



Separation and detection of apricot leaf triterpenes by high-performance thin-layer chromatography combined with direct bioautography and mass spectrometry

Ágnes M. Móricz^{a,*}, Péter G. Ott^a

^a Plant Protection Institute, Centre for Agricultural Research, ELKH, Herman O. Str. 15, 1022 Budapest, Hungary

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ABSTRACT

Prunus armeniaca leaf extract was screened for antibacterial compounds by high-performance thin-layer chromatography (HPTLC)-direct bioautography using a Gram-positive *Bacillus subtilis* bacterium. Six chromatographic zones exhibited characteristic bioactivity. Five of them also appeared after derivatization with vanillin-sulfuric acid reagent and could be characterized with HPTLC-electrospray ionization (ESI)-mass spectrometry (MS), suggesting the presence of triterpenoids and the fatty acids linolenic and palmitic acid. To confirm the identification of triterpenoids an HPTLC method using *in situ* pre-chromatographic derivatization with iodine was developed to separate the closely related triterpenoids. After development, the iodine could be eliminated from the chromatogram (verified by HPTLC-MS), making it suitable for the *B. subtilis* assay. Ursolic acid, oleanolic acid, betulinic acid, corosolic acid, and maslinic acid were discovered for the first time as antibacterial components of *P. armeniaca* leaves. Their presence was proved also by 2D-HPTLC combined with intermediate *in situ* derivatization by iodine.

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1. Introduction

High-performance thin-layer chromatography (HPTLC) is a flexible technique that enables various pre-chromatographic derivatization, sample preparation, and post-chromatographic detection, all *in situ* in the same stationary phase [1–3]. Compared to the high-performance liquid chromatography (HPLC), HPTLC has benefits, such as the ease of its performance, the unnecessary of complex sample preparation, analysis of more samples at the same time, exclusion of cross-contamination, and a wide range of applicable detection methods, including the use of chemical reagents, enzymes, and viable cells [4–6]. The bio-profiles of the samples obtained by HPTLC-effect-directed analysis (EDA) allow a subsequent targeted characterization of the compounds run in a parallel HPTLC track, directly by HPTLC-attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) [7], HPTLC-FT-Raman [8] or HPTLC-mass spectrometry (MS) [9,10], or after elution off-line, e.g., by gas chromatography (GC)-MS [11] or nuclear magnetic resonance (NMR) [12,13]. HPTLC-direct analysis in real time-MS as a technique to analyze volatilizable compounds enables the scanning of the bioautograms [14]. The discrimination

of the bioactive compounds from the co-migrating other compounds can be achieved by improving the separation, e.g., with a spherical stationary phase [15], forced-flow layer chromatographic technique [16], or two-dimensional separation [17]. Another solution can be pre-chromatographic derivatization [18,19], but for EDA the preservation of the native molecules is essential, so only reversible modifications can be adopted. The online heart-cutting HPTLC-HPLC-(UV)-MS using an on-line elution head-based interface is suitable to determine if more compounds are coeluted in the bioactive HPTLC zone [20–23]. Installation of guard columns (RP, RP/IE, or Phenyl-X) and photodiode array (PDA) in this two-dimensional separation system permits to analyze the inhibition zones in the HPTLC-*Aliivibrio fischeri* bioautograms directly by MS [22,23]. An orthogonal HPLC system with a higher separation efficiency is helpful for the discovery of the compounds present in the zones of interest, but in the case of coeluting compounds, it does not point to the active one(s).

Apricot, *Prunus armeniaca* L., is a widely domesticated fruit tree species whose wild form is endemic to the Central-Asian Tien Shan Mountains [24]. There are other related apricot species [25] from which *P. mume* has been studied the most. Fruits and kernels of apricots have multiple uses, mainly as food and medicine ingredients [25]. *P. armeniaca* leaves acquired less scientific attention than the other parts, although they have their role in traditional Chi-

* Corresponding author.

