Phytochemical analysis and biological evaluation of *Lagochilus* species from Uzbekistan

Davlat Kh. Akramov,[a] Markus Bacher,[b] Stefan Böhmdorfer,[b] Thomas Rosenau,[b] Gokhan Zengin,[c] Antje Potthast,[b] Lutfun Nahar,[d,e] Satyajit D. Sarker,[d] Nilufar Z. Mamadalieva[a, *]

[a]Institute of the Chemistry of Plant Substances of the Academy Sciences of Uzbekistan, Mirzo Ulugbek Str 77, Tashkent 100170, Uzbekistan

[b]University of Natural Resources and Life Sciences, Vienna (BOKU University), Department of Chemistry, Institute of Chemistry of Renewable Resources, Konrad-Lorenz-Straße 24, A-3430 Tulln, Austria

[c]Department of Biology, Selcuk University, Science Faculty, Konya, Turkey

[d]Centre for Natural Products Discovery (CNPD), School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, UK

[e]Laboratory of Growth Regulators, Institute of Experimental Botany ASCR & Palacký University, Šlechtitelů 27, 78371 Olomouc, Czech Republic

***Corresponding author**: Dr. Nilufar Z. Mamadalieva

Institute of the Chemistry of Plant Substances, Academy of Sciences of Uzbekistan, Tashkent 100170, Mirzo Ulugbek Str 77, Uzbekistan

Tel/Fax: +99871 2627300, +99871 2627348; E-mail: nmamadalieva@yahoo.com

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ABSTRACT

The species of the genus *Lagochilus* (Lamiaceae) are widespread in Central, South-Central, and Eastern Asia. Some of these species are used for their medicinal and therapeutic effects, in particular as hemostatic, anti-inflammatory and anti-epileptic agents. A new iridoid, glucoside 7- cinnamoyllamalbide, along with known compounds lagochilin, 5-hydroxy-7,4'-dimethoxyflavone, daucosterol, β-sitosterol, 8-acetylharpagide were isolated from *L. gypsaceus.* The high-performance thin-layer chromatography (HPTLC) method was used to determine the chemical fingerprints of 7 different *Lagochilus* species (*L. acutilobus, L. gypsaceus*, *L. inebrians*, *L. olgae*, *L. proskorjakovii*, *L. setulosus*, *L. vvedenskyi*). Among the tested species, lagochilin content was highest in the endemic species *L. inebrians* collected from the Djizzakh region of Uzbekistan. In free radical scavenging and reducing power assays, *L. inebrans* and *L. vvedenskyi* exhibited the strongest abilities. Regarding cholinesterases, amylase and glucosidase inhibition abilities of the tested samples, 5-hydroxy-7,4'-dimethoxyflavone was the most active compound.

Keywords: *Lagochilus;* iridoids; lagochilin; HPTLC; antioxidant; enzyme inhibitory activity

1. Introduction

The genus *Lagochilus* (Lamiaceae) is native to Central, South-Central, and Eastern Asia. It consists of 46 species, 33 of them growing in Central Asia. In Uzbekistan Flora, this genus is represented by 13-18 species (Vvedenskiy, 1961), basically occurring throughout the territory of Uzbekistan, starting from the deserts to the Tian-Shan and Pamir-Alay mountains (Shomurodov et al., 2014). *L. proskorjakovii* Ikram and *L. olgae* R. Kamelin are strictly endemic to the Nuratau ridge. The species of *L. setulosus* Vved. occurs in the South-West of Tian-Shan while the 4 species of *L. vvedenskyi* R. Kam. et Zucker., *L. acutilobus* (Ledeb.) Fisch. et C. A. Mey., *L. gypsaceus*

Vved. and *L. inebrians* Bunge (endemic) are found in the Turanian lowland. Two species (*L. gypsaceus* and *L. inebrians*) have their common ground in the Turanian and Pamir-Alay lowland (Shomurodov et al., 2014). Some species of the genus *Lagochilus* (*L. olgae, L. vvedenskyi, L. inebrians* and *L. proskorjakovii*) are considered as rare and endangered plants, are listed in the Red Book of Uzbekistan (Red Data Book of Republic Uzbekistan, 2016).

