Angiotensin I-converting Enzyme (ACE) Inhibitory Activity and Chemical Composition of Alchemilla Viridiflora Rothm.

Jelena Radović
University of Belgrade Faculty of Pharmacy
Relja Suručić
University of Banja Luka: Univerzitet u Banjoj Luci
Marjan Niketić
Natural History Museum, Belgrade
Tatjana Kundakovic-Vasovic (ktatjana@pharmacy.bg.ac.rs)
DIT-Faculty of Pharmacy: DIT University Faculty of Pharmacy https://orcid.org/0000-0001-5777-8612

Research Article

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Abstract

Alchemilla viridiflora Rothm., Rosaceae is a herbaceous plant widespread in central Greece, Bulgaria, North Macedonia and Serbia with Kosovo. LC-MS analysis leads to the identification of 20 compounds in methanol extract, mainly ellagitannins and flavonoid glycosides. Considering that different plant extracts were traditionally used for treatment of hypertension and that some of the analyzed methanol extract constituents possess beneficial cardiovascular effects, we hypothesized that some of these effects are achieved through inhibition of angiotensin I-converting enzyme (ACE). The dose-dependent activities ACE inhibitory activity of *A. viridiflora* and miquelianin were observed with an IC_{50} of 2.51 ± 0.00 µg/ml of *A. viridiflora* compared to IC_{50} of 2.59 ± 0.00 µg/mL for miquelianin. Contribution of the single compounds to the tested activity was further analyzed through the *in silico* experimental approach. Computational docking results showed that tiliroside, ellagic acid pentose and galloyl-HHDP-glucose exhibited even better binding affinity for ACE active site than miquelianin, which ACE activity was confirmed by an *in vitro* assay.

Introduction

Systemic arterial hypertension is a common disorder and the most important risk factor for general morbidity and mortality, also associated with increased risk for cardiovascular disease development. About 1.13 billion people worldwide have hypertension, especially in developing countries. If not treated properly, it significantly increases the risk of heart thrombosis, heart attack, or renal failure [1, 2].

According to the latest recommendations, the drugs of choice in the treatment of hypertension are ACE inhibitors (angiotensin converting enzyme inhibitors), which could be combined with other antihypertensive drugs. Growing interest and various completed and ongoing studies on natural substances ACE inhibitory potential is primarily motivated by safety concerns regarding synthetic ACE inhibitors [3].

Examination of substances of plant origin with ACE inhibitory activity shows that flavonoids and peptides are the most active ACE inhibitors [4]. Regarding essential oils, few studies have studied the effects of these secondary metabolites on ACE inhibition [5-8]. Also, tannins are found to be non-specific inhibitors of ACE, through sequestration of the enzyme metal cofactor (Zn^{2+}), precipitation of protein or as non-specific enzyme (trypsin and chymotrypsin) inhibitors [9].

Alchemilla L. species are rich in phenolic compounds, tannins and flavonoid glycosides with potent antioxidant and free radical scavenging activity [10-12]. The most studied species of this genus is a common lady's mantle, *Alchemilla vulgaris* L., Rosaceae. *A. vulgaris* and *A. mollis* Rothm. have been reported to possess ellagitannins, principally the dimers agrimoniin and laevigatin F, the monomer pedunculagin, and sanguiin H-10 and its derivatives [13]. Quercetin-3-O-glucuronide (miquelianin) was detected in aceton-water extracts of *A. vulgaris* and *A. mollis* leaves and stalks, as well as its methyl

derivative [13], in *A. coriacea* Buser, *A. filicaulis* Buser, *A. glabra* Neygenf., *A. xanthochlora* Rothm. [14], *A. speciosa* Buser [15] and *A. achtarowii* Pawł.[11].

The ethnopharmacological studies suggested the use of *A. vulgaris* herbal tea for treatment of hypertension [16, 17]. Takir et al. [18] studied vasorelaxant and blood pressure lowering effect of methanol and aqueous extract of *A. vulgaris* on isolated rat aorta precontracted with noradrenaline and K^+. Methanol and aqueous extract showed opposite effects, where methanol extract induces relaxation and aqueous extract increases the contraction of rat aorta. Same extracts were used in to study vasorelaxant and blood pressure lowering effect on endothelium intact mesenteric arteries contracted with prostaglandin F2α or potassium chloride. Similarly, methanol extract produced concentration-dependent relaxation in mesenteric arteria precontracted with K^+, while the aqueous extract induced contraction. Also, methanol extract reduced increased systolic blood pressure in L-NAME (N (gamma)-nitro-L-arginine methyl ester) hypertensive rats after oral administration of 300 mg/kg/day for 2 weeks, while aqueous extract was inactive. The authors concluded that the difference in responses using two different spasmogens may be related to the difference in chemical composition: quercetin and gallic acid as major vasoactive compounds in both extracts, but the content of flavonoids was higher in the methanol extract. Similarly, low flavonoid and high gallic acid content explain ineffectiveness of aqueous extract in hypertensive rats [19].

Considering that one of the commonly used species *A. vulgaris* exhibited *in vitro* antihypertensive activity, the main research interest was to analyze the chemical composition of methanol extract of *A. viridiflora* and its potential to inhibit ACE activity *in vitro* [19]. Contribution of the single compounds to the tested activity was further analyzed through the *in silico* experimental approach.

Alchemilla viridiflora Rothm. (*A.* sect. *Calycinae*), Rosaceae is an herbaceous plant widespread in mountainous areas of central Greece, Bulgaria, North Macedonia and Serbia with Kosovo [20, 21]. This is up to 80 cm tall plant with suborbicular leaves and greenish flowers with epicalyx-segments are as long as sepals. The whole plant is dense patent hairy. This species has not been studied before concerning biological activity (except anti-*Helicobacter pylori*) or chemical composition (except the content of ellagic acid) [22].

