH_2)	1.65, m	23.7 (CH_2)	1.47, m
2b		1.14, m		1.08, m
3	80.7 (CH)	4.52, d (6.4)	80.9 (CH)	4.51, dd (5.9)
4	37.7 (C)	–	38.1 (C)	–
5	55.2 (CH)	0.8, m	55.4 (CH)	0.80, m
6a	17.9 (CH_2)	1.60, m	18.2 (CH_2)	1.5, m
6b		1.38, m		1.45, m
7a	34.4 (CH_2)	1.55, m	34.2 (CH_2)	1.4, m
7b		1.41, m		
8	43.1 (C)	–	40.9 (C)	–
9	51.0 (CH)	1.33, m	50.3 (CH)	1.35, m
10	37.2 (C)	–	37.1 (C)	–
11a	20.9 (CH_2)	1.55, m	21.0 (CH_2)	1.5, m
11b		1.40, m		
12a	26.0 (CH_2)	1.64, m	25.1 (CH_2)	1.60, m
12b		1.25, m		
13	38.2 (CH)	2.26, d (11.0)	38.1 (CH)	1.66, s
14	40.5 (C)	–	43.0 (C)	–
15a	27.2 (CH_2)	1.72, m	27.4 (CH_2)	1.46, m
15b		1.25, m		1.00, m
16	38.2 (CH_2)	1.70, m	35.6 (CH_2)	1.5, m
17	34.2 (C)	–	42.8 (C)	–
18	167.5 (C)	–	48.3 (CH)	1.35, m
19	129.7 (CH)	4.86, s	48.0 (CH)	2.38, ddd (10.0)
20	32.2 (C)		151.0 (C)	–
21a	33.3 (CH_2)	1.60, m	29.8 (CH_2)	1.92, m
21b		1.38, m		1.30, m
22a	37.3 (CH_2)	1.49, m	40.0 (CH_2)	1.45, m
22b				1.22, m
23	28.9 (CH_3)	0.86, s	28.1 (CH_2)	0.86, s
24	16.6 (CH_3)	0.87, s	16.7 (CH_3)	0.89, s
25	16.4 (CH_3)	0.91, s	16.2 (CH_3)	0.87, s
26	15.8 (CH_3)	1.08, s	16.0 (CH_3)	1.02, s
27	14.3 (CH_3)	0.74, s	14.5 (CH_3)	0.98, s
28	25.0 (CH_3)	1.02, s	18.0 (CH_3)	0.80, s
29a	28.8 (CH_3)	0.97, s	109.3 (CH_2)	4.69, d (5.8)
29b				4.57, s
30	31.2 (CH_3)	0.94, s	19.3 (CH_3)	1.69, s
31	167.7 (CO)	–	167.9 (CO)	–
32	129.1 (C)	–	129.3 (C)	–
33	136.1 (CH)	6.84, q (5.8)	136.5 (CH)	6.83, q (7.1)
34	14.1 (CH_3)	1.79, d (5.8)	14.3 (CH_3)	1.78, d (7.1)
35	11.9 (CH_3)	1.83, s	12.1 (CH_3)	1.83, s

viously identified from the leaves and stems of *A. sericifera* [3], whereas virbutinol (**21**), trigonelline (**22**), conduritol F (**23**), and allantoin (**26**) were found in this plant for the first time. Representative ^{1}H NMR spectra of the leaf, stem, and fruit methanolic extracts are depicted in ▶ **Fig. 3**, along with an assignment of the most significant metabolites.

In order to gain insight about how the identified compounds were distributed in the different plant parts, they were quantified from leaves, stems, and fruits (**Fig. 9S**, **Supporting Information**), and an unsupervised multivariate analysis in the form of principal component analysis (PCA) was performed. The resulting score and loading plots from the PCA of the GC-MS and ^{13}C NMR data from nonpolar extracts are represented in ▶ **Fig. 4**. While the score plot (▶ **Fig. 4a and c**) gives information about how the different samples cluster based on their metabolic composition, the loading plot (▶ **Fig. 4b and d**) indicates which compounds are more present in each sample group. As we can see, a similar clustering was obtained by both analytical techniques. In both cases, large amounts of alkanes, saturated fatty acids/esters were associated with the stem extracts, while larger quantities of unsaturated fatty acids/esters, linoleic and linolenic acids and derivatives were found in the leaves. Cis-polyisoprene (**20**) and squalene (**5**) were identified only in the leaves, which also had greater amounts of β-sitosterol (**9**) and lupeol (**11**) than the other aerial parts. However, fruit extracts stood out for possessing germanicol (**10**) and large quantities of triterpene esters including germanicol-3-acetate (**13**), lupeol-3-acetate (**14**), lupeol-3-cinnamate (**15**), germanicol-3-propionate (**16**), 3-O-tigloyl germanicol (**18**), and 3-O-tigloyl lupeol (**19**).