Aerial parts and roots of *L. inebrians* has been used in Uzbek traditional medicine for spasm and stomach pain and as styptic and sedative (Eisenman et al., 2013; Sezik et al., 2004). This traditional use of the plant dates back centuries. People of Central Asia have used these plants during celebrations for their intoxicating and sedative effects (Pratov et al., 2006). Infusions and decoctions of *L. gypsaceus* have been used as a sedative tea, and to stop bleeding as well. This plant is also used in modern medicine as therapeutic and preventive agents for different kinds of hemorrhage (pulmonary, traumatic, nasal, uterine, hemorrhoidal and lung) and bleeding disorders (Akopov, 1981; Eisenman et al., 2013).

Despite their wide applications in folk and traditional medicine, the chemistry of the genus *Lagochilus* is still rather poorly understood. Several phytoconstituents from the species of *Lagochilus*, belonging to diterpenoids, flavonoids, polysaccharides, sterols and iridoids, have been isolated (Taban et al., 2009). Some *Lagochilus* species growing in Uzbekistan were examined for their chemical constituents, which included lagochilin and its acetates (*L. inebrians, L. pubeseens*), lagohirsin and acetyllagohirsin (*L. hirsutissimus, L. setulosus, L. gypsaceus, L. olgae*), polysaccharides, pectin (*L. zeravschanicus, L. usunachmaticus*), iridoids, such as harpagide and 8-*O*-acetylharpagide (*L. inebrians, L. platycalyx*), and phenylpropanoids (*L. platycalyx*) (Zainutdinov et al., 2002). So far, there were no reports on the biological activity of *Lagochilus* species in Uzbekistan. Only the diterpenoids lagochilin, lagochirsine and some of their synthetic derivatives were studied as hemostatics (Zainutdinov et al., 2002). Our study was aimed to evaluate the chemical content and *in vitro* biological activities of the species from the *Lagochilus* genus

and to carry out HPTLC (High-Performance Thin-Layer Chromatography) -based fingerprinting of seven species of *Lagochilus* (*L. acutilobus, L. gypsaceus, L. inebrians, L. olgae, L. proskorjakovii, L. setulosus, L. vvedenskyi*).

2. Materials and methods

2.1. Plant materials

Aerial parts (flowers, leaves and stems) of *L. olgae* (dry mass 38 g, herbarium code N454) and *L. proskorjakovii* (70 g, N1656) were collected from the Djizzakh region of Uzbekistan, *L. inebrians* (N1768) from two different regions, the Djizzakh (LiD) and Surkhandarya regions (LiS) (each 80 g), and *L. acutilobus* (35 g, N465), *L. vvedenskyi* (22 g, N759), *L. gypsaceus* (470 g, N1656), *L. setulosus* (25 g, N273) from the Karakalpakstan (Ustyurt plato), Bukhara, Surkhandarya and Tashkent regions, respectively. *L. inebrians* and *L. setulosus* were collected by D. Akramov, while *L. acutilobus, L. gypsaceus, L. olgae, L. proskorjakovii, L. vvedenskyi* were collected and verified by Dr. A. Akhmedov. Plant species were collected during the summer season of 2017. Plant materials were air-dried in shadow and powdered in a mortar before use.

2.2. Preparation of the methanolic extracts

Powdered aerial parts of *L. acutilobus, L. gypsaceus, L. inebrians* from Djizzakh region (LiD) and Surkhandarya regions (LiS), *L. olgae*, *L. proskorjakovii*, *L. setulosus*, *L. vvedenskyi* (each 12 g) were soaked in methanol (200 ml) at room temperature for 24 h, providing extractive yields of 9.3%, 14.0%, 16.0%, 8.5%, 12.3%, 13.1%, 17.4%, and 11.7%, respectively. The extracts were filtered, and the filtrate was evaporated under vacuum (40°C) and yielding crude MeOH extract. The residual powders stored in airtight containers under frozen condition until further use.

2.3. Isolation of the compounds

Air-dried powdered aerial parts of *L. gypsaceus* (0.4 kg) were macerated in methanol (3×2 L) at room temperature. Solids were filtered off and the solvent was evaporated to dryness at 40°C to

give 56 g of dry methanolic extract. This extract was dissolved in distilled water (1:1, v/v) and further fractionated using chloroform (5×200 mL) followed by *n*-butanol (5×200 mL). The combined chloroform and butanol fractions were concentrated at 40°C under reduced pressure to yield 43.7 g and 5.8 g, respectively. The dried butanol fraction of *L. gypsaceus* (5.5 g) was mixed with silica gel and chromatographed (column size 10×60 cm) with a gradient of $CHCl_3$:MeOH to afford 58 fractions (Fr.1 - Fr.58), monitored by TLC on silica gel F_{254} plates (Merck, Germany). Spots were visualized under UV light (λ=254 and 366 nm) and by spraying with anisaldehyde solution followed by heating at 105°C for 5 min.