Materials And Methods

Plant material

Aerial parts of *A. viridiflora* were collected in July 2013 at Mt. Suva Planina in subalpine pastures in the spruce zone at 1750 m s.m., on carbonate soil. This is also a new site of the species in the far north of its range. Voucher specimens (20130708/1-2,) was deposited in Natural History Museum (Belgrade, Serbia) and identification of plant material was done by dr Marjan Niketić.

Preparation of extracts

The plant material was air-dried at room temperature. The air-dried, powdered material (320 g of *A. viridiflora*) was extracted with methanol for two days to yield methanol extract (80.94 g).

Chemical composition of *A. viridiflora* methanol extract

The content of total polyphenols was determined spectrophotometrically [23]. The content of polyphenols was expressed µg of the standard substance (Gallic acid, GA)/mg of dry extract.

The assays for determination of content of tannins and flavonoids were followed according to the corresponding monograph of general chapter of the European Pharmacopoeia (Ph. Eur. 10.0) [24].

The methanol extract solution (5 mg/mL) was further studied using liquid chromatography-mass spectrometry (LC-MS) method. The analysis was performed on liquid-mass chromatograph Agilent Technologies HPLC1260 Infinity with automatic injector, diode array and single quadrupol mass detectors (Singlequad MS detector 6130) with *Zorbax SB Aq*-C18 column (3.0 x 150 mm; 3.5 µm), temperature 25 °C, and binar mobile phase (A: 0.1% solution of formic acid in water; B: acetonitril). Gradient program with flow rate 0.3 mL/min was used: 0-30 min from 10 to 25% B; 30-35 min from 25 to 70% B, 35-40 min - return to 10% B. Injection volume was 3 µL. Detection wavelengths were at 280 and 350 nm and in negative mode in a range of 50-2000 *m/z*. Electrospray ionisation under athmospheric pressure was done under pressure of 40 psi, temperature 350 °C and nitrogen flow 10 L/min. The deprotoned molecule signals and fragmented ions were obtained under fragmentation voltage of 100 V and 250 V in full-scan. Compounds were identified tentatively, by comparison with literature data, based on the comparison of their UV and MS spectra to those of commercially available standards or previously isolated compounds. All organic solvents were HPLC grade and were purchased from J.T.Baker (Deventer, The Netherlands).

In vitro ACE inhibitory activity

To investigate ACE inhibitory activity of methanol extract of *A. viridiflora* ACE Kit-WST (Dojindo, Japan) was used. Briefly, the sample was dissolved in absolute ethanol and diluted with a borate buffer pH 8.3. Different concentrations of sample solution (0.0016 - 5.00 mg/mL) were added to 96-well microplate together with 20 µL of borate buffer and 20 µL of deionised water. The enzymatic reaction started by adding enzyme solution 3-hydroxybutyrylglycylglycyl-glicine and aminoacylase. After the incubation period of 60 min at 37°C, 200 µL of indicator solution from was added to each sample well and once more incubated at room temperature for 10 min. Absorbance was measured at 450 nm using a Evolution 300 absorbance microplate reader. Parallel to sample, ACE inhibitory activity of different concentrations of standard miquelianin (0.00032 - 1 mg/mL) was investigated following the same method. Miquelianin standard was purchased from Sigma-Aldrich.

The inhibitory activity of samples was calculated according to the following equation: Inhibitory activity (%) = ((absorbance without an inhibitor - absorbance with an inhibitor)/ (absorbance without an inhibitor-blank absorbance)) x 100

ACE inhibitory activity *in silico*

Prediction of the identified compounds from studied *A. viridiflora* methanol extract potential for ACE inhibition was performed by an *in silico* experimental approach. The molecular docking calculations were prepared using the Yet Another Scientific Artificial Reality Application (Yasara) Structure package. A rectangular box with dimensions 30 Å × 30 Å × 30 Å was centred around lisinopril molecule complexed with ACE (crystal structure PDB:1O86). Some ligand molecules were downloaded as 3D molecules from Pubchem (brevifolin carboxylic acid, ellagic acid 4-O-xylopyranoside, galloyl-HHDP-glucose, HHDP-hexoside, miquelianin and tiliroside), while others were downloaded as 2D molecules (agrimoniin, pedunculagin, sanguiin H-10, tellimagrandin I and II) which were later converted in 3D molecules by CORINA online service. All ligands were energetically optimised by Yasara. The ligand-receptor pairs with the lowest binding energy were considered to have the best docking conformations. The binding energy values of each tested ligand were compared with the result of lisinopril (confirmed ACE inhibitor) and only better results than lisinopril were presented and further discussed.

Results And Discussion

Chemical composition of *A. viridiflora* methanol extract

The major phenolic compounds found in genus *Alchemilla* belonged to the ellagitannins (5-8%), principally the dimers agrimoniin (3.5-3.8%) and laevigatin F (0.9%) and the monomer pedunculagin (1.2%) [25]. Ellagitannins are hydrolysable tannins esterified with hexahydroxydiphenic acid (HHDP) and a most often glucose. Typical neutral losses of ellagitannins during MS fragmentation are galloyl (152 Da), gallic acid (170 Da), hexahydroxydiphenic acid, HHDP, (302 Da), galloylglucose (332 Da), HHDP glucose (482 Da), and galloyl-HHDP-glucose (634 Da) residues and characteristic UV-vis spectra at 254 nm and 360-368 nm. Flavonoids are also often found in the genus *Alchemilla.* Those are dominantly derivatives of flavonols, quercetin and kaempferol. Quercetin and its derivate have absorption maximum at 354 nm and characteristic MS fragmentation ion at *m/z* 301 (negative mode), while kaempferol and its derivate have λ_{max} at 348 nm and characteristic MS fragment ion at *m/z* 285 (negative mode) [11, 12, 14, 26, 27]. Phenolic acids, such as gallic, 3, 4-dixydroxy-benzoic acid, chlorogenic and caffeic acidare also commonly present [28].