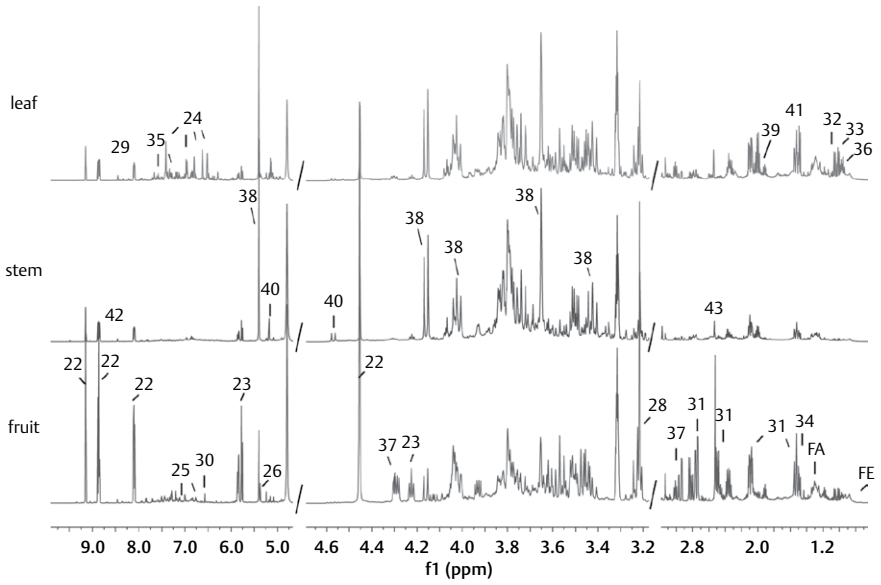
For the polar metabolites, we quantified the identified compounds by ^{1}H NMR in the same set of samples of the three plant aerial parts and also performed PCA to compare the metabolite contents. The resulting score and loading plots are represented in ▶ **Fig. 5**. We observed that the stem extracts stand out for their high content in sugars such as sucrose (**38**) and glucose (**40**). Larger amounts of branched amino acids such as isoleucine (**32**), leucine (**33**). and valine (**36**) proved to be characteristic of the leaf extracts. Flavonoid luteolin-7-glucoside (**24**) was also significantly increased in the leaves. The metabolic composition of fruits differed vastly from that of the stems and leaves, standing out for high concentrations of viburtinol (**21**), trigonelline (**22**), conduritol F (**23**), serotonin (**25**), choline (**28**), succinate (**43**), or fumarate (**30**).

Thus, our study lays bare that *A. sericifera*, a climbing weed that competes with crops, contains several bioactive compounds with beneficial applications. Accordingly, nonpolar extracts possess high levels of triterpen-3-ol and their esters, especially in leaves and fruits, respectively. Naturally occurring and synthetic pentacyclic triterpenes exhibit a variety of unique biological activities, including antitumor, antiviral, antidiabetic, anti-inflammatory, antimicrobial, antiparasitic, cardio-, hepato- and gastro-protection, and analgesic and wound healing effects, among others. In fact, they are receiving ever-increasing interest as therapeutic agents in pharmacological research [18]. Lupeol (**11**) has the ability to inhibit α-amylases [19] and α-glucosidases [20] and possesses hypoglycemic and antidiabetic properties. In addition, germanicol (**10**) and lupeol (**11**) display anti-inflammatory [21] and antidyslipidemic effects [19]. Among the polar secondary metabolites of *A. sericifera*, trigonelline (**22**), which is abundant in leaves, has shown hypogly-



▶ **Fig. 2** Identification of nonpolar metabolites from leaves, stems, and fruits of *A. sericifera* containing principally triterpen-3-ols and/or their esters. **a** A section of total ion GC-MS chromatograms of the TMS-derivatized hexanic extracts. **b** ^{13}C NMR spectra of hexanic extracts at 600 MHz. Assignments: **8**, tritiacontane; **9**, *β*-sitosterol; **10**, germanicol; **11**, lupeol; **12**, *β*-amyrin-3-acetate; **13**, germanicol-3-acetate; **14**, lupeol-3-acetate; **15**, lupeol-3-cinnamate; **16**, germanicol-3-propionate; **17**, germanicol-3-butyrate; **18**, 3-*O*-tigloyl germanicol; **19**, 3-*O*-tigloyl lupeol; **20**, *cis*-polyisoprene.