E-mail address: moricz.agnes@agrar.mta.hu (Á.M. Móricz).

nese medicine and healthcare [26]. Indeed, the water extract from the leaves had potent antibacterial and antifungal activity, including *Bacillus* species [26] but the active ingredient(s) has not been identified. A phytochemical investigation of *P. armeniaca* leaf extracts revealed the presence of alkaloids, volatile oil, saponin glycosides, condensed tannins, terpenoids, steroids, flavonoids, and flavanoids, the latter possibly responsible for the hepatoprotective effect of the extract [27]. More recently, an anti-leishmanial effect was attributed to 1,2-benzenedicarboxylic acid, diisooctyl ester found in the ethanolic extract [28]. In an attempt to popularize apricot leaves as a rich source of bioactive components, this by-product of cultivation was characterized in terms of polyphenolics content and its antioxidant, anti-obesity, and anti-inflammatory actions were revealed [29]. *P. armeniaca* in Europe suffers from several infectious diseases. A relatively recent but potentially deadly disease is European Stone Fruit Yellow (ESFY), caused by a phytoplasma '*Candidatus* Phytoplasma prunorum'. Phytoplasmas are cell wall-less non-culturable Gram-positive bacteria. Interestingly, they appear more closely related to Firmicutes (encompassing important species like *Clostridium* or *Bacillus*) than to the other wall-less group, the mycoplasmas [30]. Thus, *Bacillus* may be used as a substitute *in vitro* test organism instead of the unculturable phytoplasma. Currently, it is largely unknown how plants resist phytoplasma infections.

The aim of this study was the screening for and identification of antibacterial compounds in *P. armeniaca* leaf extract by HPTLC-*B. subtilis* assay and HPTLC-MS. The separation of the closely related triterpenes, expected according to preliminary experiments, was planned using *in situ* pre-chromatographic derivatization that does not disturb the post-chromatographic bioassay.

2. Experimental section

2.1. Materials

HPTLC silica gel 60 F₂₅₄ plates (20 cm × 10 cm, #105642) were obtained from Merck Millipore (Darmstadt, Germany). Oleanolic acid (≥95%), ursolic acid (≥98%), corosolic acid (≥85%), maslinic acid (≥98%), and 2,3,24-trihydroxy-12-ursen-28-oic acid (≥98%) were purchased from BioSynth (Bratislava, Slovakia). Analytical grade solvents and gradient grade methanol were from Molar Chemicals (Halásztelek, Hungary). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Carl Roth (Karlsruhe, Germany). Iodine and vanillin were purchased from Reanal (Budapest, Hungary). The soil inhabitant *Bacillus subtilis* strain F1276 was a gift from Jőzsef Farkas (Central Food Research Institute, Budapest, Hungary).

2.2. Standard solutions and sample preparation

Oleanolic acid, ursolic acid, corosolic acid, maslinic acid, and 2,3,24-trihydroxy-12-ursen-28-oic acid were dissolved in ethanol (1 mg/mL each). Apricot (*Prunus armeniaca*) leaves (100 g) were collected in Budapest, Hungary, in October 2020. The leaves were dried at 25 °C in the dark and pulverized in a coffee grinder (Bosch MKM6000, Stuttgart, Germany). The milled leaves (150 mg) were macerated for 24 h with 1 mL ethanol in a glass vial. The supernatant was directly used for HPTLC. For flash chromatography 5 g of pulverized sample was macerated for 24 h with 30 mL ethanol.

2.3. High-performance thin-layer chromatography

Samples were applied onto the HPTLC layer as 5 mm bands with an 8 mm distance from the bottom (ATS3, CAMAG, Muttenz, Switzerland or manually for 2D-HPTLC with a 10-μL Hamilton syringe) in the range of 1–8 μL. One-dimensional HPTLC separa-

tions were achieved in a 10 cm × 10 cm twin trough chamber (CAMAG) with the mixtures of chloroform – ethyl acetate – methanol (20:3:2, 85:9:6, or 15:2:3 V/V/V) as a mobile phase. Saturation, if used, took 10 min. The chromatographic plates developed to the distance of 70 mm were dried for 5 min by a cold air stream of a hair-dryer. The *in situ* pre-chromatographic derivatization of the triterpenes was performed by applying 10 μL iodine solution (2% in chloroform) on the band of the deposited sample, covering the layer for 10 min with a glass sheet, and drying it for 1 min by a cold air stream of a hair-dryer before development. The 2D-HPTLC method consisted of two developments in an orthogonal direction up to 60 mm and an intermediate *in situ* derivatization with iodine. The developed layers were dried for 10 min by a cold air stream.

