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Secondary Metabolites of *Alchemilla persica* Growing in Iran (East Azarbaijan)

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Phytochemical investigations of *Alchemilla persica* Rothm. growing in Iran were performed taking into account both the volatile and polar constituents. The hydrodistilled essential oil was analysed by GC-MS that revealed the presence of diterpenoids (19.6 %) and sesquiterpenes (17.1%) as the major constituents, while tannins and flavonol glycosides were identified as the most abundant constituents of the methanol extract by HPLC-MS. *A. persica* can be a valuable source of ellagitannins and polyphenols.

Keywords: *Alchemilla persica*, Essential oil, Polar constituents, Phenolic compounds, Tannins, GC-MS, HPLC-MS.

The genus *Alchemilla* L., belonging to the Rosaceae family, includes more than one thousand species distributed especially in northeastern Anatolia (Turkey), north-west of Iran and the north of Iraq [1,2]. This genus, with its common English names "Lady`s Mantle "or "lion`s foot", is documented in the Iranian flora as 24 species, of which 14 are considered endemic [3,4]. In Iran, the genus *Alchemilla* is mainly represented by *A. persica* Rothm.

Previous reports on *Alchemilla* species, especially *A. vulgaris* L. (the most commonly used species), revealed that they exhibit various pharmacological properties such as astringent, antihemorrhagic, antidiarrheal [5], anti-inflammatory, antiseptic [6,7], antimicrobial [1] and antioxidant [8-10]. Externally, they have been used in bath preparations, for wound healing, skin bruises, skin rashes and eczema [11,12]. In addition, *Alchemilla* species have been used as a traditional remedy to modify the hormonal levels of the body in the case of menopause [13]. In particular, methanolic extracts of *A. persica* were effective in the treatment of endometriosis [14].

According to the literature, the genus *Alchemilla* is rich in tannins (ellagitannins such as pedunculagin and alchemillin), flavonoids (orientin, quercetin, quercetin-3-arabinopyranoside, quercetin 3-*O*-β-(2"-*O*-α-L-rhamnopyranosyl)-glucopyranoside uronic acid, kaempferol 3-*O*-β-(2"-*O*-α-L-rhamnopyranosyl)-glucopyranoside uronic acid, rutin, vitexin, miquelianin and hyperoside) and coumarins (esculetin) [15-22]. Previous works have been carried out on the chemical composition of the essential oils of *A. xanthochlora* Rothm. and *A. alpina* L. [23,24], but to the best of our knowledge there are no available reports on the chemical composition of the essential oil and polar constituents of *A. persica* yet.

Therefore, in the present study, we report the phytochemical polar constituents of the flowering aerial parts of *A. persica* collected in Iran (East Azarbaijan province), along with the volatile components contained in the hydro-distilled essential oil.

Hydro-distillation of the aerial parts of *A. persica* growing in Iran gave a yellow oil with a yield of 0.025 %, w/w, based on the dry mass. The chemical composition of the essential oil is reported in Table 1, where the components are listed in order of their elution on an HP-5MS column. A total of 16 volatiles were identified, corresponding to 77.6% of the total composition.

The major constituents were phytol (19.6 %), *n*-tricosane (12.7 %), (*E*,*E*)-α-farnesene (11.1 %) and *n*-heptacosane (8.4 %). The main classes occurring in the oil were alkanes (27.8%), diterpenes (19.6%) and sesquiterpene hydrocarbons (17.1%). Other minor groups were oxygenated sesquiterpenes (4.9%), aromatics (3.4%), norisoprenoids (2.6%) and oxygenated monoterpenes (2.1%).

A literature review showed that there is no previous report on the chemical constituents of *A. persica* essential oil. Previous studies conducted on other *Alchemilla* species showed that *Z*-3-hexenol, linalool, α-terpineol and nonanal are the main volatile constituents [23,24]. These components were not detected in our examined oil. Concerning the main constituent detected in our oil, i.e. phytol, it was not found in *A. xanthochlora*, while it was present in low levels (2.6%) in *A. alpina*. Moreover, the sesquiterpene hydrocarbon (*E*,*E*)-α-farnesene was reported for the first time as a major volatile constituent in the genus *Alchemilla*.