cemic, hypocholesterolemic, antitumoral, and antiseptic properties besides playing an essential role in the resistance process of plants against several pathogens [22]. Concerning the two natural cyclitols viburnitol (**21**) and conduritol F (**23**), compound **21** acts as a glycosidase inhibitor [23] and compound **23** has potential in the treatment of metastatic cancer and diabetes because of its



▶ **Fig. 3** Representative ^{1}H NMR spectra of leaf, stem, and fruit methanolic extracts of *A. sericifera*. Metabolite keys are given in **Table 1S**, **Supporting Information**. Assignments: **22**, trigonelline; **23**, conduritol F; **24**, luteolin-7-glucoside; **25**, serotonin; **26**, allantoin; **28**, choline; **29**, formate; **30**, fumarate; **31**, L-asparagine; **32**, L-isoleucine; **33**, L-leucine; **34**, L-threonine/lactate; **35**, L-tryptophan; **36**, L-valine; **37**, malate; **38**, sucrose; **39**, γ-butyric acid; **40**, glucose; **41**, L-alanine; **42**, adenosine; **43**, succinate; **FA**, fatty acids; **FE**, fatty esters. ■Please remove ,,a" from figure, as there is no ,,b"■

ability to inhibit type I α-glucosidase [24]. Luteolin-7-O-glucoside (**24**) has an important nutraceutical application value thanks to its numerous biological properties, including antioxidant activity [25].