Compound **2** (18 mg) (Figure S1) was obtained from Fr.4 to 18 (1.2 g) by recrystallization from MeOH. Fr.19 to 27 (1.7 g) was re-chromatographed, eluting with solvent system $CHCl_3$:MeOH (15:1, v/v) and collecting 9 fractions (A1-9). The fractions A2 to 4 were re-chromatographed using a gradient of hexane:ethyl acetate which resulted in 12 fractions (B1-12). Fractions B5 to 8 were combined and partitioned with repeated PTLC using hexane:ethyl acetate (1:6) to obtain compound **3** (7 mg). Fr.28 to 41 (0.5 g) was separated by column chromatography (CC) with $CHCl_3$:MeOH (20:1, v/v) to yield compounds **1** (8 mg) and **5** (26 mg). Fr.42 to 58 (1.4 g) was fractionated by CC in the $CHCl_3$:MeOH (9: 1, v/v) and PTLC to yield **4** (9 mg) and **6** (12 mg).

2.4. General experimental procedures

Analytical grade solvents and reagents were used for the study, which were acquired from Merck (Vienna, Austria). Ultraviolet (UV) spectra were recorded on a SF-2000 spectrophotometer (ZAO OKB Spectrum, Russia) and IR spectra on a Perkin Elmer FT-IR spectrometer (Scheltec AG, Russia). NMR experiments were performed on a Bruker Avance II 400 spectrometer (resonance frequencies 400.13 MHz for ^1H and 100.63 MHz for ^{13}C, respectively) equipped with a 5 mm observe broadband probe head (BBFO) with z-gradients at room temperature with standard Bruker pulse programs. Chemical shifts are presented in parts per million (δ/ppm) and referenced to residual solvent signals ($CDCl_3$: 7.26 ppm for ^1H, 77.0 ppm for ^{13}C; CD_3OD: 3.31 ppm for ^1H, 49.0 ppm for ^{13}C; DMSO-d_6: 2.49 ppm for ^1H, 39.6 ppm for ^{13}C). Coupling constants (*J*) are

reported in Hz. HR-ESI-MS spectra were recorded on an Orbitrap HF mass spectrometer coupled to a Vanquish HPLC (Thermo Fisher Scientific).

2.5. Compound characterization

***7-Cinnamoyllamalbide* (1).** $C_{26}H_{32}O_{14}$, yellowish amorphous powder. ^{1}H (400 MHz) and ^{13}C NNR (100 MHz) in CD_3OD see Table 1. HR-ESI-MS: $[M+H]^+$ *m/z* 569.18488 (calcd. *m/z* $C_{26}H_{33}O_{14}$, 569.18648). Spectra are available in the Supplementary file (Fig. S1-S16).

***5-Hydroxy-7,4'-dimethoxyflavone* (2).** $C_{17}H_{14}O_5$, yellow crystalline substance, mp. 173-174°C. IR (KBr, ν/cm^{-1}): 3509, 2845, 2920, 1667, 1605, 1442, 1383, 1271, 1162, 834. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 6.56 (1H, s, H-3), 12.80 (1H, s, 5-OH), 6.35 (1H, d, *J* = 2.3, H-6), 6.47 (1H, d, *J* = 2.3, H-8), 7.83 (2H, d, *J* = 9.0, H-2', H-6'), 7.00 (2H, d, *J* = 9.0, H-3', H-5'), 3.89 (3H, s, 4'-OMe), 3.87 (3H, s, 7-OMe). ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 163.99 (C-2), 104.33 (C-3), 182.42 (C-4), 105.55 (C-4a), 162.19 (C-5), 98.02 (C-6), 165.43 (C-7), 55.75 (7-OMe), 92.60 (C-8), 157.69 (C-8a), 123.57 (C-1'), 128.01 (C-2', C-6'), 114.49 (C-3', C-5'), 162.59 (C-4'), 55.50 (4'-OMe). Spectra are available in the Supplementary file (Fig. S17-21, 51-52).