The chromatographic zones were derivatized with vanillin-sulfuric acid reagent (40 mg vanillin, 10 mL ethanol, and 200 μL concentrated sulfuric acid; heating at 110 °C for 5 min) by dipping with a homemade device (immersion time 2 s and immersion speed 3 cm/s) and documented at white light illumination (Vis, transmittance mode, Eglo 98,025 Salobrena LED panel, digital camera Sony Cybershot DSC-HX60), or evaluated via the *B. subtilis* assay.

2.4. HPTLC-direct bioautography

Bioassays were performed with *B. subtilis* test bacteria, utilizing the slightly modified TLC-DB method previously described [11]. Briefly, the developed and dried chromatoplates were immersed for 8 s in the bacterial cell suspension, and after 2 h incubation (at 37 °C, 100% humidity), the bioautograms were dipped into MTT solution (water, 1 mg/mL), incubated again for 0.5 h, and documented by a Cybershot DSC-HX60 digital camera. The metabolically active cells can reduce the yellow MTT to the bluish MTT-formazan, so the antibacterial compounds appear as bright spots against a bluish background.

2.5. HPTLC-MS

A binary pump (LC-20AB, Shimadzu, Kyoto, Japan), a TLC-MS Interface (CAMAG) with an oval elution head (4 mm × 2 mm), and a single quadrupole electrospray ionization mass spectrometer (LCMS-2020, Shimadzu) were combined online for the mass spectrometric analysis of the HPTLC zones of interest. Elution of the compounds was achieved with methanol (gradient grade) at a flow rate of 0.2 mL min⁻¹. The mass spectrometric conditions were as follows: nebulizer gas (N₂) flow rate, 1.5 L min⁻¹; drying gas (N₂) flow rate, 10 L min⁻¹; interface temperature, 350 °C; heat block temperature, 400 °C; desolvation line temperature, 250 °C; detector voltage 4.5 kV. The full mass scan spectra were recorded in the positive and negative ionization mode, in the *m/z* range of 200–1200. The instrument control and data acquisition were performed using the LabSolutions 5.42v software (Shimadzu).

2.6. Flash chromatography

To avoid the initial band broadening and focus the triterpenoids at the start of the column, the injection of the sample was carried out in chloroform. For this reason, 20 mL ethanol extract was dried, re-suspended in chloroform and then directly injected to a RediSep Rf Gold silica gel column (20–40 μm, 12 g; Teledyne Isco). Fractionation was performed with a Combiflash NextGen 300 (Teledyne Isco, Lincoln, NE, USA) flash chromatography system at a flow rate of 30 mL/min with a gradient of *n*-hexane (A) and acetone (B): 0–0.5 min, 0% B; 0.5–10 min 0–50% B; 10–10.2 min, 50–100% B; 10.2–11 min, 100% B. The chromatogram was monitored by absorbance measurement at 220 nm.

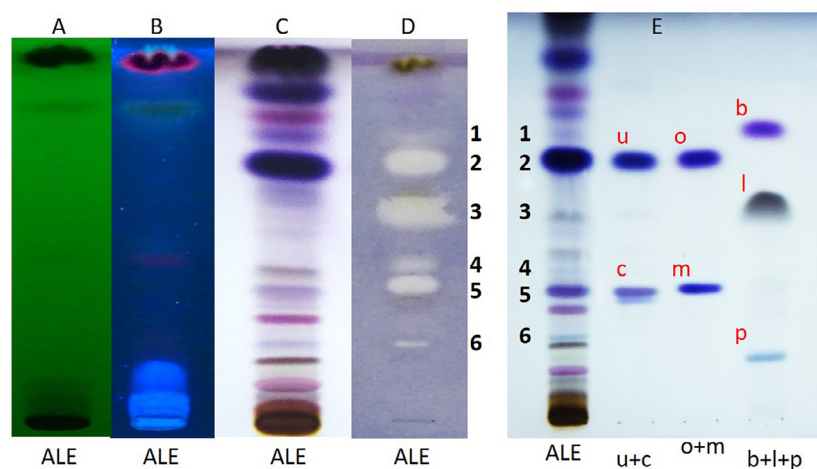


Fig. 1. HPTLC chromatograms of apricot (*Prunus armeniaca*) leaf extract (ALE) and triterpenes (u – ursolic acid, o – oleanolic acid, b – betulinic acid, c – corosolic acid, m – maslinic acid, p – pygenic acid B, and l – linolenic acid) at UV 254 nm (A) and 365 nm (B), and after derivatization with vanillin-sulphuric acid reagent at white light illumination in transmittance mode (C, E), and bioautogram after *B. subtilis* assay (D). Separation was performed with chloroform – ethyl acetate – methanol 20:3:2 (A–D) or 85:9:6 (E) (V/V/V) in a saturated chamber.