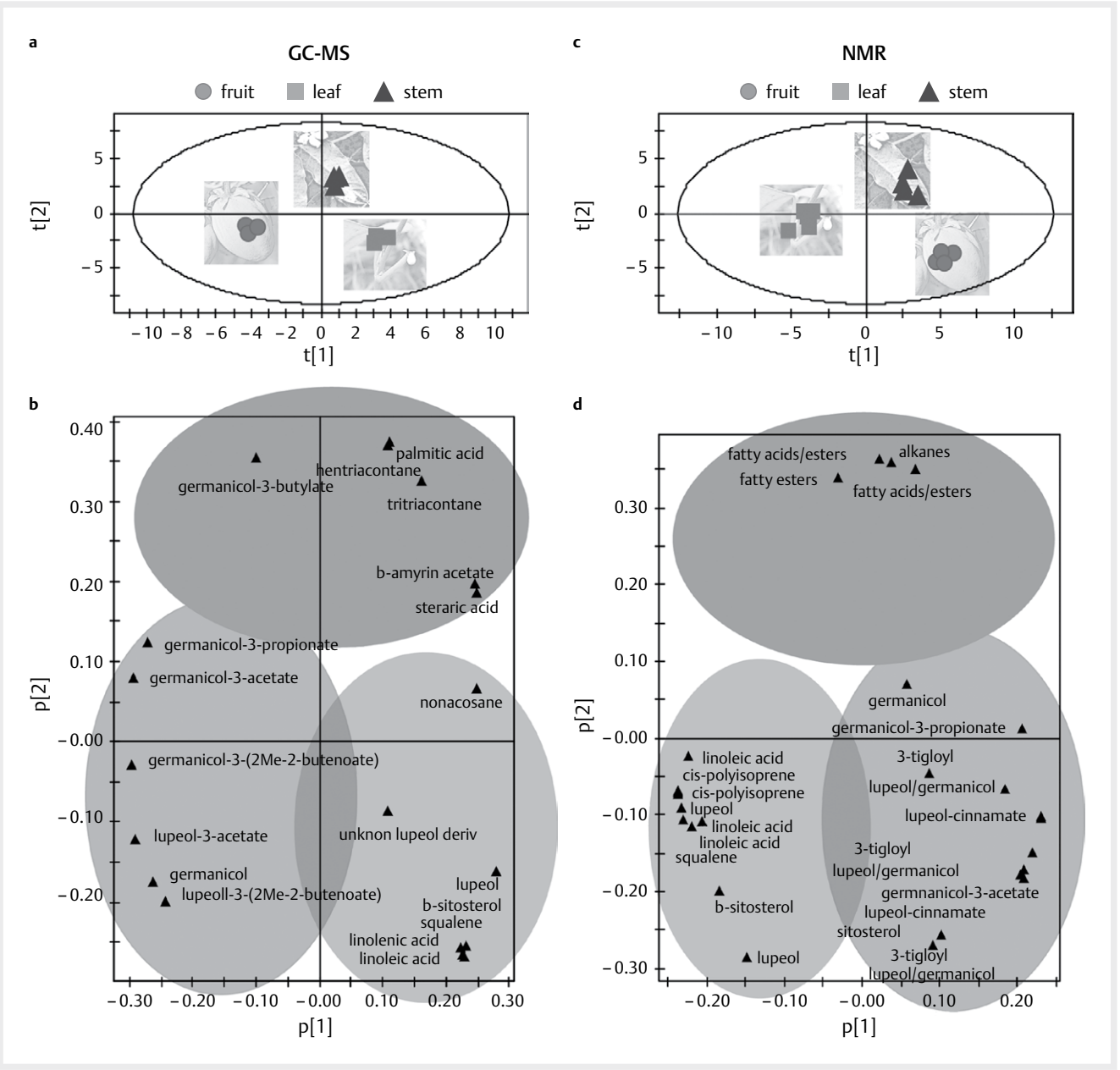
In order to evaluate the cytotoxic activity of *A. sericifera* plant extracts and the pentacyclic triterpenes, MTS assays were performed on human breast carcinoma (MDA-MB-453 and MCF-7) and human colon carcinoma (HCT-116) cell lines. As a result, the fruit hexanic extract of *A. sericifera* showed a significant cell growth reduction of HCT-116 and MCF-7 cells, while the effect produced by leaf extracts was more modest but with a higher impact against the aggressive cell line MDA-MB-453 (▶ **Fig. 6a and b**). These results are coherent with the fact that the most bioactive compounds reported from nonpolar extracts occurred in these two plant organs. The cytotoxic activity for fruit hexanic extracts may be attributed to germanicol derivatives [26]. In leaves, high amounts of lupeol, a known inhibitor of proliferation [27], can induce cytotoxicity. In order to find out if 3-O-tigloyl lupeol (**19**) contributes to the cytotoxic activity of the nonpolar fruit and leaf extracts, it was semisynthesized by esterification between lupeol and tiglic acid. Nevertheless, compounds **15** and **19** did not show significant cell growth reduction at the tested concentrations, which was only obtained for lupeol (**11**) against the three cell lines (▶ **Fig. 6c**). 3-O-Tigloyl germanicol (**18**) could not be assayed as not enough quantity was available. In the case of polar extracts of *A. sericifera*, a significant growth reduction was only detected in leaf extracts (▶ **Fig. 6d**). This effect may be mainly due to the presence of luteolin-7-O-glucoside, which was previously described to inhibit proliferation of cancer cells [28].

In summary, our study of different aerial parts of *A. sericifera* by GC-MS and NMR spectroscopy allowed for the identification of interesting compounds with potential pharmaceutical and/or industrial uses. Several secondary metabolites have been identified in this plant for the first time, and two new triterpen-3-ol esters, 3-O-tigloyl germanicol (**18**) and 3-O-tigloyl lupeol (**19**), were characterized from the hexanic extracts. A systematic analysis of an array of plants showed that the major bioactive compounds were distributed in fruits and leaves. Accordingly, the new compound 3-O-tigloyl lupeol (**19**) did not show any cytotoxicity against the cancer cell lines at the tested concentrations. Therefore, the moderate cytotoxicity exhibited for both fruit and leaf hexanic extracts against the breast cancer model MCF-7 and the human colon carcinoma HCT-116 may be attributed to lupeol (**11**).

Materials and Methods

Cell lines, chemicals, and biochemicals

MDA-MB-453, MCF-7 (human breast carcinomas), and HCT-116 (human colon carcinoma) cell lines were provided by M. Orzáez (CIPF) [29], and grown at 37 °C in a humidified 5 % CO_2, 95 % air incubator. All the standard compounds and reagents (purities ≥ 94 %) were purchased from Sigma-Aldrich with the exception of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) (purity > 98 %), which was purchased from Acros. Hexane, dichloromethane, methanol, and pyridine were analytical grade and purchased from Scharlab SL. Chloroform-d with 0.05 % v/v trimethylsilane (TMS), methanol-d_4, and trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; purities > 99 %)were purchased from Deutero GmbH.