Preliminary studies have shown the presence of polyphenolic compounds (233.41 ± 3.29 μg GA/mg of dry extract), flavonoids (0.30 ± 0.05%) and tannins (3.74% ± 0.98) in methanol extract of *A. viridiflora*. LC-MS analysis leads to the identification of 20 compounds in methanol extract, mainly ellagitannins and flavonoid glycosides (Fig. 1, Table 1). This was the first time that chemical analysis of *A. viridiflora* was done. Also, some compounds were identified for the first time in *Alchemilla* species.

Table 1 Results of LC-MS chemical analysis of methanol extract *Alchemilla viridiflora*

Peak	Rt (min)	Molecular formula¶	*Λmax* (nm)	MW	$[M-H]^-$ (*m/z*) (100 V)	MS data (*m/z*) (250V)	Compound name
1	3.567	$C_{20}H_{18}O_{14}$	236, 314	482	481	301, 275	HHDP-hexoside†
2	6.138	$C_{34}H_{24}O_{22}$	232	784	783	481, 301	Pedunculagin I isomer†
3	9.188	C34H24O22	232	784	783	481, 301	Pedunculagin I isomer
4	12.247	$C_{27}H_{22}O_{18}$ $C_{27}H_{28}O_{18}$	222, 274 258, 352	634 640	633 639	463, 301	Galloyl – HHDP-glucose† Quercetin-hexoside-glucuronide†
5	14.140	$C_{13}H_8O_8$	278, 360	292	291	247, 219, 190	Brevifolin carboxylic acid†
6	16.256	$C_{34}H_{26}O_{22}$	218, 274	786	785	755, 301	Tellimagrandin I§
7	18.865	$C_{68}H_{48}O_{44}$	230, 278	1568	783	1265, 1103, 935, 631, 301	Sanguiin H-10 isomer†
8	20.945	$C_{41}H_{28}O_{26}$	224, 278	936	935	633, 301	Galloyl-bis-HHDP-glucose†
9	22. 167	$C_{68}H_{48}O_{44}$	230, 278	1568	783	1265, 1103, 935, 631, 301	Sanguiin H-10 isomer†
10	23.906	$C_{41}H_{30}O_{26}$	222, 280	938	937	433, 301	Tellimagrandin II§
11	25.544	$C_{21}H_{18}O_{13}$	254, 308, 354	478	477	301	Miquelianin‡
12	26.383	$C_{82}H_{54}O_{52}$	254, 306 sh, 368	1870	934	1567, 1265, 1085, 934, 633, 301	Agrimoniin†
13	28.121	$C_{19}H_{14}O_{12}$	256, 354	434	433	301	Ellagic acid pentose†
14	29.331	$C_{41}H_{21}O_{26}$	220, 280	940	939	787, 769, 635	Pentagalloylglucose†
15	30.965	$C_{22}H_{20}O_{13}$	252, 356	492	491	315, 301, 275	Quercetin methyl ether glucuronide†

16	33.735	$C_{23}H_{22}O_{13}$	254, 358	506	505	329, 301, 269	Quercetin dimethyl ether glucuronide†
17	36.518	-	n.d.	712	711	665, 503, 465	Formate aduct of triterpene acid hexoside†
18	37.173	-	n.d.	710	709	663, 501	Formate aduct of triterpene acid hexoside†
19	37.592	$C_{30}H_{26}O_{13}$	226, 266, 314	594	593	447, 283	Tiliroside‡
20	37.933	-	n.d.	696	695	649, 487	Formate aduct of triterpene acid hexoside†

HHDP - hexahydroxydiphenic acid.

¶source PubChem®

†tentatively identified by comparing with literature data

‡identified comparing with commercial standard

§identified comparing with previously isolated compound

Peak 1 produced a $[M- H]^-$ ion at *m/z* 481 and generated fragment ion *m/z* 301 $[M - 180 - H]^-$ (loss of hexose) corresponding to an HHDP residue, and *m/z* 275 by decarboxylation of the HHDP moiety [29]. This compound has been identified as HHDP-hexoside. Monohexosides of HHDP were identified before in Rosaceae family, in leaves methanol extract of wild blackberries*, Rubus grandifolius* L. [30], but were never identified or isolated from *Alchemilla* species.

Peaks 2 and 3 correspond to isomeric compounds, with the $[M - H]^-$ ion at *m/z* 783, yielding main fragment ions at *m/z* 481 $[M - 302 - H]^-$ (loss of HHDP) and 301 $[M - 482 - H]^-$ (loss of HHDP-glucose), whose fragmentation pattern corresponds to a bis-HHDP-glucose structure presumably pedunculagin I isomers, which were previously reported in acetone-water extract of aerial parts of both *A. vulgaris* and *A. mollis*, as well as in methanol extract of aerial parts of *A. persica* Rothm. [13, 31].

Peak 4 had $[M - H]^-$ at both *m/z* 633 and 639 with main fragments at *m/z* 463 $[M - 170 - H]^-$ (loss of gallic acid) and 463 $[M - 176 - H]^-$ (loss of glucuronide unit) and *m/z* 301 $[M - 332 - H]^-$ (loss of galloylglucose) and 301 $[M - 338 - H]^-$ (loss of hexoside-glucuronic unit) and. The same mass $[M -H]^-$ at *m/z* 633 can be seen in m-galloyl-HHDP-glucose, but this compound has a main fragment at *m/z* 481 $[M - 152 - H]^-$ (loss of galloyl moiety) which suggests that the galloyl unit is probably bonded via an m-

depside bond, and not attached directly to the glucose core. On the contrary, loss of gallic acid in Peak 4 indicates that the galloyl unit is attached directly to the glucose [27]. Accordingly, peak 4 is identified as galloyl-HHDP-glucose (presumably corilagin isomer). In the case of compound with ion at *m/z* 639 it clearly indicates that the compound can be identified as quercetin-hexoside- glucuronide. These compounds were both previously identified in *A. vulgaris* and *A. mollis* as well as *A. persica* [13, 31].