***β-Sitosterol* (3).** $C_{29}H_{50}O$, white powder, mp. 137-138°C. IR (KBr, ν/cm^{-1}): 3347, 2932, 2869, 1647, 1448, 1371, 1040, 970. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 1.85 (1H, m, H-1a), 1.08 (1H, m, H-1b), 1.84 (2H, m, H-2a, H-16a), 1.51 (1H, m, H-2b), 3.52 (1H, m, H-3), 2.30 (ddd, *J* = 13.1, 5.1, 1.9, H-4a), 2.25 (dm, *J* = 13.1, H-4b), 5.35 (1H, m, H-6), 1.98 (1H, m, H-7a), 1.54 (1H, m, H-7b), 1.46 (1H, m, H-8), 0.93 (2H, m, H-9, H-24), 1.50 (1H, m, H-11a), 1.46 (1H, m, H-11b), 2.01 (1H, m, H-12a), 1.16 (1H, m, H-12b), 1.00 (1H, m, H-14), 1.58 (1H, m, H-15a), 1.07 (1H, m, H-15b), 1.27 (1H, m, H-16b), 1.12 (1H, m, H-17), 0.68 (3H, s, H-18), 1.01 (3H, s, H-19), 1.36 (1H, m, H-20), 0.92 (3H, d, *J* = 6.7, H-21), 1.33 (1H, m, H-22a), 1.02 (1H, m, H-22b), 1.17 (1H, m, H-23), 1.25 (2H, m, H-24^1), 0.85 (3H, t, *J* = 7.4, H-24^2), 1.67 (1H, m, H-25), 0.82 (3H, d, *J* = 7.0, H-26), 0.84 (3H, d, *J* = 7.0, H-27). ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 37.28 (C-1), 31.70 (C-2), 71.82 (C-3), 42.34 (C-4, C-13), 140.78 (C-5), 121.71 (C-6), 31.91 (C-7), 31.93 (C-8), 50.17

(C-9), 36.53 (C-10), 21.10 (C-11), 39.80 (C-12), 56.79 (C-14), 24.31 (C-15), 28.24 (C-16), 56.09 (C-17), 11.86 (C-18), 19.39 (C-19), 36.15 (C-20), 18.79 (C-21), 33.98 (C-22), 26.14 (C-23), 45.88 (C-24), 23.10 (C-24^1), 11.99 (C-24^2), 29.20 (C-25), 19.05 (C-26), 19.81 (C-27). Spectra are available in the Supplementary file (Fig. S22-28, 53-54).

***Daucosterol* (4).** $C_{35}H_{60}O_6$, white powder, mp. 281-283°C. IR (KBr, ν/cm^{-1}): 3438, 2919, 2850, 1636, 1464, 1383, 1043. ^{1}H-NMR (400 MHz, $CDCl_3$:MeOD=1:1, *δ*, ppm, *J*/Hz): 5.33 (m, 1H; H-6), 4.37 (d, 1H, *J* = 8.0, H-1'), 3.81 (dd, 1H, *J* = 12.0, 3.0, H-6'a), 3.70 (dd, 1H, *J* = 12.0, 5.0, H-6'b), 3.55 (m, 1H, H-3), 3.37 (m, 2H, H-3', H-4'), 3.25 (m, 1H, H-5'), 3.19 (m, 1H, H-2'), 2.37 (ddd, 1H, J = 13.2, 4.6, 2.1, H-4a), 2.23 (m, 1H, H-4b), 1.99 (m, 1H, H-12a), 1.95 (m, 1H, H-7a), 1.89 (m, 1H, H-2a), 1.83 (m, 1H, H-1a), 1.82 (m, 1H, H-16a), 1.63 (m, 1H, H-25), 1.58 (m, 1H, H-2b), 1.55 (m, 1H, H-15a), 1.52 (m, 1H, H-7b), 1.46 (m, 2H, H-11), 1.42 (m, 1H, H-8), 1.33 (m, 1H, H-20), 1.31 (m, 1H, H-22a), 1.25 (m, 1H, H-16b), 1.23 (m, 2H, H-24^1), 1.13 (m, 3H, H-12b, H-23), 1.09 (m, 1H, H-17), 1.04 (m, 2H, H-1b, H-15b), 0.99 (m, 1H, H-22b), 0.98 (s, 3H, H-19), 0.98 (m, 1H, H-14), 0.90 (m, 2H, H-9, H-24), 0.90 (d, 3H, *J* =6.6, H-21), 0.81 (t, 3H, *J* = 7.7, H-24^2), 0.80 (d, 3H, *J* =7.0, H-27), 0.78 (d, 3H, *J* =7.0, H-26), 0.66 (s, 3H, H-18). ^{13}C-NMR (100 MHz, $CDCl_3$:MeOD=1:1, *δ*, ppm): 140.78 (C-5), 122.41 (C-6), 101.56 (C-1'), 79.46 (C-3), 76.97 (C-3'), 76.37 (C-5'), 74.02 (C-2'), 70.72 (C-4'), 62.23 (C-6'), 57.18 (C-14), 56.48 (C-17), 50.64 (C-9), 46.30 (C-24), 42.71 (C-13), 40.18 (C-12), 39.05 (C-4), 37.67 (C-1), 37.10 (C-10), 36.52 (C-20), 34.34 (C-22), 32.31 (C-8), 32.29 (C-7), 29.96 (C-2), 29.56 (C-25), 28.59 (C-16), 26.47 (C-23), 24.63 (C-15), 23.43 (C-24^1), 21.44 (C-11), 19.97 (C-27), 19.55 (C-19), 19.21 (C-26), 19.02 (C-21), 12.14 (C-24^2), 12.09 (C-18). Spectra are available in the Supplementary file (Fig. S29-37, 55).

