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Bearberry identification by a multidisciplinary study on commercial raw materials

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Herbal species different from the official bearberry, *Arctostaphylos uva-ursi,* are sold through conventional markets and also through non-controlled Internet websites, putting consumer safety at risk owing to the lack of quality control. Recently, *Arctostaphylos pungens* has become one of the most used species as a raw material for herbal medicines and dietary supplements in the place of official bearberry, a plant used for the treatment of various urinary disorders. A fingerprint identification based on an integrated application of different analytical techniques (HPTLC, NMR, HPLC-DAD and LC-ESI-MS) is here described to distinguish *A. uva-ursi* from *A. pungens*. The HPTLC and HPLC-DAD fingerprints resulted the simplest methods to differentiate the two species, whereas LC-ESI-MS was more useful to quantify arbutin, the main component of bearberry, and to evaluate its different content in the two species. This multidisciplinary study showed for the first time a specific phytochemical fingerprint of the new species *A. pungens*.

Keywords: bearberry; *Arctostaphylos uva-ursi*; *Arctostaphylos pungens*; HPTLC fingerprint; HPLC-DAD fingerprint; NMR; LC-ESI-MS

1. Introduction

Owing to the increase of the global herbal market, new species are being introduced for therapeutic and nutritional uses instead of those traditionally utilised and scientifically recognised. They are sold through conventional markets as well as through non-controlled Internet websites; therefore, quality controls on the new species and their derivatives are urgently needed. The distinction between raw materials of taxonomically near botanical entities constitutes an analytical challenge, in the past limited to the scientific community, nowadays, involving important economic consequences. Ordinary approaches, such as the study of the macroscopic and microscopic characters of the herbal drugs alone, can be of low or without utility. However, recent progresses in phytochemistry offer a set of powerful devices helpful to explore the world of natural substances based on the molecular and metabolomic approach (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Mattoli et al., 2006).

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Bearberry, *Arctostaphylos uva-ursi* (L.) Spreng. (Ericaceae), is a well-known medicinal plant, widely used to treat a variety of urinary disorders (Blumenthal et al., 1998; Cui, Nakamura, Ma, Li, & Kayahara, 2005; European Scientific Cooperative on Phytotherapy, 1997). The used part of bearberry is the leaf and arbutin (AR) (Figure S1) is considered the principal active compound (Bundesinstitut für Arzeneimittel und Medizinprodukte. Kommission E, 1994). The beneficial effects of this plant are related to its antimicrobial activity *in vitro* (Bundesinstitut für Arzeneimittel und Medizinprodukte. Kommission E., 1994; Panaiotov, 1951), although pure AR shows a lower antimicrobial efficacy (Chauhan et al., 2007). It is assumed that the glucoside AR is adsorbed from the gastrointestinal tract unchanged and it is excreted in urine where it is hydrolysed to hydroquinone (Figure S1) at alkaline pH. In the urinary tract, the free hydroquinone exerts its antiseptic and astringent effects (Yarnell, 2002), to be then eliminated as glucuronic and sulphuronic acids conjugates (Glockl, Blaschke, & Vei, 2001).

Bearberry is considered safe for oral medicinal use, but there is a concern regarding its long-term utilisation, mainly due to the mutagenic and carcinogenic effects of hydroquinones. These compounds, used at high doses, can cause several side effects (Brinker, 1998; Pizzorno, 1999) and are reported as oxytocic (inducing or increasing the rapidity of labour) (Brooks, 1995). Their chronic use, especially in children, can cause liver impairment due to their metabolites (Brooks, 1995).

The increasing demand for bearberry leaf causes the introduction on the market of herbs alternative to the traditionally used and thoroughly studied *A. uva-ursi* (Table S1) (Chukarina, Vlasov, & Eller, 2007; Lamien-Meda, Lukas, Schmiderer, Franz, & Novak, 2009; Radulovic, Blagojevic, & Palic, 2010). In particular, *Arctostaphylos pungens* H.B. & K. (=*Arctostaphylos glauca* S. Wats.) (Royal Botanic Gardens Kew, 1997) is frequently sold as bearberry raw materials. It has morphological characters similar to *A. uva-ursi,* but it originates from Mexico, where it is wildly collected and marketed at the lowest cost. The few phytochemical data include contradictory information about the presence of AR (Applequist, 2006). Some other minor bearberry adulterations are reported, like those involving the European cowberry, *Vaccinium vitis-idaea* L., and other Mexican Ericaceae spp., e.g. *Arctostaphylos tomentosa* Lindl., *Arctostaphylos mucronifera* D.C., *Arctostaphylos polifolia* H.B. & K., *Arbutus xalapensis* H.B. & K. and *Vaccinium uliginosum* L. (Applequist, 2006; Mills & Bone, 2005).