Peak 5 exhibited the $[M - H]^-$ ion at *m/z* 291 and fragment ions at *m/z* 247 $[M - 45 - H]^-$ (loss of carboxylic acid) which corresponds to brevifolin, ion *m/z* 219 $[M - 73 - H]^-$ which corresponds to the loss of C = OCOOH and *m/z* 191, which are characteristic fragment ions for brevifolin carboxylic acid according to literature. This compound has never been identified in *Alchemilla* species before. Brevifolin carboxylic acid is considered as an end product of ellagitannin hydrolyses, and it can be commonly found in whether fruits, leaves, flowers or heartwood of pomegranate, *Punica granatum* [32]. Nevertheless, this ellagitannin has previously been reported in species from Rosaceae family. It has been isolated from raspberry (*Rubus idaeus* L.) juice and whole plant of *Duchesnea indica* (Andrews.) Focke. [33, 34].

Peak 6 $[M - H]^-$ ion at *m/z* 785 with fragment 392 $[M - 2H]^{2-}$ was reported for the first time in *Alchemilla* species. This compound was identified as tellimagrandin I using a standard compound previously isolated from flowers of *Filipendula vulgaris* Moench. [35].

Peaks 7 and 9 were tentatively identified as sanguiin H-10 isomers, previously reported in *Alchemilla* species (*A. vulgaris, A. mollis* and *A. persica)* with fragment ion at *m/z* 783. This dimeric ellagitanninis composed of galloyl-bis-HHDP-glucose (*m/z* 935) and galloyl-HHDP-glucose (*m/z* 783). The proposed fragment ions at *m/z* l265 $[M-302-H]^-$ (loss of HHDP), 1103 $[M- 464- H]^-$ (loss of HHDP-glucose), 933 $[M-634- H]^-$ (loss of galloyl-HHDP-glucose), 631 $[M-936-H]^-$ (loss of HHDP-glucose-galloyl-HHDP) and 301 $[M- 1266- H]^-$ (loss of galloyl-HHDP-glucose-galloyl-HHDP-glucose) [13, 31].

Peak 8 $[M - H]^-$ ion at *m/z* 935 and fragment ions at *m/z* 783 $[M - 152 - H]^-$ (loss of galloyl unit), 633 $[M - 302 - H]^-$ (loss of HHDP) and 301 $[M - 634 - H]^-$ (loss of galloyl-HHDP-glucose) is identified as galloyl-bis-HHDP-glucose. This compound is a commonly present unit in structure of many ellagitannins, such as dimeric ellagitannin agrimoniin, and it has been previously identified in *Alchemilla vulgaris* and *A. mollis* species [13].

Peak 10 is identified first time in *Alchemilla* species as tellimagrandin II using a standard compound previously isolated from flowers of *Filipendula vulgaris* $[M - H]^-$ ion at *m/z* 937 with fragment ions *m/z* 767 $[M - 170- H]^-$ (loss of galloyl unit), *m/z* 468 $[M-2H]^{2-}$ and 301$[M-634-H]^-$ (loss of galloyl-HHDP-glucose) [36].

Peak 11 corresponds to the mass spectra of used standard substance quercetin-3-O-β-glucuronide (miquelianin) with $[M - H]^-$ ion at *m/z* 477, with a fragment ion at *m/z* 301 $[M - 176 - H]^-$ (loss of

glucuronide), which corresponds to quercetin. As mentioned before, miquelianin is a commonly found flavonoid in *Alchemilla* species.

Peak 12 corresponds to agrimoniin, common ellagitannin is *Alchemilla* species, since it has been recognized as a marker compound of the Rosaceae family [13]. It showed a $[M–H]^-$ ion at *m/z* 1869 and fragment ions at *m/z* 1567 $[M– 302– H]^-$ (loss of an HHDP unit), 1265 $[M– 604– H]^-$ (loss of bis-HHDP), 1085 $[M– 784– H]^-$ (loss of bis-HHDP-glucose) and the main fragment at m/z 935 $[M– 2H]^{2-}$, corresponding to one galloyl-bis-glucose unit, followed then by fragmentation ions at *m/z* 633 (935 – 302 , loss of HHDP unit) and 301 (633 – 332, loss of galloyl-glucose residue) [37].

Peak 13 has $[M – H]^-$ ion at *m/z* 433 and fragment ion at *m/z* 301 $[M – 132 – H]^-$ (loss of a pentose), corresponding to the both ellagic acid and quercetin. As mentioned before, ellagic acid and it derives show characteristic UV spectra with λ_{max} at 254 nm and 360-368 nm, while quercetin and its glycosides are with absorption maximum at about 354 nm. Hence, this compound is proposed to be ellagic acid pentose. Ellagic acid is a common constituent of *Alchemilla* species, while its conjugates are reported for the first time in this paper. These metabolites can be found in other species of Rosaceae family, such as in acetone extract of strawberry fruits (*Fragaria x ananassa*) [27].

Peak 14 shows $[M– H]^-$ at *m/z* 939 and a fragment ion at *m/z* 769 $[M– 170– H]^-$ corresponding to loss of gallic acid moiety. In addition, the appearance of fragment ions at *m/z* 787 and 635 corresponds to the loss of the first galloyl moiety $[M– 152– H]^-$, and the second one $[M– 304– H]^-$ respectively, thus this compound can be interpreted as pentagalloylglucose, found in methanol extract from leaves of *Eucalyptus globulus* Labill. [38].