Phytol is a natural linear diterpene alcohol often produced from degradation of chlorophyll. It possesses a balsamic olfactive note which is used in the manufacture of synthetic vitamins E and K, and in cosmetic applications, such as soap, detergent and beauty care products [25]. (*E*,*E*)-α-farnesene is one of the two naturally occurring stereoisomers of α-farnesene, an acyclic sesquiterpene hydrocarbon that was found in the coating of apples and other pomoidea fruits within the Rosaceae family. This compound is responsible for the characteristic green apple odor, and is used for enhancing the aroma or taste of foodstuffs, chewing gums, medicinal products and toothpastes, as well as in perfumery, as an ingredient of perfume compositions and colognes [29].

Table 1: Chemical composition of the essential oil hydro-distilled from the aerial parts of *A. persica*.

No.	Compounds[a)]	Calc. LRI[b)]	Lit. LIR[c)]	% [e)]	ID [f)]
1	Myrtenol	1187	1194	2.1	RI,MS
2	1,2-Dihydro-1,1,6-trimethyl-naphthalene	1338	1340	3.4	RI,MS
3	(*E*)-β-Damascenone	1374	1383	0.8	RI,MS
4	(*E*)-Caryophyllene	1402	1417	5.7	RI,MS
5	Germacrene D	1465	1484	0.3	RI,MS
6	(*E*)-β-Ionone	1476	1487	1.8	Std
7	(*E*,*E*)-α-Farnesene	1502	1505	**11.1**	Std
8	Caryophyllene oxide	1564	1582	2.4	Std
9	Phytone	1837	1845	2.5	RI,MS
10	*n*-Heneicosane	2100	2100	0.3	RI,MS
11	Phytol	2104	2107	**19.6**	Std
12	*n*-Docosane	2200	2200	0.2	Std
13	*n*-Tricosane	2300	2300	**12.7**	Std
14	*n*-Tetracosane	2400	2400	0.7	Std
15	*n*-Pentacosane	2500	2500	5.6	Std
16	*n*-Heptacosane	2700	2700	**8.4**	Std
	Total Identified (%)			77.6	
	Oil yield (%)			0.025	
	Grouped compounds (%)				
	Alkanes			27.8	
	Oxygenated monoterpenes			2.1	
	Sesquiterpene hydrocarbons			17.1	
	Diterpenoids			19.6	
	Oxygenated sesquiterpenes			4.9	
	Norisoprenoids			2.6	
	Aromatics			3.4	

[a] Compounds reported in order of their elution from a HP-5 MS capillary column. [b] Retention index (*RI*) on HP-5 MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c] Literature *RI* published by Adams [26] and /or listed in the NIST08 mass-spectral library [27]. [e] Contents are given as means of three determinations; the relative standard deviations for the main components were below 10% in all cases. [f] Identification method: Std, based on the comparison with authentic compound; MS, based on the comparison of mass spectra with those listed in the Adams, Wiley, and NIST08 mass spectral libraries; *RI*, based on the comparison of *RI*s with those reported by Adams [26], NIST08 [27] and FFNSC2 [28].

Alkanes, especially long chain *n*-alkanes, comprised the highest contribution, representing 27.8% of the essential oil, with the linear odd carbon atom series C23-C27 as dominant (*n*-tricosane, *n*-pentacosane and *n*-heptacosane accounting for 12.7, 5.6 and 8.4%, respectively). Previous studies displayed that *n*-alkanes distribution in waxy coatings on leaves and other organs of plants might follow A and B patterns: the A pattern includes a Gaussian-like distribution of even and odd *n*-alkanes in equivalent amounts (around C22-C28) and originating from parenchymatic parts; the B pattern, produced by epidermal tissues in the cuticular waxes, shares an alternation in chain length distribution and the odd *n*-alkanes are dominant (C25-C33) [30,31]. In the case of *A. persica*, the B pattern of *n*-alkanes distribution is clearly shown.