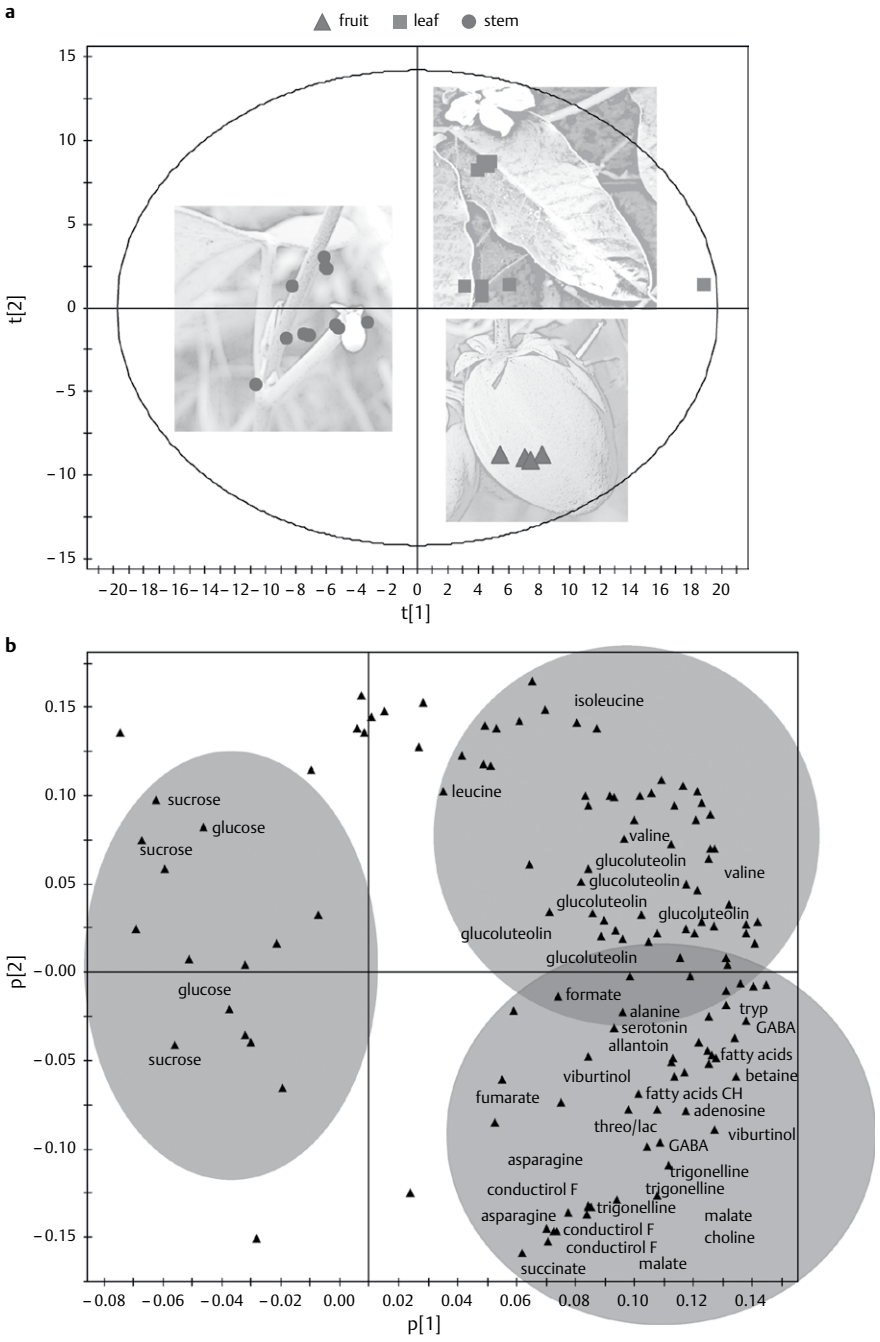
▶ **Fig. 4** PCA of metabolites in the hexanic extract of leaves, stems, and fruits of *A. sericifera* analyzed by GC-MS and ^{13}C NMR spectroscopy. **a** Score plot of GC-MS analysis, 2 components R2X (cum) = 0.89, Q2 (cum) = 0.79. **b** Loading plot of GC-MS analysis. **c** Score plot of ^{13}C NMR analysis 2 components R2X (cum) = 0.87, Q2 (cum) = 0.77. **d** Loading plot of ^{13}C NMR analysis.