3. Results and discussion

3.1. HPTLC-effect-directed analysis of apricot leaf extract

The extraction solvent and the components of the mobile phase were selected based on the HPTLC fingerprints. HPTLC-UV/FLD chromatograms were not informative, but the derivatization with the universal vanillin-sulfuric acid reagent gave characteristic colorful bands (Fig. 1). Next to the HPTLC chemical fingerprints, the bio-profile obtained by HPTLC-*B. subtilis* assay enabled the choice of ethyl acetate and ethanol to extract the apricot leaves as they provided the same inhibition zones with similar intensity. Ethyl acetate gave less matrix among the more polar compounds, however, ethanol extracted less fatty acid, which was beneficial. Furthermore, the less polar *n*-hexane could not extract the same bioactive compounds with appropriate efficiency to reach detectable ap-

plied amounts. Six anti-*B. subtilis* zones were separable with mixtures of chloroform, ethyl acetate, and methanol (20:3:2 or 85:9:6, V/V/V). The bioactive zones were not UV-active but detectable after derivatization with vanillin-sulfuric acid reagent; zones 3 and 4 gave dark gray color, and all others appeared as bluish bands (Fig. 1).

3.2. Characterization of the bioactive zones by hptlc-ms

The six bioactive zones were further characterized by HPTLC-ESI-MS using an elution head-based interface. Compounds in all zones of interest gave characteristic mass signals (Table 1) except zone 4. For zone 3, two intense mass signals were obtained in negative ionization mode at m/z 255 and m/z 277. Comparing the chromatographic, chemical, and antibacterial features of these compounds with previous data [31–33], the presence of linolenic