Analysis of the methanolic extract revealed the presence of several phenolic constituents which can be referred to as caffeic acid esters with sugars, flavonoids glycosides, catechin and epicatechin, condensed tannins related to gallic acid, such as pedunculatin/ pedunculagin, agrimoniin, casuarictin, castalagin/vescalagin, and sanguiin H-10 isomers. Their structures were identified on the basis of the exact mass, fragmentation pathways, comparison with reference compounds when available and comparison with the literature [32-34]. From a quantitative point of view, sanguiin and pedunculatin are the most abundant constituents. The obtained extract contains an high amount of phenolics, accounting for 24% of the total extract on the basis of dry weight and shows the high levels of such constituents in this medicinal plant

In conclusion, current work on *A. persica* grown in Iran describes both the volatile constituents as well as the most abundant phenolic compounds which are present in this medicinal plant. Gallotannins and ellagitannins are nowadays highly considered for their proposed health promoting effects. In particular, these phytochemicals show

Table 2: Identified phenolic constituents from the methanol extract of *A. persica*; identification of compounds was achieved on the basis of HR-MS Q-TOF measurement through calculation of molecular formula (M), comparison of literature data related to fragmentation in MS-Ion trap (L), comparison with reference compounds, when available (S).

	Exact mass	Observed fragments	Molecular formula	Identification	%
Tetra-acetyl hexose	377.0839	341-179	$C_{15}H_{22}O_{11}$	M, L	0.16±0.01
Gallic acid 4-glycoside	331.0565	169	$C_{13}H_{16}O_{10}$	M, L,S	0.17±0.01
Gallic acid methoxy glycoside	345.0444	169	$C_{14}H_{18}O_{10}$	M, L	0.10±0.01
Gallic acid-*O*-glycoside	331.0566	169	$C_{13}H_{16}O_{10}$	M, L	0.19±0.01
Pedunculatin isomer 1	783.0685	301-257-229	$C_{34}H_{24}O_{22}$	M, L	0.21±0.01
Chlorogenic acid	353.0873	191	$C_{16}H_{18}O_9$	M,L,S	0.11±0.02
Gallic acid	169.0149	125	$C_7H_6O_5$	M,L,S	0.17±0.01
Catechin	289.0718	245-205-179	$C_{15}H_{14}O_9$	M,L,S	0.30±0.02
Galloyl-HHDP-hexoside isomer 1	633.0721	463-481-301	$C_{27}H_{22}O_6$	M, L	0.27±0.01
Pedunculatin isomer 3	783.0712	301 257 229	$C_{34}H_{24}O_{22}$	M, L	0.77±0.01
Galloyl-HHDP-hexoside isomer 2	633.0721	463-481-301	$C_{27}H_{22}O_{18}$	M, L	0.37±0.01
Quercetin-3-*O*-glucuronide	477.0721	301	$C_{21}H_{18}O_{13}$	M,L,S	0.20±0.01
Epicatechin	289.0718	245-205-179	$C_{15}H_{14}O_9$	M,L,S	0.10±0.02
Pedunculatin isomer 4	783.0703	301 257 229	$C_{34}H_{24}O_{22}$	M, L	1.23±0.01
Sanguiin H-10 isomer 1	1567.1401	783	$C_{68}H_{47}O_{44}$	M, L	1.06±0.02
Sanguiin H-10 isomer 2	1567.1412	783	$C_{68}H_{47}O_{44}$	M, L	8.21±0.01
Casuarictin	935.0753	301	$C_{48}H_{23}O_{21}$	M, L	1.94±0.01
Agrimonin	1870.1611	915-783-301	$C_{82}H_{54}O_{52}$	M, L	0.80±0.01
Procyanidin B1	577.2611	288-406	$C_{30}H_{26}O_{12}$	M,L,S	1.26±0.01
Digalloyl-galloyl galloside	1084.0666	783-633-301	$C_{48}H_{29}O_{30}$	M, L	1.98±0.01
Aromadendrin glucoside derivative	693.3812	287	$C_{37}H_{57}O_{12}$	M, L	0.13±0.01
Kaempferol-3-*O*-rutinoside	593.1277	285	$C_{27}H_{30}O_{15}$	M,L,S	1.99±0.01
Methyl gallate	183.0112	169-125	$C_8H_8O_5$	M, L	0.03±0.01
Ellagic acid	301.0341	229-185-173	$C_{14}H_6O_8$	M,L,S	0.62±0.01
Total amount					24

~~Identification of compounds was achieved on the basis of HR-MS Q-TOF measurement through calculation of molecular formula (M), comparison of literature data related to fragmentation in MS-Ion trap (L), comparison with reference compounds when available (S).~~

biological effects mainly related to the prevention of cardiovascular diseases and some types of cancer [34]. Oxidative stress has been reported to play a crucial role in cardiovascular pathologies such as atherosclerosis, hypertension and myocardial infarction. Therefore, the 'antioxidant activity' exerted by compounds such as tannins and polyphenols has been linked to potential cardioprotective effects of such compounds. *A. persica* can be considered as a good source of such constituents and the phytocomplex of this plant may be further investigated due to its abundant level of phytoconstituents and also due to its high consideration in oriental traditional medicine.