***Lagochilin* (5).** $C_{20}H_{36}O_5$, crystalline white powder, mp. 167-168°C. IR (KBr, ν/cm^{-1}): 3489, 3384, 2925, 1664, 1635, 1468, 1450, 1052, 999. ^{1}H-NMR (400 MHz, $CDCl_3$+DMSO, *δ*, ppm, *J*/Hz): 1.42 (1H, m, H-1a), 1.35 (1H, m, H-1b), 1.56 (1H, m, H-2a), 1.49 (1H, m, H-2b), 3.50 (1H, dd, *J* = 10.5, 4.9, H-3), 1.43 (1H, m, H-5), 1.30 (2H, m, H-6), 1.25 (2H, m, H-7), 1.63 (1H, m, H-8), 1.93 (1H, m, H-11a), 1.52 (1H, m, H-11b), 1.73 (1H, m, H-12a), 1.59 (1H, m, H-12b), 1.87 (2H,

m, H-14), 3.68 (2H, t, *J* = 5.5, H-15), 3.50 (1H, d, *J* = 11.0, H-16a), 3.37 (1H, d, *J* = 11.0, H-16b), 0.77 (3H, d, *J* = 6.8, H-17), 3.51 (1H, d, *J* = 10.4, H-18a), 3.28 (1H, d, *J* = 10.4, H-18b), 0.76 (3H, s, H-19), 0.83 (3H, s, H-20). ^{13}C-NMR (100 MHz, $CDCl_3$+DMSO, *δ*, ppm): 30.38 (C-1), 26.46 (C-2), 75.76 (C-3), 41.84 (C-4, C-10), 41.44 (C-5), 21.47 (C-6), 31.17 (C-7), 36.12 (C-8), 93.23 (C-9), 28.91 (C-11), 35.45 (C-12), 85.58 (C-13), 39.98 (C-14), 59.02 (C-15), 66.39 (C-16), 17.87 (C-17)*, 71.27 (C-18), 11.24 (C-19), 17.84 (C-20)* (* - interchangeable). Spectra are available in the Supplementary file (Fig. S38-43, 56-57).

8-O-Acetylharpagide **(6).** $C_{17}H_{26}O_{11}$, white powder, mp 154-156ºC. IR (KBr, ν/cm^{-1}): 3434, 2917, 1711, 1652, 1375, 1238, 1076. ^{1}H-NMR (400 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 6.07 (1H, d, *J* = 1.3, H-1), 6.38 (1H, d, *J* = 6.4, H-3), 4.91 (1H, dd, *J* = 6.4, 1.6, H-4), 3.72 (1H, dd, *J* = 4.7, 1.6, H-6), 2.17 (ddd, *J* = 15.1, 1.2, 1.2, H-7a), 1.95 (dd, *J* = 15.1, 4.5, H-7a), 1.46 (3H, s, 8-CH_3), 2.86 (1H, br.s, H-9), 4.59 (1H, d, *J* = 8.0, H-1'), 3.20 (1H, dd, *J* = 9.2, 8.0, H-2'), 3.39 (1H, t, *J* = 9.2, H-3'), 3.30 (1H, m, H-4'), 3.31 (1H, m, H-5'), 3.89 (1H, dd, *J* = 12.0, 1.7, H-6'a), 3.69 (1H, dd, *J* = 12.0, 5.4, H-6'b), 2.01 (3H, s, Ac-CH_3). ^{13}C-NMR (100 MHz, CD_3OD, *δ*, ppm): 94.56 (C-1), 143.84 (C-3), 106.96 (C-4), 73.31 (C-5), 77.68 (C-6)*, 46.07 (C-7), 88.62 (C-8), 22.50 (8-CH_3), 55.56 (C-9), 99.92 (C-1'), 74.58 (C-2'), 77.71 (C-3')*, 71.74 (C-4'), 78.20 (C-5'), 62.89 (C-6'), 22.19 (CO$\underline{C}H_3$), 173.29 ($\underline{C}OCH_3$), (*- interchangeable). Spectra are available in the Supplementary file (Fig. S44-50, 58-59).