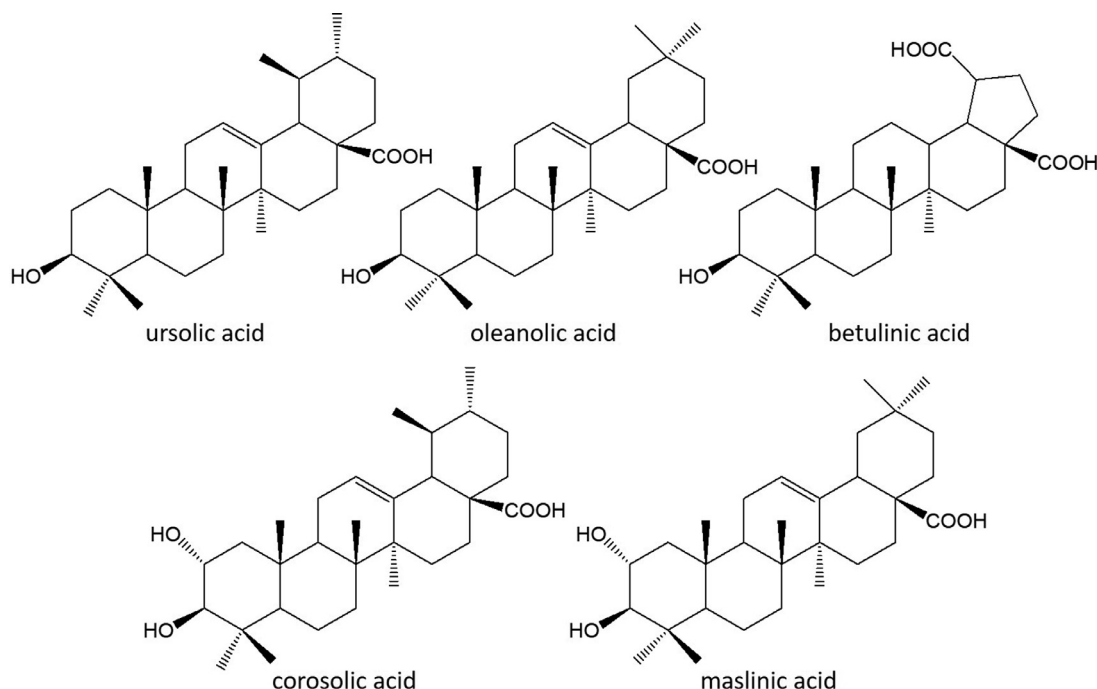


Fig. 2. Chemical structure of ursolic acid, oleanolic acid, betulinic acid, corosolic acid, and maslinic acid triterpenoids.

Table 1
HPTLC-MS analysis of the zones of interest.

HPTLC zones	HPTLC-ESI-MS signals <i>m/z</i> and assignment	Molar mass (g/mol)	Tentatively identified compounds
1 and 2	455 [M-H] ⁻ , 911 [2M-H] ⁻ , 933 [2M-2H+Na] ⁻ , 479 [M+Na] ⁺ , 501 [M-H+2Na] ⁺ , 511 [M+CH ₃ OH+Na] ⁺ , 935 [2M+Na] ⁺	456	ursolic acid and/or oleanolic acid and/or betulinic acid
3	277 [M ₁ -H] ⁻ , 255 [M ₂ -H] ⁻	278, 256	linolenic acid, palmitic acid
5	471 [M-H] ⁻ , 495 [M+Na] ⁺ , 527 [M+CH ₃ OH+Na] ⁺	472	pygenic acid A (corosolic acid) and/or maslinic acid (crategolic acid)
6	487 [M-H] ⁻ , 511 [M+Na] ⁺ , 543 [M+CH ₃ OH+Na] ⁺	488	triterpene with molecular formula of C ₃₀ H ₄₈ O ₅ ?

and palmitic acids are supposed. The same migration distance of linolenic acid standard to zone **3** and its HPTLC-MS spectrum confirmed its identity (Fig. 1E).

The HPTLC-ESI-MS spectra of zones **1** and **2** were very similar. In negative ionization mode the deprotonated molecule at *m/z* 455, the deprotonated dimer at *m/z* 911, and its sodium adduct at *m/z* 933 were detected. In positive mode, the sodium, the disodium, and the sodium-methanol adducts, as well as the sodium adduct of the dimer were recorded at *m/z* 479, at *m/z* 501, at *m/z* 511, and at *m/z* 935, respectively. According to literature data [19,21], the blue color after derivatization as well as the obtained mass signals indicated the presence of isomeric triterpenoids, ursolic, oleanolic, and/or betulinic acids (Fig. 2). The co-chromatography of these standards with the extract showed that betulinic acid had the same retardation factor as zone **1**, and oleanolic and ursolic acid were not separated and migrated as zone **2** (Fig. 1E). HPTLC-MS analysis of the standards also approved the candidature of these triterpenes.

In zones **5** and **6** the presence of further triterpenes was expected. In both zones the deprotonated ion (*m/z* 471 and *m/z* 487, respectively), the sodium adduct (*m/z* 495 and *m/z* 511, respectively), and the sodium-methanol adduct (*m/z* 527 and *m/z* 543, respectively) were detected by HPTLC-MS. Literature data of *Prunus* species [34–37] suggested the possible presence of corosolic acid (pygenic acid A, Fig. 2) and/or maslinic acid (crategolic acid, Fig. 2) in zone **5** and 2,3,24-trihydroxy-12-ursen-28-oic acid (pygenic acid B) or 2,3,24-trihydroxyolean-12-en-28-oic acid (arjunolic acid) or 2,3,19-trihydroxyurs-12-en-28-oic acid (tormentic acid) in zone **6**. With co-chromatography and HPTLC-MS analysis, the co-migration of corosolic and maslinic acids, along with zone **5**, was observed, but pygenic acid B (only purchased) had a slightly lower retardation factor than zone **6** (Figs. 1 and 3).