Experimental

Plant material: The aerial parts of *A. persica* were collected near Marand (Misho mountain) at E: 45° 47', N: 38° 19' (altitude of 2036 m) in Eastern Azarbaijan province (Iran) during June 2014. The identity of the plant was confirmed by ~~anatomical~~ examination in comparison with the herbarium specimens (voucher No. Tbz-FPh-748) retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran.

Isolation of the essential oil: Plant material was dried at room temperature. Aerial parts (200 g) were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil yield was

estimated on a dry-weight basis (w/w). Once obtained, the oil was dried (Na_2SO_4), transferred into an amber glass flask, and kept at -20°C before chemical analysis.

GC-MS analysis: Chemical analysis of *A. persica* essential oil was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer using a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 mm film thickness; J & W Scientific, Folsom) capillary column. The oven temperature programme was the following: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of 65 min. Injector and detector temperatures were 280°C. He was used as the carrier gas, at a flow rate of 1 mL/min. Split ratio, 1:50; acquisition mass range, *m/z* 29–400. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV. Oil samples were diluted to 1: 100 in *n*-hexane, and the volume injected was 2 µL. Whenever possible, the essential oil constituents were identified by co-injection with authentic standards purchased from Sigma-Aldrich (Milan) (see Table 1). Phytol was previously isolated from *Onosma echioides* (L.) L. var. *columnae* Lacaita [25]. Otherwise, the peak assignment was carried out according to the recommendations of the International Organization of the Flavour Industry (http://www.iofi.org/), i.e. by the interactive combination of chromatographic linear retention indices that were consistent with those reported in the literature [26-28] for non-polar stationary phases, and MS data consisting of computer matching with the WILEY275, NIST 08, ADAMS, FFNSC2 and home-made (based on the analysis of reference oils and commercially available standards) libraries. Quantification of essential oil components was achieved by peak-area internal normalization without using correction factors.

Preparation of extracts for HPLC and UPLC-MS measurements: Dried plant material (5 g) was extracted with methanol in an ultrasound bath using 50 mL of solvent for 15 min. Solvent was removed under vacuum yielding a brown gummy residue (12.8%, w/w). The solid was then redissolved in a mixture of methanol/water (1/1) for further analysis. The plant extract was analyzed using two different LC-MS systems. First a Varian 212 chromatograph equipped with Prostar 430 autosampler and MS 500 ion trap was used. Spectra were recorded in negative ion mode (50-2000 Da) using the turbo data depending scanning (tdds) functionality of the MS 500 spectrometer that allow the observation of fragmentation patterns for analytes.

Furthermore, samples were also analyzed on an Agilent 1290 UPLC system equipped with a diode array (1290 series) and a Waters Xevo G2 Q-TOF mass spectrometer detector. The stationary phase used in both systems was an Agilent Poroshell XDB C-8 2.1 x 150 mm (2.7µm). As mobile phases, acetonitrile (A) and water with 0.1% formic acid (B) were used. Gradient elution started from 90% B and in 40 min reached 100% A. Mass spectra were acquired in full scan mode in the range 100-2000 Da and were also acquired using MS^e functionality allowing the observation of fragmentation spectra. Quantification was obtained on a DAD detector using rutin, catechin and chlorogenic acid for quantification of flavonoids, catechin and chlorogenic acid derivatives, respectively on the basis of the similarity of UV spectra. For quantification purposes calibration curves were built for each analyte (rutin 5-100 µg/mL, catechin 4.8-96 µg/mL, chlorogenic acid 1.2-120 µg/mL). Compounds were identified on the basis of their exact mass, fragmentation spectra and comparison with the literature [30-34].

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