Peak 15 and 16 exhibits $[M – H]^-$ ion at *m/z* 491 and 505, respectively, with fragment ions at *m/z* 329 and *m/z* 315 $[M – 176 – H]^-$ which clearly indicates the loss of glucuronide unit, while the fragment of *m/z* 301 in both peaks indicates to presence of quercetin aglycone ($[M – 190 – H]^-$ loss of glucuronide and methylene moiety; $[M – 204 – H]^-$ loss of glucuronide and two methylene units). The proposed compounds are tentatively identified as quercetin methyl ether glucuronide, previously found in *Alchemilla* species [13], and quercetin dimethyl ether glucuronide. This quercetin conjugate has never been reported neither in *Alchemilla* species or Rosaceae family.

Peaks 17, 18 and 20 exhibit an $[M– H]^-$ ion at *m/z* 711, 709 and 695, respectively. This form of fragmentation may correspond to triterpene components found in raspberry, *Rubus ideus* [39]. Namely, all the compounds show proposed loss of formate $[M-46-H]^-$, giving fragments at m/z 665, 663 and 649, respectively, followed by loss of hexose moiety $[M– 162– H]^-$ to form the fragments at *m/z* 503, 501 and 487, respectively. These fragments are presumably aglycones of pentacyclic triterpenoids, ursane type (asatic and madecassic acid) [40] and olean type (serjanic acid) [41]. Therefore, these peaks could be tentatively identified as formate addicts of triterpene acid-O-hexoside. These compounds were not identified in *Alchemilla* species before. Nevertheless, triterpenes, sucha as ursolic acid, 2-a-hy-

droxyursolic acid, tormentic acid, euscophic acid, and oleanolic acid, have been identified in aerial parts of *A. vulgaris, A. alpine* L. *and , A. faroënsis* **(Lange) Buser** [42].

Peak 19 exhibits an $[M- H]^-$ ion at *m/z* 593 and a fragment ion at *m/z* 285 $[M- 308- H]^-$ (loss of a coumaroyl glucoside moiety) which corresponds to kaempferol, in addition to fragment ions at *m/z* 447 $[M- 146- H]^-$ (loss of p-coumaroyl) which corresponds to kaempferol glucoside. This compound is identified based on the comparison of its UV and an MS spectrum to those of commercially available standard as kaempferol-3-O-(6-p-coumaroyl)-glucoside, tiliroside. This is the first report of presence of tiliroside in tested *Alchemilla* species. Tiliroside has been isolated before from other *Alchemilla* species, such as from methanol extracts aerial parts of *A. vulgaris*, *A. barbatiflora* Juz. and *A. mollis* [10, 12, 43].

ACE inhibitory activity

Molecules obtained from various plant isolates have gained great interest as ACE inhibitors recently. The most promising compounds based on their structure difference could be divided in tannins, flavonoids, essential oil [5, 44]. Although structurally different, all these compounds have in common presence of functional groups which serve as hydrogen (H) bond acceptors or donors, such as phenolic and carboxylic. Different concentration ranges of the *A. viridiflora* methanol extract rich in flavonoids and ellagitannins and miquelianin standard were evaluated *in vitro* for ACE activity inhibition. The results of ACE inhibitory activity of tested sample at the concentration range of 0.0016–5 mg/mL are presented in Fig. 2 and ACE inhibitory activity of miquelianin standard at the concentration range of 0.00032–1 mg/mL in Fig. 3.

The dose-dependent activities of *A. viridiflora* and miquelianin were observed in the tested concentration ranges. The result obtained from *in vitro* investigation of ACE inhibitory activity of methanol extract of *A. viridiflora* showed IC_{50} of 2.51 ± 0.00 µg/mL compared to IC_{50} of 2.59 ± 0.00 µg/mL for miquelianin standard. According to the manufacturer of ACEKit-WST (Dojindo, Japan), IC_{50} of Alacepril and Captopril are 3.62 µM and 2.14 nM, respectively. Our extract had lower IC_{50} than standard substance miquelianin, whose activity was significantly lower than captopril, but in range with alacepril, potent synthetic ACE inhibitor.

The result obtained from molecular docking simulation study showed that 3 out of 20 compounds from methanol extracts obtained from *A. viridiflora*, formed more stable complexes with amino-acid residues in receptor binding site than lisinopril, well known synthetic ACE inhibitor (positive control) under the same experimental conditions (Table 2). Further interaction analysis revealed that all complexes were stabilized by conventional H bond interactions between receptor and selected ligands. Moreover, complexes were additionally stabilized Van der Waals and π-interactions (Fig. 4).

Table 2 Summarized docking simulation results for identified constituents of studied *Alchemilla viridiflora* which formed more stable complexes than positive control lisinopril (7.564 kcal/mol)

Compound	Pubchem ID	Bind.energy [kcal/mol]	Hydrogen bonds contacting receptor residues
Tiliroside	5320686	-10.750	Glu 143, Ala 356, His 513
Ellagic acid pentose	5487461	-10.364	Ala 354, Lys 511, His 513, Tyr 520
Galloyl-HHDP-glucose	503250	-10.258	Gln 281, Ala 354, Cys 370, Glu 384, Asp 453
Miquelianin	57331105	-9.894	Thr 166, Asn 277, Gln 281, Thr 282, Glu 384
Tellimagrandin I	442690	-9.678	Asn 70, His 353, Asp 358, Tyr 360, Glu 411, Arg 522
Brevifolin carboxylic acid	9838995	-8.576	Gln 281, Asp 411

Fig. 4: 2D schematic diagrams of key receptor amino acid residues interactions with selected ligands from *A. viridiflora* sample: a) brevifolin carboxylic acid, b) ellagic acid pentose, c) galloyl-HHDP-glucose, d) miquelianin, e) tellimagrandin I and f) tiliroside

Computational docking results showed that tiliroside, ellagic acid pentose and galloyl-HHDP glucose exhibited even better binding affinity for ACE active site than miquelianin, which ACE activity was confirmed by an *in vitro* assay. The best affinity for receptor binding site was registered for tiliroside, flavonoid which demonstrated significant antihypertensive properties in a previously reported study [45].