Plant material

Aerial parts of *A. sericifera* (leaves, stems, and fruits) were harvested in September 2014 from adult plants that grew in an open field located in Moncófar (Castellón, eastern Spain). The botanical characterization of the plant was carried out by Prof. Herminio Boira from the Mediterranean Agroforesty Institute of the Polytechnic University of Valencia, and a voucher specimen was deposited at the VALA herbarium of the University Polytechnic of Valencia (Spain) with the registration number 6647.

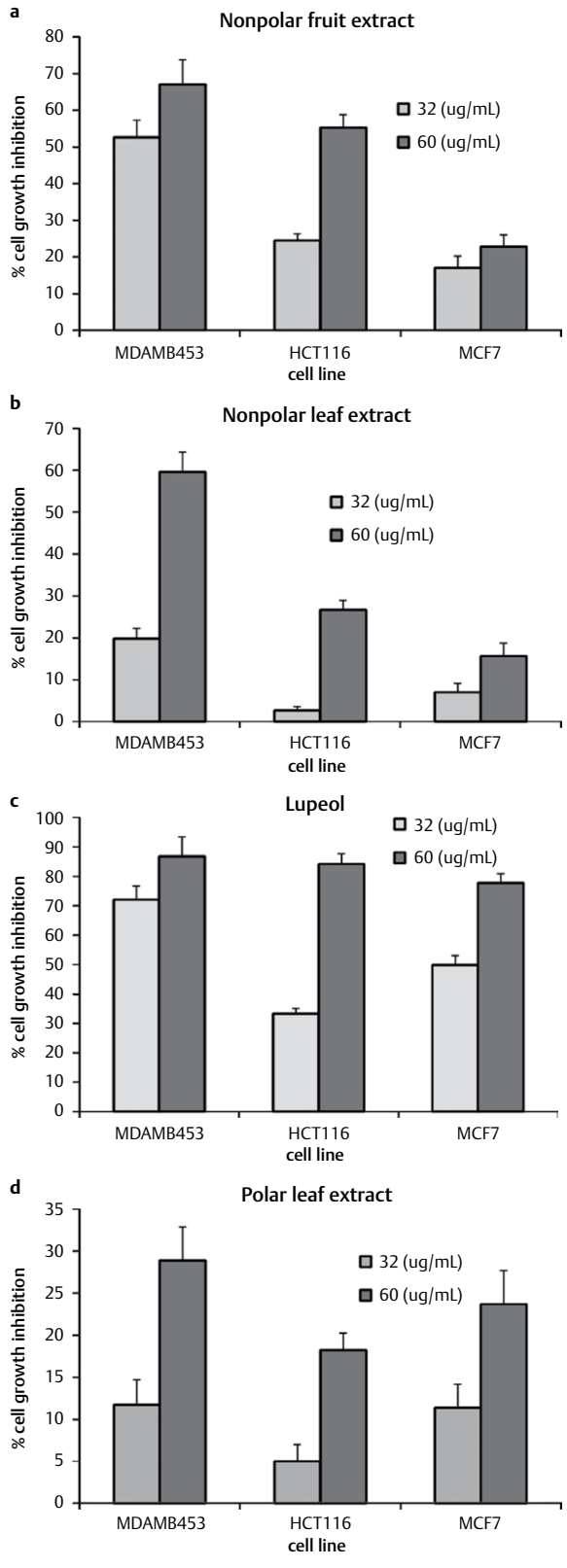
Extraction and isolation

Oven-dried leaves, stems, and fruits were separated from each plant and, in addition, the fruits were cut and seeds removed. These aerial organs were finely powdered in a mechanical grinder and extracted by an automatic Soxhlet in a Foss Tecator Soxtec™ system with a 2043 extraction unit and a 2046 Soxtec Foss Control unit. Each sample (5 g) was firstly extracted with n-hexane (50 mL) for 1 h 30 min of boiling time and 20 min of rising time, followed by MeOH (50 mL) under the same operating conditions [30]. The solvents were evaporated under reduced pressure to obtain the hexanic and methanolic extracts.



▶ **Fig. 5** Multivariate analysis of ^{1}H NMR spectra of polar methanolic extracts of *A. sericifera*, an unsupervised PCA model. **a** Score plot: two components R2X (cum) = 0.63, Q2 (cum) = 0.46. **b** Loading plot.