3.3. HPTLC separation using pre-chromatographic derivatization

To identify the compounds responsible for the antibacterial activity, flash chromatographic fractionation was carried out initially to obtain two fractions (Fig. 4) rich in the component present in zones **1** and **5**. Thus, fractions F12 and F16 were involved in the further characterization process. Betulinic acid and the two coeluting pairs oleanolic / ursolic acids as well as corosolic / maslinic acids displayed an antibacterial effect in the HPTLC-*B. subtilis* assay (Fig. 5). It has to be noted that also pygenic acid B inhibited the bacteria, but it was not in the leaf extract. It is known that oleanolic and ursolic acids are coeluted in normal phase (silica gel) separation systems [19]. Their separation could be achieved on a RP18 layer [19], by heart cutting two-dimensional hyphen-

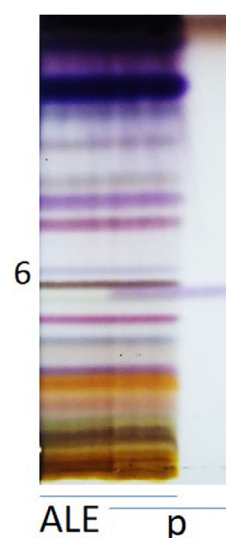


Fig. 3. HPTLC chromatogram of apricot (*Prunus armeniaca*) leaf extract (ALE) and pygenic acid B (p) in white light after vanillin-sulphuric acid derivatization. Separation was performed with chloroform – ethyl acetate – methanol 15:2:3 (V/V/V) in an unsaturated chamber.

ation HPTLC-HPLC [21], or by pre-chromatographic derivatization with iodine [38–40] or bromine [19]. Similarly to the pair of ursolic and oleanolic acids, corosolic and maslinic acids differ only in the position of one methyl group, which can explain their coelution.

The separation method was improved with a pre-chromatographic derivatization with iodine. The iodine solution (2% in chloroform) was applied onto the deposited sample, the layer was incubated under a glass sheet for 10 min, and after a short drying, the chromatogram was developed with the previously used mobile phase. Interestingly, the derivatization process did not change the retardation factor of ursolic, corosolic, and betulinic acids but made longer the migration distance of oleanolic and maslinic acids. It was observed in a previous study [38] that iodine could react with oleanolic acid at room temperature, but with ursolic acid only at a higher temperature (above 40 °C). So the latter one was found to be less reactive. Due to the similarity of their structure there is probably the same difference between the reactivity of maslinic acid and corosolic acid. Furthermore, betulinic acid without the double bond is not reactive. Thus, the pairs in question were separated from each other as well as from other components of the extracts. Their separation and pres-