To not only obtain a proper identification of a plant material, but also evaluate the influence of the environmental conditions, a fingerprint approach gives an easy discrimination of different species. In chromatography, a fingerprint is an individual track representing, as near as possible, the mixture of the characteristic constituents of a plant. HPTLC and HPLC-DAD fingerprints have been recognised by World Health Organization (2007) as official methods to identify an herbal species or its preparations when it is not possible to identify an active compound. Other techniques useful for defining metabolomic fingerprinting of herbal drugs and plant extracts are nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography coupled with mass spectrometry (LC-MS) (Bilia, Bergonzi, Lazari, & Vincieri, 2002; Kruger, Troncoso-Ponce, & Ratcliffe, 2008; Mattoli et al., 2006; Piccin, Serafini, & Nicoletti, 2009).

The aim of this study was to evaluate the quality of commercial bearberry leaf utilised as starting plant raw material (herbal substance) for herbal medicinal products or food supplements by the fingerprint approach. In particular, the objective was the fingerprint identification of *A. uva-ursi* by a multidisciplinary approach with the application of different techniques (HPTLC, NMR, HPLC-DAD and LC-ESI-MS) to the analysis of 16 samples of bearberry leaf obtained from different market suppliers (Table 1).

Table 1. Bearberry leaf raw materials from Italian suppliers.

Sample	Label name	Origin
1	Bearberry	Macedonia
2	Bearberry	Not declared
3	Bearberry leaf	Not declared
4	Bearberry leaf (tea)	Not declared
5	Bearberry uva-ursi	Mexico
6	Bearberry leaf (tea)	Latin America
7	Bearberry leaf	Balkans
8	Bearberry glauca leaf (tea)	Mexico
9	Bearberry leaf	Albania
10	Bearberry leaf	Siberia
11	Bearberry leaf (tea)	Mexico
12	Bearberry officinal (tea)	Albania
13	Bearberry officinal (tea)	Macedonia
14	Bearberry leaf (tea)	Macedonia
15	Bearberry leaf (tea)	Mexico
16	Bearberry leaf (tea)	Serbia

The ultimate intent was to detect possible substitution of bearberry leaf by other species especially by *A. pungens* which was characterised for the first time in this study.

2. Results and discussion

To evaluate the quality of 16 commercially available bearberry leaf by the fingerprint analysis, each raw material was extracted with methanol (European Pharmacopoeia 7.1., 2011) in an ultrasonic bath and the resulted dried extracts were analysed by the application of different techniques.

2.1. *HPTLC analysis*

HPTLC was used to make a preliminary and rapid screening of the 16 bearberry extracts. Two types of fingerprints were obtained. Samples 3, 4, 9, 10, 12–14 and 16 (Figure 1a) belonged to the first type, accordingly to the track of the authenticated *A. uva-ursi* reference plant sample 7, characterised by a strong spot corresponding in position and colour to that of AR standard at $R_F = 0.42$. Samples 2, 5, 6, 8, 11 and 15 (Figure 1a) belonged to the second type of fingerprints, where the AR zone was not clearly displayed. The presence of AR was confirmed by the UV spectra ($\lambda_{max} = 284$ nm) directly obtained on the spots at $R_F = 0.42$ (Figure S2) and by the AR standard addition. Sample 1 differed from both types, showing a totally different fingerprint (Figure 1).

In the next densitometric analysis, the most evident difference between the two types of fingerprint profiles was a higher peak of AR in the first than in the second typology (Figure 1b).

2.2. *NMR results*

The ^{1}H-NMR of the same 16 dried bearberry extracts gave results similar to those observed by the HPTLC analyses providing again two different sets of spectra, related to the same two groups of samples. In the first, the aromatic region of the spectra showed a net pair of doublets, assigned to the AA'BB' system of AR (6.5–7.0 ppm), also present in *A. uva-ursi* reference plant sample 7, but not evident in the second type spectra where this