2.6. High-performance thin-layer chromatography (HPTLC) analysis

The HPTLC was performed as described previously (Mamadalieva et al., 2019). Prepared a 1 mg/mL stock solution of lagochilin (**5**) in MeOH. The MeOH extracts (20 mg/mL) of 7 species of *Lagochilus* were dissolved in $CHCl_3$-MeOH-H_2O (4:4:1, v/v/v). The solution of the extracts was applied to an HPTLC plate and analyzed according to the conditions described by Mamadalieva et al. (2019). Densitometric detection of lagochilin was executed out after derivatization (at 330 nm) (Figure S60).

2.7. Profile of bioactive compounds

198 The total bioactive compounds namely total phenolic and flavonoid were determined
199 calorimetrically as described previously (Zengin and Aktumsek, 2014). The results were expressed
200 as mg of standard compounds (gallic acid for TPC; and rutin for) per g of dried extract. Samples
201 were analyzed in triplicate.

202 ***2.8. Determination of antioxidant potential***

203 The metal chelating (MC), phosphomolybdenum (PPBD), ferric reducing power (FRAP), Cupric
204 reducing antioxidant capacity (CUPRAC), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic
205 acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) activities of the extracts were evaluated
206 following the methods described by Grochowski et al. (2017). The antioxidant activities were
207 reported as Trolox equivalents, whereas ethylenediaminetetraacetic acid (EDTA) was used for
208 metal chelating assay. Samples were analyzed in triplicate.

209 ***2.9. Determination of enzyme inhibitory effects***

210 The possible enzymatic inhibitory activities of the extracts and individual compounds against
211 acetylcholinesterase (AChE), butyrylcholinesterase (BChE) (by Ellman's method), tyrosinase,
212 α- amylase and α-glucosidase were assessed using standard *in vitro* bioassays (Grochowski et al.,
213 2017). Samples were analyzed in triplicate.

214 ***2.10. Statistical Analysis***

215 The results were evaluated by ANOVA assay (with Tukey's test, significant value: $p < 0.05$). The
216 Correlation analysis (Pearson) was performed between total bioactive components and biological
217 activity results. The statistical analysis was performed by Xlstat 2017.

218 **3. Results and discussion**

219 ***3.1. Phytochemical composition***

220 The species of *Lagochilus* are mainly used traditionally for their hemostatic and sedative effects.
221 The phytochemical and biological properties of the species from the genus *Lagochilus* are not well
222 studied, in particular of *L. gypsaceus*. Previous studies with TLC analyses showed the presence of

two diterpenes lagochilin and lagochirsine in this species (Matchanov et al., 2017; Zainutdinov et al., 1994). Therefore, *L. gypsaceus* was investigated to get more detailed information about the chemical composition, which should be related to its most relevant biological properties. The butanol fraction of *L. gypsaceus* was subjected to column chromatography and afforded a new iridoid glucoside (**1**), apart from the five known constituents 5-hydroxy-7,4'-dimethoxyflavone (**2**), β-sitosterol (**3**), daucosterol (**4**), lagochilin (**5**), 8-acetylharpagide (**6**) (Fig. 1). 5-Hydroxy-7,4'-dimethoxyflavone (**2**) has previously been detected in *L. proskorjacovii* and *L. pubescens* (Mavlyankulova et al., 1989; Zainutdinov et al., 1975), β-sitosterol (**3**) in *L. pubescens* (Zainutdinov et al., 1975) and 8-O-acetylharpagide (**6**) in *L. platycalyx*, *L. inebrians*, and *L. setulosus* (Kotenko et al., 1994). Compounds **2-4** and **6** were isolated and identified for the first time from *L. gypsaceus*. All structures were established by means of IR, UV, 1D and 2D NMR spectroscopy (see Suppl. file S1-59).