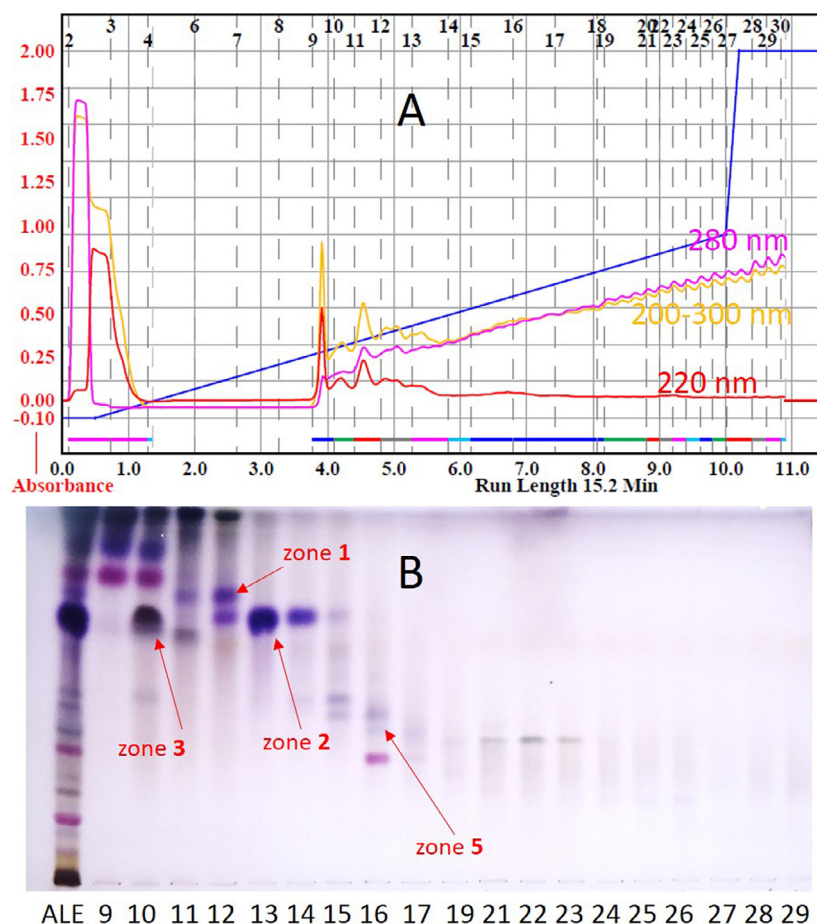


Fig. 4. HPTLC chromatograms (B) under white light illumination after derivatization with the vanillin-sulphuric acid reagent of apricot leaf extract (ALE) and its fractions (9–29) obtained by preparative normal phase flash chromatography of the crude extract (A). HPTLC separation was performed with chloroform – ethyl acetate – methanol 20:3:2 (V/V/V) in a saturated chamber.

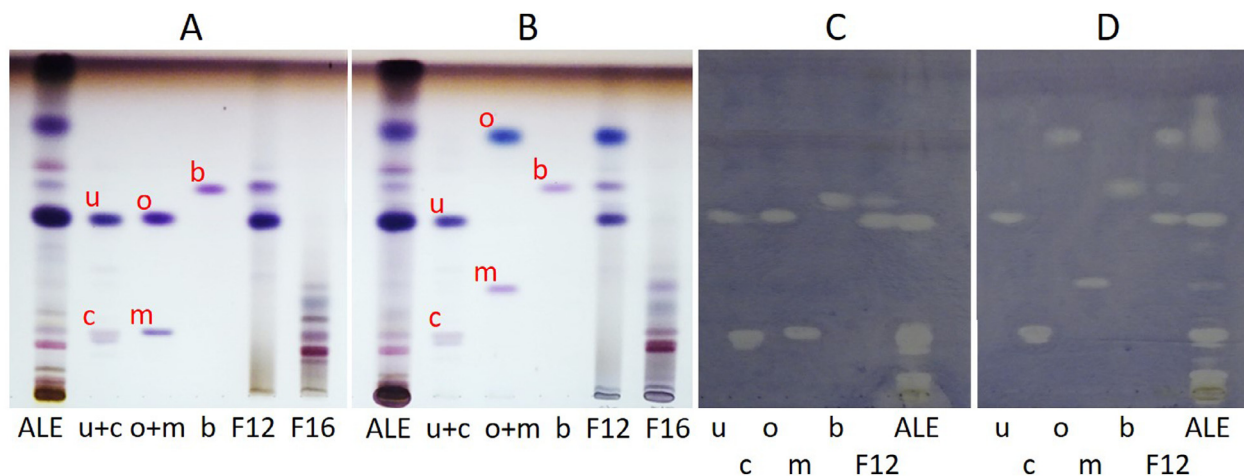


Fig. 5. HPTLC chromatograms of apricot (*Prunus armeniaca*) leaf extract (ALE), its flash fractions (F12 and F16), and active compounds (u – ursolic acid, o – oleanolic acid, b – betulinic acid, c – corosolic acid, and m – maslinic acid) after derivatization with vanillin-sulphuric acid reagent at white light illumination in transmittance mode (A, B), and bioautograms after *B. subtilis* assay (C and D). Separation was performed with chloroform – ethyl acetate – methanol 85:9:6 (V/V/V) without (A and C) and after pre-chromatographic derivatization with iodine (B and D).

ence in the extract were confirmed also by 2D-HPTLC combined with the intermediate derivatization by iodine (Fig. 6). Moreover, the pre-chromatographic derivatization did not influence the HPTLC-MS and 2D-HPTLC-MS signals. That is, the derivatization was only temporary, and iodine was eliminated during the drying of the developed chromatogram. This could explain why

there was no disturbance in the HPTLC-bioassay when we used pre-chromatographic derivatization, and the native triterpenoids could be analyzed. Thus, the presence of all five triterpenes in the apricot leaf extract was confirmed with the combination of pre-chromatographic derivatization, HPTLC separation, and detection with vanillin-sulfuric acid derivatization (Fig. 5B), MS, or B.