Investigation of potential inhibitory effects on ACE activity of 11 *Cuphea* spp. crude extracts along with pure compounds showed that polyphenol miquelianin exhibited inhibitory activity comparable to captopril, well known ACE inhibitor which was the case in our study also [46].

Interactions with amino acid residues: Glu 162, His 353, Ala 354, Asp 377, Glu 384, Lys 511, His 513, Tyr 520, Tyr 523 in ACE active site is considered as crucial for ACE enzyme activity inhibition [47]. Tiliroside, tellimagrandin I, galloyl-HHDP and ellagic acid pentose from *A. viridiflora* methanol extract stabilized the most favorable molecule orientation through conventional H bonds interactions with above mentioned residues.

Conclusion

To summarize, the investigation of chemical composition of methanol extract of *Alchemilla viridiflora* proposed high content of total polyphenols, and 20 of them were identified. Tannins, with a content of 3% of total polyphenols, were identified mainly as ellagitannins, while flavonoids (0.30%) were identified as hexosides of quercetin and kaempferol. The extract showed dose-dependent *in vitro* ACE inhibitory activity, which mainly attributed to the presence of flavonoids and tannins. The *A. viridiflora* extract and miquelianin showed dose-dependent *in- vitro* ACE inhibitory activity with IC_{50} values 2.51 µg/mL and 2.59 µg/mL, respectively. These result suggested that beside miquelian, some other extract constituents also

contributed to this activity. Furthermore, molecular docking simulation results showed that 3 more compounds from extract formed more stable complexes with receptor binding site than lisinopril.

Flavonoid tiliroside, showed better affinity for receptor than miquelianin. Among ellagitannins, galloyl-HHDP and ellagic acid pentose also showed better affinity for receptor than miquelianin. Nevertheless, further studies are required to find out whether the use of extract of *A. viridiflora* is related to blood pressure lowering activity, and whether the proposed mechanism of ACE inhibition is mainly responsible for that effect.

Declarations

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Authors' contributions All authors contributed to the study conception and design. All authors read and approved the final manuscript

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Consent to participate Not applicable