A new iridoid glucoside - 7-cinnamoyllamalbide (**1**) - was isolated and identified from *L. gypsaceus*. Compound **1** was obtained as a yellowish amorphous powder and had a molecular formula of $C_{26}H_{32}O_{14}$ deduced from its HR-ESI mass spectrum, exhibiting the $[M+H]^+$ ion peak at *m/z* 569.18488 (calcd. 569.18648). The ^{1}H NMR spectrum showed the presence of a cinnamoyl moiety, with the resonances of the *p*-substituted benzene at δ_H at 7.48 and 6.81, and those of the double bond as doublets at δ_H 7.70 and 6.44 with a coupling constant of *J*=16.0 Hz, characteristic for *E*-configuration. A doublet at δ_H 4.63 was identified as the anomeric proton of an glucose residue by its H,H-COSY correlations and the corresponding ^{13}C shifts deduced from the HSQC spectra. Additional to these units, the ^{1}H NMR spectrum in combination with the ^{13}C and HSQC spectra revealed signals of one methoxyl group (δ_H 3.74, δ_C 51.92), one aliphatic singlet methyl group (δ_H 1.30, δ_C 22.40), a strongly delocalized olefinic proton at δ_H 7.45 / δ_c 153.02, an anomeric proton at δ_H 5.65 / δ_c 94.7, two oxymethin protons – a triplet at δ_H 4.18, and a doublet at δ_H 4.89 - and finally two aliphatic methin protons (δ_H 3.06 and 2.90). Detailed analyses of the 2D NMR spectra identified the core structure built from these signals as being identical with that of

lamalbide. In the COSY spectrum, the spin system could be deduced by starting from the anomeric proton H-1 *via* coupling to H-9 and further to H-5, H-6, and H-7, respectively. Crosspeaks in the HMBC spectra from the aliphatic methyl group protons to C-9, C-7 and the quaternary carbon C- 8 as well as NOESY data proved the presence of the lamalbide skeleton, whereas a crosspeak from H-7 to the cinnamoyl carbonyl carbon at δ_C 168.76 located the cinnamoyl group at position C-7. Therefore, the structure of compound **1** was elucidated as shown in Fig. 1 and named 7- cinnamoyllamalbide. Spectra are available in the Supplementary file (Fig. S1-16).

In MeOD as the solvent, the ^{1}H NMR spectrum of **1** showed signals of around 10% of a second compound (**1a**). After 5 hours the ratio was nearly 1:1 and after 48 hours a stable ratio of **1**:**1a** = 1:2 was obtained. In compound **1a** the H-6 experienced a low field shift to δ_H 5.13, whereas H-7 was shifted to higher fields at δ_H 3.79 (Table 1). Moreover H-6 revealed a long-range crosspeak in the HMBC spectra to the cinnamoyl carbonyl carbon. Evidently, transesterification of 7-cinnamoyllamalbide to 6-cinnamoyllamalbide occurred in methanol (Fig. 2).

3.2. High-performance thin-layer chromatography (HPTLC) investigations

Lagochilin (**5**) is a main component of the total extractives of many species of the genus *Lagochilus*. It has already been identified in *L. inebrians*, *L. setulosus*, *L. gypsaceus* (Zainutdinov et al., 1994), *L. hirsutissimus* (Nurmatova et al., 1979), *L. proskorjacovii* (Mavlyankulova et al., 1989) and *L. pubescens* (Mavlyankulova et al., 1976). In this study, HPTLC fingerprint patterns have been elaborated for the methanolic extracts of 7 species of *Lagochilus* (Fig. S60), showing significant differences in the chemical natures of these plant materials. The presented HPTLC method can successfully separate the bioactive compound lagochilin in the extracts of *Lagochilus* species. The major difference was the presence of the marker compound lagochilin (**5**) in *L. acutilobus, L. gypsaceus, L. inebrians* from Djizzakh region (LiD) and Surkhandarya regions (LiS), *L. setulosus*, its very low content in *L. olgae* and *L. vvedenskyi*, and its absence in *L. proskorjakovii*. Among the *Lagochilus* species studied, lagochilin was highest in *L. inebrians* from Djizzakh region (LiD) (Fig. 3). This species can be considered a potential candidate for

obtaining lagochilin (**5**) in higher amounts for pharmacological studies. However, *L. inebrians* is an endangered species due to overexploitation and as part of the conservation efforts this species has to be cultivated.