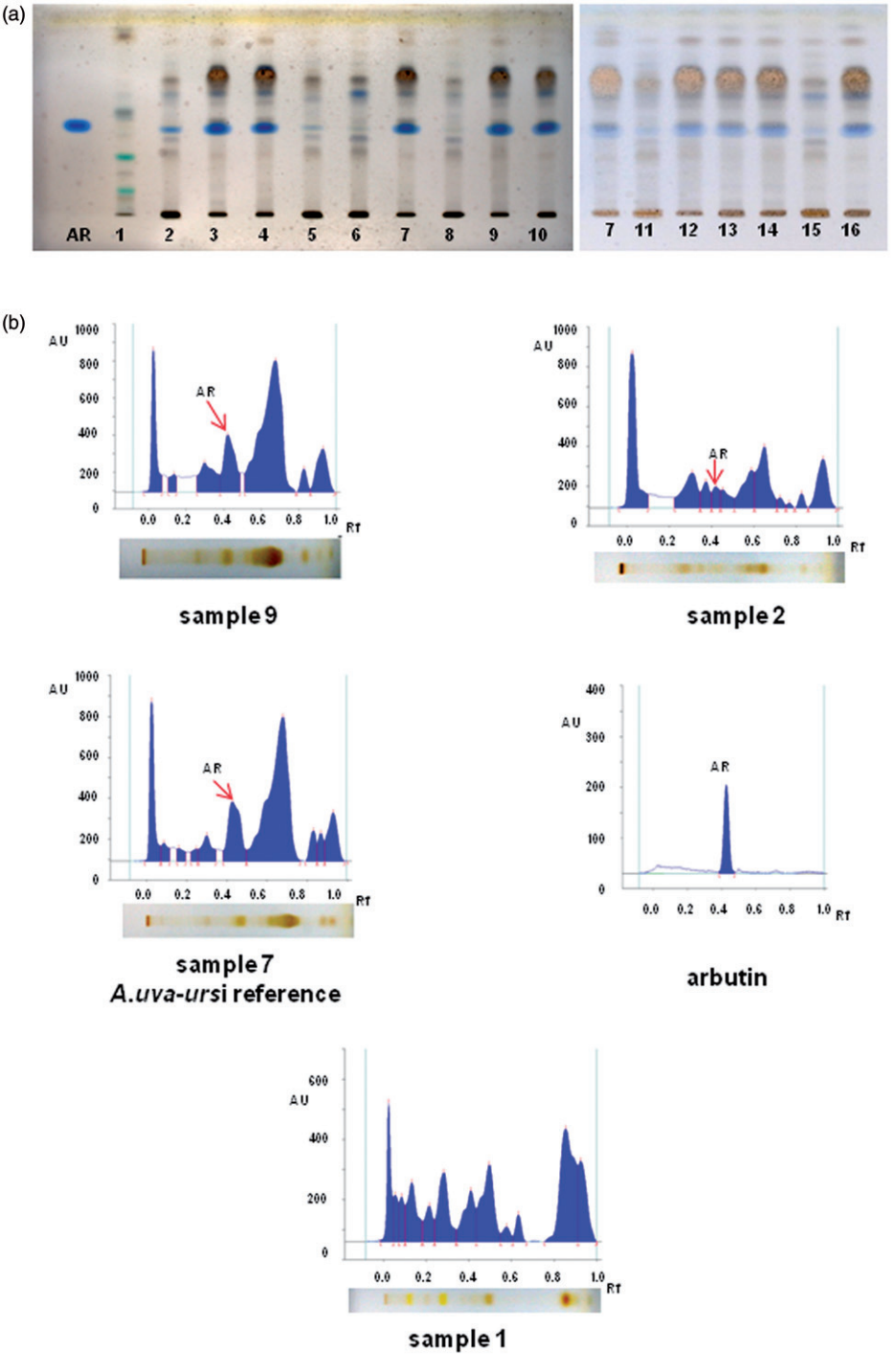

Figure 1. (a) HPTLC of samples 1–16 and AR after visualisation with Gibbs reagent. (b) Densitograms of *A. uva-ursi* and *A. pungens* (samples 9 and 2, respectively) compared with AR and *A. uva-ursi* reference plant sample 7; densitogram of the unidentified plant sample 1.

region was crowded of peaks, probably due to flavonoids (Figure S3). In the ^{1}H-NMR spectrum of sample 1, the characteristic peaks of AR and flavonoids were not present.

2.3. *HPLC-DAD analysis*

As for HPTLC and NMR, also with HPLC it was possible to discriminate two types of chromatograms, related to the same two different groups of samples. The fingerprints (Figure S5) in the *Rt* range 4.2–9.0 min showed groups of recurring peaks among which AR (4.7 min) and hydroquinone (7.9 min) were confirmed by the addition of the standards in either sets of samples. The intensity of AR peak is higher in the first typology, corresponding to *A. uva-ursi* reference plant sample 7 (Parejo, Viladomat, Bastida, & Codina, 2001), than in the second typology. In the *Rt* range 17.0–20.0 min, a group of peaks corresponding to flavonoids were observed and confirmed by the addition of rutin reference standard (17.8 min) chosen as flavonoid marker compound. In the second typology, the flavonoid peak group was much crowded than in the first one. Again, sample 1 did not show characteristic peaks of the two categories and differed from both species (Figure S5).

2.4. *Analysis of flavonoids*

The relevant presence of flavonoids in the second type samples, as it resulted from ^{1}H-NMR and HPLC-DAD analysis, was confirmed by the column chromatography isolation of two compounds from methanol extract of sample 6, chosen as an example. The two isolated substances were identified as myricetin and a diglucopyranoside of kaempferol, acetylated in position 6 of the glucose, i.e. 3-*O*-(6″-*O*-acetylglucoside)-7-*O*-glucoside-kaempferol (Harborne, 1994; He et al., 2009).