Dry leaf hexanic extracts (100 mg) were dissolved in MeOH (4 mL) and centrifuged at 3000 rpm for 8 min at 22 °C in order to partially remove the rubber content. The supernatant was separated and the pellet was dissolved in MeOH (4 mL) and then centrifuged again. This MeOH centrifugation procedure was performed three times for each sample [10]. The supernatants were combined and dried by nitrogen stream to give a residue of leaf hexanic extract (100 mg) that was subjected to flash column chromatography on silica gel 60 (40–63 µm) using a stepwise gradient soln. solution from 100 % hexane to 100 % ethyl acetate and yielding five fractions. The major fraction F-2 (hexane/EtOAc, 9:1, 13 mg) was purified by a semipreparative reversed-phase HPLC instrument (Waters 600E system) equipped with a solvent delivery pump unit (Waters 600E) coupled to a photodiode array detector (Waters 2996 PDA) and an evaporative light scattering detector (Waters 2420 ELSD). The separation of metabolites was carried out using a Phenomen-



▶ **Fig. 6** Cell growth inhibition obtained by the MTS assay against three different cell lines at two different concentrations.

ex Luna C18 (2) column (25.0 × 1 cm, 5 µm) and 100 % methanol as the mobile phase (flow of 3 mL/min) to afford $SF_{hex}2$-1 ($t_{R=}$30 min), $SF_{hex}2$-2 ($t_{R=}$35 min), $SF_{hex}2$-3 ($t_{R=}$42 min), $SF_{hex}2$-4 ($t_{R=}$45 min), $SF_{hex}2$-5 ($t_{R=}$48 min), and $SF_{hex}2$-6 ($t_{R=}$52 min). On the other hand, a residue of the fruit methanolic extract (85 mg) was subjected to the solid-phase extraction (SPE) C_{18} cartridge (2 g, 12 ml) model ExtraBond (Scharlab) with 100 % H_2O followed by 100 % MeOH. Fraction F-2 (100 % MeOH) was purified by semipreparative reversed-phase HPLC using a Phenomenex Luna C18 (2) column (25.0 × 1 cm, 5 µm) and 100 % H_2O as the mobile phase (flow of 3 mL/min) to afford the $SF_{meth}2$-1 ($t_{R=}$6 min), $SF_{meth}2$-2 ($t_{R=}$9 min), and $SF_{meth}2$-3 ($t_{R=}$11 min). All purified fractions from both hexanic and methanolic extracts were dried, derivatized, and analyzed by GC-MS and NMR spectroscopy.

3-O-Tigloyl germanicol (**18**): white amorphous powder; m.p. 244-249 °C; $[\alpha]_D^{25}$ + 27.2 (c 0.5, $CHCl_3$); IR (film): 2940, 1710, 1634, 1446 cm^{-1}; ^1H and ^{13}C NMR, see ▶ **Table 1**; HRESIMS m/z 409.3832 $[(M\text{-}C_5H_7O\text{-}H_2O) + H]^+$ (calcd. for $C_{30}H_{48}$, 409.3829).

3-O-Tigloyl lupeol (**19**): white amorphous powder; m.p. 233–237 °C; $[\alpha]_D^{25}$ + 42.5 (c 0.5, $CHCl_3$); IR (film): 2987, 1710, 1654, 1457 cm^{-1}; ^1H and ^{13}C NMR, see ▶ **Table 1**; HRESIMS m/z 409.3829 $[(M\text{-}C_5H_7O\text{-}H_2O) + H]^+$ (calcd. for $C_{30}H_{48}$, 409.3829).

Derivatization and GC-MS analysis

Samples of the hexanic extracts were derivatized according to Van Beek [31]. Trimethylsilyl derivatives of hexanic extract samples (5 mg) were prepared by the addition of 100 µL of dry CH_2Cl_2 and 100 µl of BSTFA with 1 % TMCS. Next, the mixture was stirred overnight at room temperature. Samples of the methanolic extracts were derivatized according to Herebian et al. [32]. Derivatives of the methanolic extracts were prepared by a two-step procedure involving a methoximation-trimethylsilylation process. For methoximation, the extract samples (5 mg) were treated with methoxyamine hydrochloride soln. solution (20 mg mL^{-1} in pyridine) (100 µL) and stirred overnight at room temperature. Next, BSTFA with 1 % TMCS (100 µL) was added as a silylation reagent and stirred for 3 h at room temperature. Derivatized samples were directly analyzed by GC-MS on a PerkinElmer Clarus® 500 gas chromatograph-mass spectrometer, operating in the electron impact mode (EI) and equipped with a ZB-5 MS (30 m × 0.25 mm × 0.25 µm particle size) capillary column (Phenomex Inc). GC-MS parameters, peaks identification, and quantification procedure are described in more detail in the **Supporting Information**.