Consent for publication Not applicable

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Figures

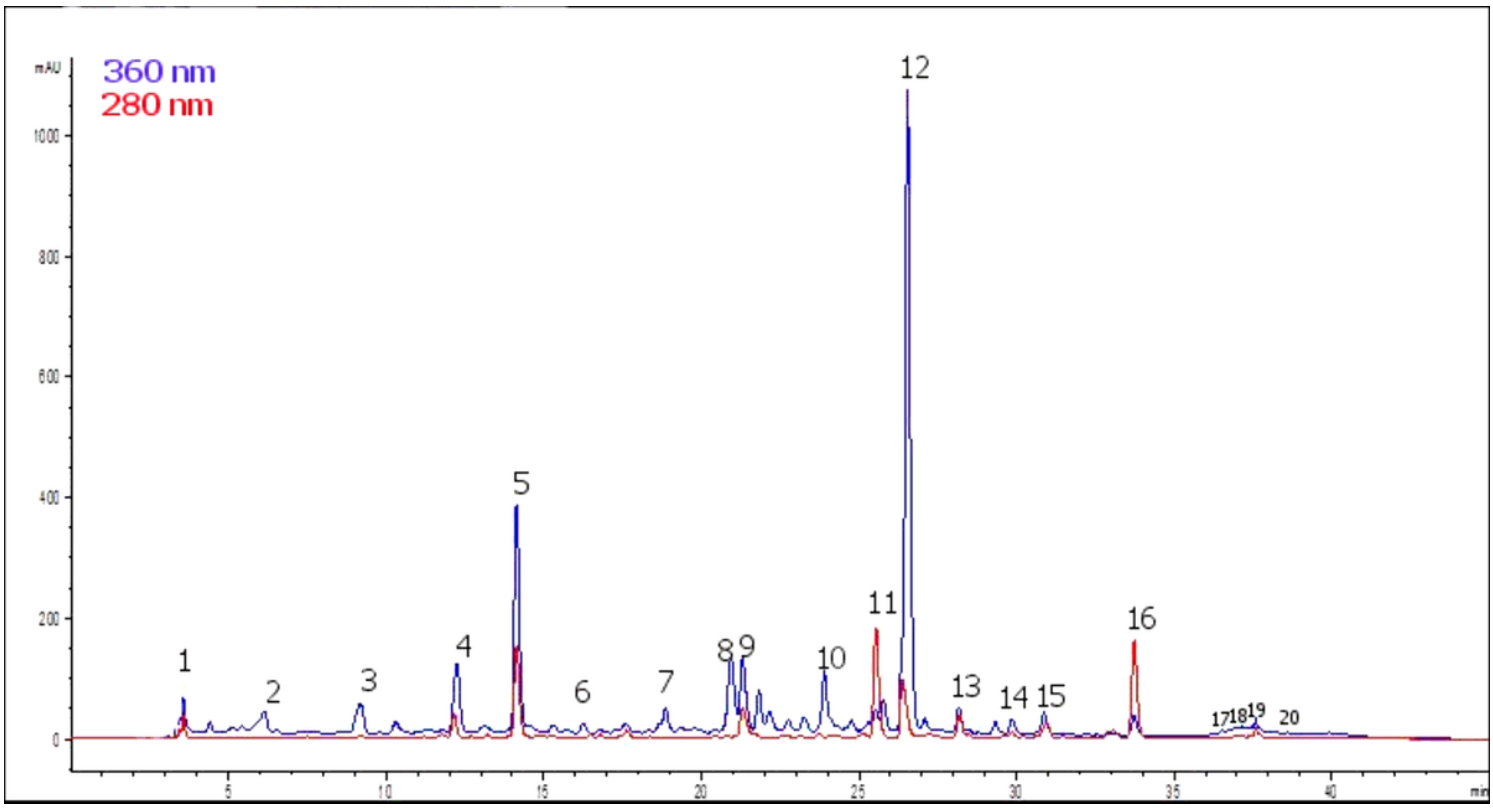

Figure 1

HPLC chromatogram of methanol extract of A. viridiflora at 280 and 350 nm

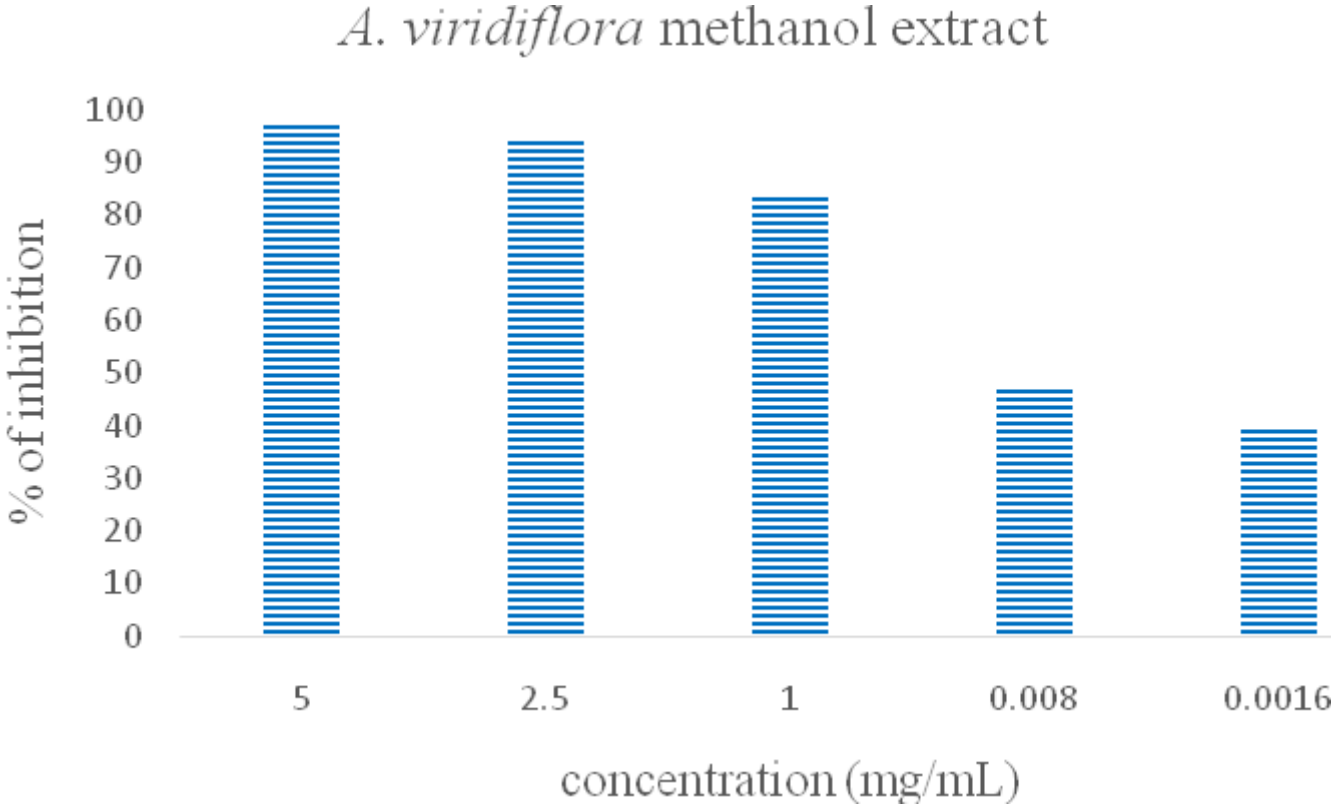

Figure 2

The ACE inhibition of A. viridiflora methanol extract

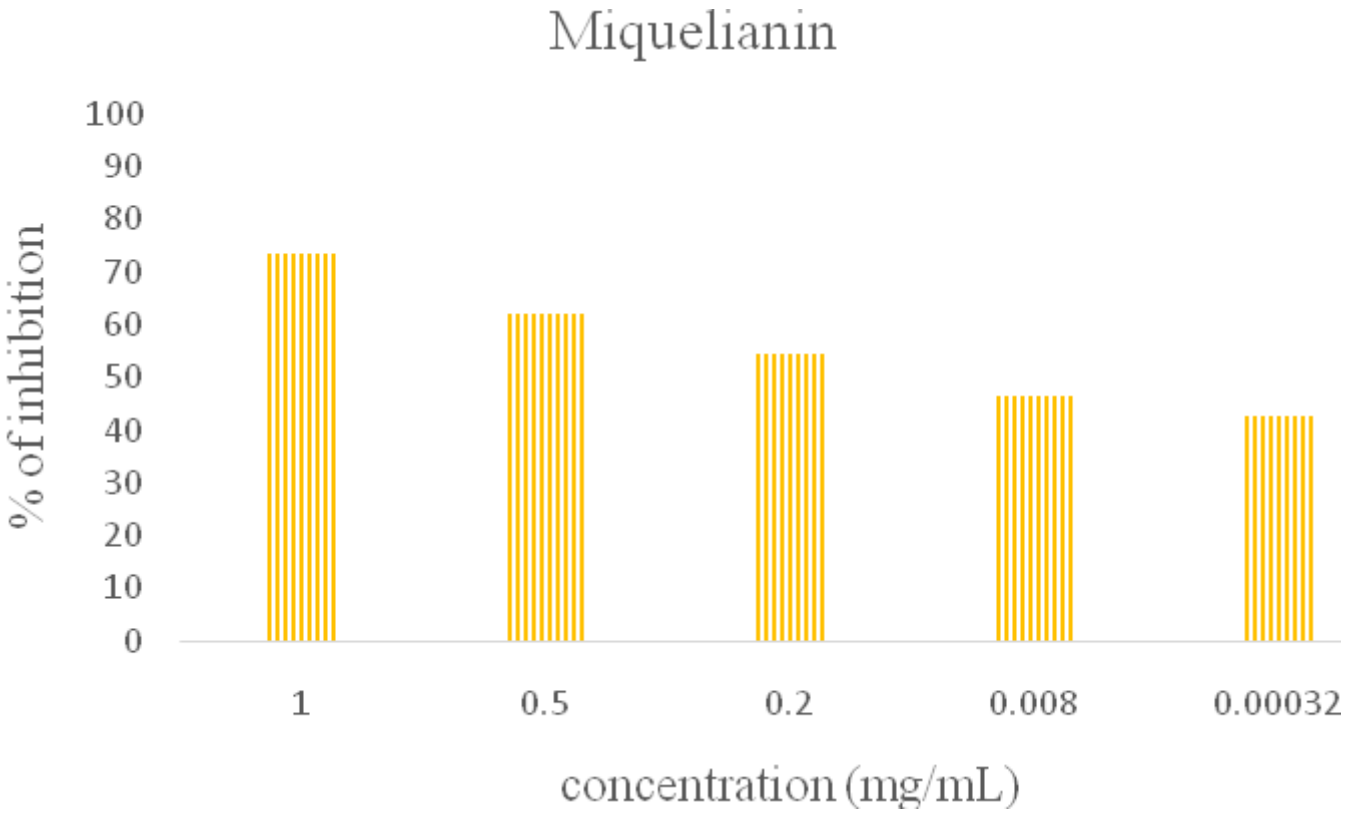