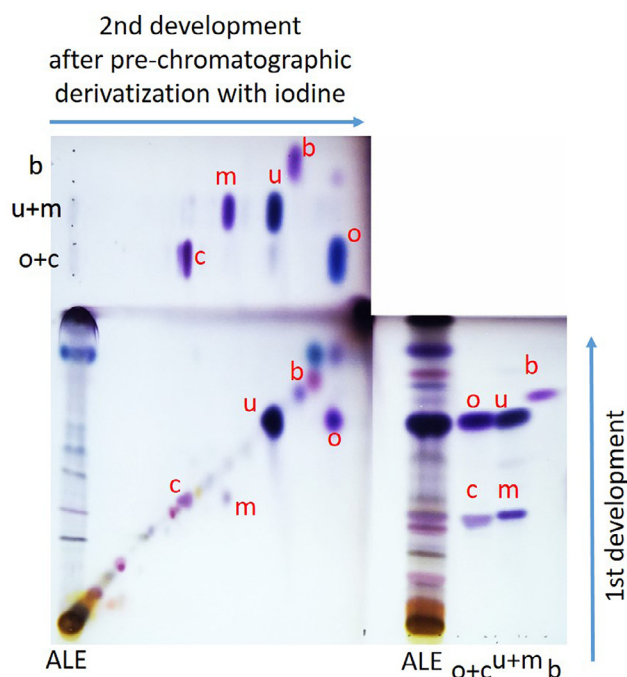


Fig. 6. Detection of triterpenes (u – ursolic acid, o – oleanolic acid, b – betulinic acid, c – corosolic acid, and m – maslinic acid) of apricot (*Prunus armeniaca*) leaf extract (ALE) by 2D-HPTLC combined with iodine derivatization before the 2nd development and visualization with vanillin-sulphuric acid reagent. Separation was performed with chloroform – ethyl acetate – methanol 20:3:2 (V/V/V) in a saturated chamber, in both directions.

subtilis assay (Fig. 5D). Various bioactivities have been previously attributed to all five compounds, e.g., antibiotic [41,42], including anti-*Bacillus subtilis* [43–45], cytotoxic [46], and antidiabetic ones [47].

The identified triterpenes have already been discovered as the constituents of several *Prunus* species, like corosolic and ursolic acids in fresh leaves of *P. zippeliana* [37], ursolic, oleanolic, betulinic, and corosolic acids in *P. dulcis* hulls [35,36,48], ursolic, and corosolic acids in *P. serrulata* var. *spontanea* dried leaves [34], and oleanolic, ursolic, and maslinic acids in *P. africana* bark [49]. Oleanolic [50], ursolic [50,51], and corosolic [51] acids have been discovered in the fruit of *P. mume*, the closest relative of *P. armeniaca*. A study using the TLC-anisaldehyde reagent demonstrated a pink zone in the chromatogram of the *P. armeniaca* bark extract at the same height as the pink spot of ursolic acid [52]. However, the presence of ursolic acid was not confirmed. Furthermore, the stationary phase was silica gel where ursolic and oleanolic acids were not separable. To the best of our knowledge, this is the first report to present the five triterpenoids in *P. armeniaca*.

4. Conclusions

The screening of apricot (*P. armeniaca*) leaf extract for antibacterial components with HPTLC hyphenations (EDA, chemical derivatization, MS) revealed fatty acids (linolenic and palmitic acids) and triterpenes as compounds of interest. HPTLC combined with pre-chromatographic derivatization and post-chromatographic bioassay enabled the separation and identification of five triterpenoids, the structural isomers ursolic, oleanolic, and betulinic acids, as well as corosolic acid and maslinic acid isomers. The 2D-HPTLC-MS analysis confirmed that the pre-chromatographic derivatization altered the migration distance only of oleanolic and maslinic acids. The derivatization was found to be temporary: after development, the derivatizing agent (iodine) was

eliminated by drying, so the native compounds could be analyzed with HPTLC-*B. subtilis* assay. This method was suitable for the separation, effect-directed detection, and identification of closely related isomers that are not separable in normal phase silica gel in a conventional way.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ágnes M. Móricz: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Péter G. Ott:** Conceptualization, Methodology, Resources, Writing – review & editing.

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