UPLC-Q-TOF analysis

High-resolution mass (HRESIMS) spectra for the new compounds were obtained from a Triple TOF™ 5600 hybrid quadrupole time-of-flight (TOF) LC-MS/MS system (AB SCIEX) and a Waters Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 µm). UPLC-Q-TOF parameters are described in more detail in the **Supporting Information**.

General procedure for the preparation of 3-tigloyl lupeol (19)

The esterification procedure was carried out according to Liu et al. [33]. Lupeol (6.0 mg, 1 equiv) was added to a soln. solution of trans-2-methyl-2-butenoic acid (1.4 mg, 1 equiv), DCC (5.8 mg, 2 equiv), and DMAP (3.4 mg, 2 equiv) in dry CH_2Cl_2 (2 ml), and the reaction

mixture was shaken for 24 h. Next, the solvent was removed under reduced pressure to give a white solid that was redissolved in CH_2Cl_2, washed with 5 % $NaHCO_3$, dried over Na_2SO_4, and concentrated under reduced pressure to obtain a crude residue. The residue was purified by semipreparative reverse-phase HPLC-PDA-ELSD using a Phenomenex Luna C18 (2) column (25.0 × 1 cm, 5 µm) and 100 % methanol as the mobile phase with a flow rate of 3 mL min^{-1}. 3-O-Tigloyl lupeol (**19**) was obtained in a 63 % yield as white powder.

NMR spectroscopy

NMR spectra were recorded at 25 °C on a Bruker AVII-600 using a 5-mm TCI cryoprobe (^{13}C spectra) and a Bruker AVIII-500 using a 5-mm TBI probe (all the other experiments) and processed using Topspin 3.17 software (Bruker GmbH). Nonpolar extracts (20 mg) were dissolved in 500 µL of 99.8 % chloroform-d with 0.05 % v/v TMS. Polar extracts were dissolved in 500 µl of 50 % methanol-d_4 in buffer [90 mM KH_2PO_4, pH = 6, 1 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP)]. Chemical shifts (δ) of ^1H and ^{13}C NMR are given in ppm. The acquisition parameters of NMR spectra and quantification procedure are described in more detail in the **Supporting Information**.

Principal component analysis

Variable sized bucketing was performed of the ^1H and ^{13}C NMR spectra, excluding the regions corresponding to $CDCl_3$ (in nonpolar samples) and to D_2O and MeOD resonances (in polar samples). After normalization and univariate scaling, data were subjected to PCA, an unsupervised pattern recognition method [34], using the software Simca-P + 12.0. PCA score plots show clustering trends between samples, how metabolite concentrations are related, and if there are any strong outliers. The discriminant metabolites were identified from the corresponding loading plots.

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]assay with cancer model cell lines

The effect of organic leaf, fruit, and stem extracts and lupeol (purity ≥ 94 %; Sigma), lupeol-cinnamate, and 3-O-tigloyl lupeol compounds was evaluated by the standard procedure of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell viability assay [35] in HCT-116, MFC-7, and MDA-MB-453 cells lines. A stock solution of extracts and compounds (2 mg/mL) in DMSO was prepared. This stock solution was diluted in culture medium (DMEM for HCT-116 and MFC-7, or DMEM/F12 for MDA-MB-453) to obtain of 32, 64, 96, and 120 µg/µL solutions (DMSO, maximal concentration 0.1 %). A solution of medium containing only DMSO at 0.1 % was also prepared. Cells were harvested in their logarithmic phase and seeded at concentrations of 12500 (HCT-116), 5000 (MCF-7), and 10000 (MDA-MB-116) cells per well in 96-well microtiter plates and incubated for 18 h in 50 µL of their respective media at 37 °C and 5 % CO_2. Cells were then observed under a light microscope (20 ×) to check if they were attached to the plates. Fifty µL of medium were added with the absence (only DMSO at a final concentration of 0.05 %) or presence of compounds/extracts to yield final compound/extract concentrations of 16, 32, 48, and 60 µg/µL per well, and cells were incubated for 72 h. MTS was freshly prepared at 5 mg/mL in PBS, and 20 µL of a mixture of MTS solution and phenazine methosulfate (20:1) were added to each well and incubated at 37 °C for another 3 h. Finally, the absorbance was measured with a spectrophotometer (VICTOR2 1420 Multilabel HTS Counter) at 595 nm. Results are the mean of three independent experiments (n = 3).

Supporting information: Details on extraction and properties of the extracts are available as **Supporting Information**.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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