Figure 3

The ACE inhibition of miquelianin

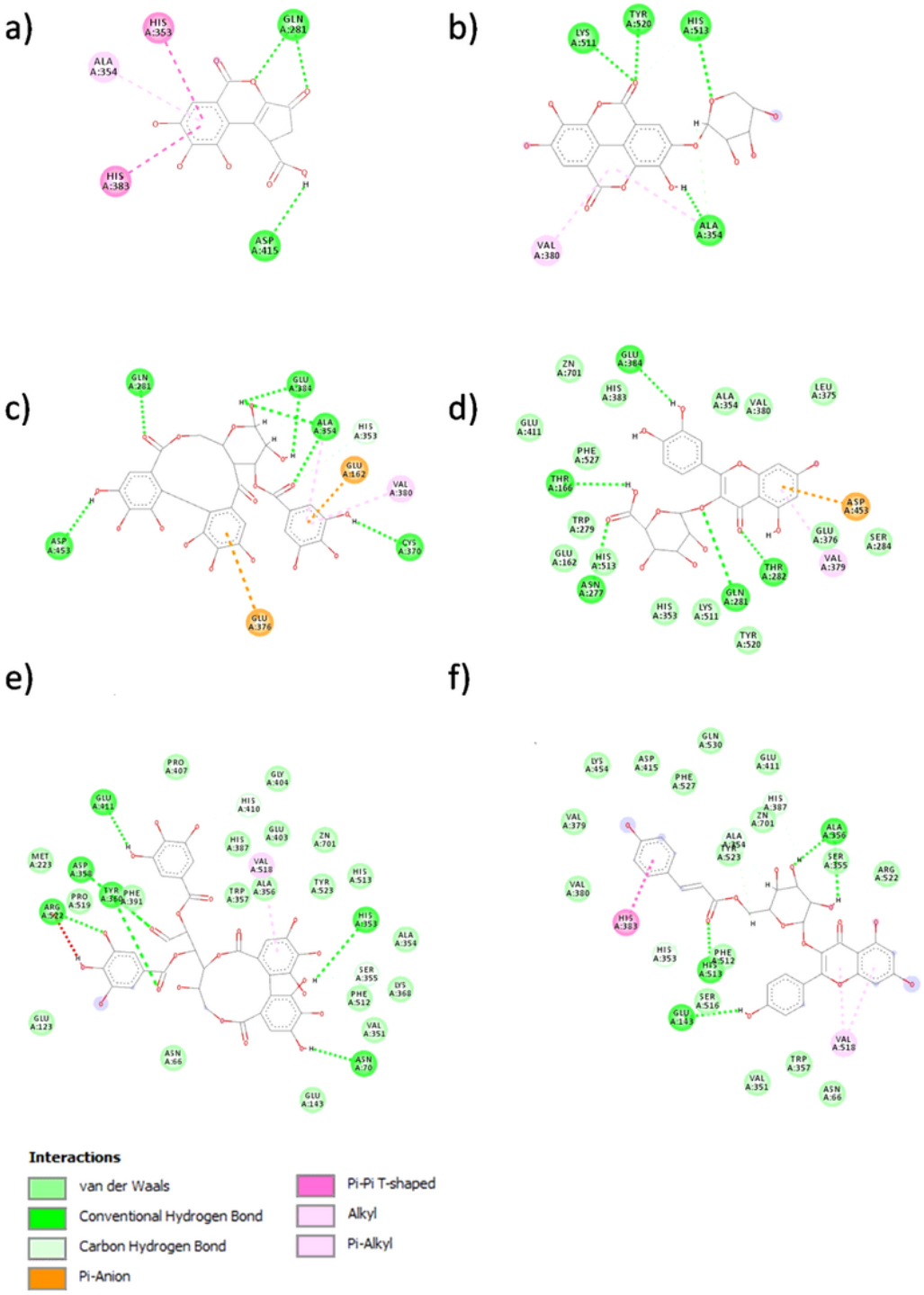

Figure 4

2D schematic diagrams of key receptor amino acid residues interactions with selected ligands from A. viridiflora sample: a) brevifolin carboxylic acid, b) ellagic acid pentose, c) galloyl-HHDP-glucose, d) miquelianin, e) tellimagrandin I and f) tiliroside