2.5. *LC-ESI-MS data*

Under the optimised conditions, retention times of the reference AR and internal standard (IS) were 3.1 and 8.2 min, respectively. The method was applied to quantify AR in all dried extract samples prepared as described in experimental section (Supplementary online material). In Figure S6, a representative single ion monitoring trace, relative to AR and IS, obtained from the *A. uva-ursi* reference plant sample 7 was displayed. LC-ESI-MS investigation was able to detect unequivocally the different AR amounts consistently with the results obtained with the other techniques. AR content ranged from 8% to 16% (w/w) in samples of the first type (samples 3, 4, 9, 10, 12–14 and 16 together with the *A. uva-ursi* reference plant sample 7) and it was under 2% in the second type (samples 2, 5, 6, 8, 11 and 15) (Panusa, Petrucci, Gallo, Multari, & Marrosu, 2011).

On the basis of the two different fingerprint sets obtained by HPTLC and HPLC-DAD, and of the two different AR content by LC-ESI-MS, it was possible to distinguish the two species of *Arctostaphylos*. The leaf samples of the first type were assigned to *A. uva-ursi* according to the authenticated reference plant sample 7, whereas the leaves of the second type samples presented the morphological characters reported for *A. pungens* (Applequist, 2006). Sample 1 leaves belonged neither to *A. uva-ursi* nor to *A. pungens* species and their assignment is still under investigation.

The HPTLC analysis gave evident and quickly interesting results about the two examined species. The further derivatisation of the layers with Gibbs reagent evidenced the AR zone due to its specificity for phenolic subunits (Figure 1a). On the other hand, the HPTLC failed in the absolute determination of the AR content in *A. pungens,* due to the overlapping of AR spot with other related substances. However, the efficient use of HPTLC densitometry for quantification of AR in *A. uva-ursi* was recently reported,

confirming the importance of this technique in natural product assay (Alam, Alqasoumi, Shakeel, & Abdel-Kader, 2011). In this study, the differences in AR content between *A. uva-ursi* and *A. pungens* were better evidenced by the higher sensitivity and selectivity of LC-ESI-MS. In particular, among nine samples belonging to *A. uva-ursi* species (first type), three samples presented a content of AR up to 12%, and six within 7.7–10%. Among the six examined samples belonging to *A. pungens* (second type), five presented an AR content less than 1% (w/w) and one under 2% (Panusa et al., 2011).

These results are not a secondary aspect because, as reported in European Pharmacopoeia 7.1. (2011), bearberry leaf, defined as dried leaf of *A. uva-ursi* (L.) Spreng. useful for pharmaceutical purposes, must have an AR amount not inferior to 7%, although this cannot be applied to food supplements. For this reason, the substitution of the bearberry (*A. uva-ursi*) (Bundesinstitut für Arzeneimittel und Medizinprodukte. Kommission E., 1994) with cowberry (*V. vitis-idaea*) can be allowed because in the last plant the AR content is about 5–7% (National Toxicology Program, 2006; Pyca, Bober, & Stolarczyk, 2007). Taking in account the lower quantities of AR recovered in the examined *A. pungens* samples, a replacing of *A. uva-ursi* with *A. pungens* would lead to a decline of activity. However, the relevant presence of flavonoids evidenced in *A. pungens,* as well as also isolated in sample 6, could compensate the low AR content in this species because their antibacterial and anti-inflammatory activities in urinary infections have been recently described (de la Iglesia, Milagro, Campión, Boqué, & Martínez, 2010; Liao et al., 2011). On the light of the existing literature about the negative effects of hydroquinones (US Public Health Service, 1989; World Health Organization, 1994), and the analytical results here described a scientific validation of *A. pungens* is needed to support its current use on the market.

3. Conclusions

Two typologies of HPTLC fingerprints, confirmed by NMR and HPLC-DAD, permitted to establish an immediate difference between two *Arctostaphylos* species and they can be used to quickly detect the substitution of *A. uva-ursi* with *A. pungens* in bearberry products.

The results evidenced that HPTLC fingerprint was easier to obtain and to be interpreted, for a parallel comparison of herbal samples, than the other techniques. In fact, although less sensitive than HPLC-DAD and LC-ESI-MS, it allows a quick identification of raw material and an easy data interpretation. Furthermore, it is more cost-effective.

For all these reasons, HPTLC is emerging as a powerful tool for analysis of complex mixtures of natural substances from plants and their derivative herbal products. In fact, while the separation of a single characteristic component from a plant is a hard task, the fingerprint approach allows visualisation of the whole characterising chemical profile for each individual species.

Supplementary material

Experimental details relating to this article are available online, alongside Table S1 and Figures S1–S6.

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