3.3. The total phenolic and flavonoid content of **Lagochilus** *extracts*

The total phenolic and flavonoid content of the tested *Lagochilus* MeOH extracts was determined (Table 2). The highest amount of total phenolic compounds was observed in *L. inebrans* (from Djizzakh), followed by *L. vvedenskyi* and *L. proskorjakovii*. *L. gypsaceus* contained the lowest level of phenolics. Regarding total flavonoid content, *L. acutilobus* and *L. olgae* had more flavonoids as compared with other *Lagochilus* extracts. Interestingly, the minimum level of flavonoids was detected in *L. inebrans* (from Djizzakh).

3.4. Antioxidant assays

Regarding quenching of DPPH radical activity, the observed abilities decreased in the order: *L. inebrans* (from Djizzakh)> *L. vvedenskyi*> *L. olgae*> *L. setulosus*> *L. proskorjakovii*> *L. gypsaceus*> *L. acutilobus*>*L. inebrans* (from Surkhandarya region) (Table 2). Similar to DPPH, the best cupric (CUPRAC) and ferric reducing power (FRAP) ability was determined by *L. inebrans* (from Djjzzakh), followed by *L. vvedenskyi*, which follows the same trend as the total phenolic content. We also observed strong correlation between total phenolic content and antioxidant (DPPH, CUPRAC and FRAP) properties of the tested extracts (Table 3). In the phosphopmolybdenum assay, *L. proskorjakovii* exhibited the strongest ability with 2.00 mmolTE/g, while *L. inebrians* (from Surkhandarya) was the weakest. In the ferrozine assay the metal chelating ability of *L. acutilobus* was the best, followed by *L. olgae* and *L. setulosus*. Interestingly, *L. inebrians* (from Djizzakh) exhibited the weakest ability, although it was the richest in terms of phenolics. Evidently, the presence of non-phenolic compounds (peptides, polysaccharides, etc.) is governing the metal chelating ability for the tested extracts rather than the phenolics content (Islam et al., 2016; Rahman et al., 2018).

3.5. Enzyme inhibition potential

As far as we know, no studies have been reported on the enzyme inhibitory properties of the members of *Lagochilus* so far. We investigated the enzyme inhibitory properties of *Lagochilus* extracts and some isolated compounds. Compound **2** exhibited the strongest inhibitory effects on both AChE and BChE, while compound **6** had the weakest effect on these enzymes (Table 4). From the extracts, *L. olgae* and *L. gypsaceus* were the most active on these enzymes, respectively. In an earlier study conducted by Sawasdee et al (2009), several flavones were investigated for cholinesterase inhibition. In their study, the number and position of methoxy and hydroxyl groups were effected their inhibition position. Based on their results, a methoxy group at C-3 could reduce inhibitory effects, while a 4-methoxy group in ring B could increase the inhibitory effects. In agreement with our results, several researchers have reported some flavones as anti-cholinesterase agents (Uriarte-Pueyo and Calvo 2011; Khan et al., 2018). Regarding tyrosinase inhibition, the highest inhibitory effect was found for *L. inebrians* (from Djizzakh) with 70.29 mgKAE/g, followed by *L. acutilobus* and *L. olgae*. Similar to cholinesterases, compound **2** was also the most active in the case of tyrosinase. From these results, the observed tyrosinase inhibitory effects of the *Lagochilus* species could be attributed to the presence of flavones. Analogously to cholinesterase inhibitory assays, tyrosinase inhibitory effect could change the numbers and position of hydroxyl and methoxyl groups in flavonoid rings (Gao et al., 2007). In the amylase inhibitory assay, *L. acutilobus* and compound **2** showed the best inhibitory effects and the weakest ability was once more observed for compound **6**. *L. inebrians* extracts exhibited stronger glucosidase inhibitory effects than other species and again compound **2** was the most active of the isolated compounds. To sum up, we suggest that compound **2** is a main active compound in inhibition of the tested enzymes and that the tested species could be a potential source of natural enzyme inhibitory agents.

4. Conclusion

Our chemical studies of *L. gypsaceus* have isolated and identified iridoids, diterpenes, flavonoids and sterols. For the first time we quantified lagochilin in 7 species of *Lagochilus* by HPTLC. Results of HPTLC fingerprinting have shown both clear similarity and distinct difference between the components in methanolic extracts from the 7 species of *Lagochilus* collected from Uzbekistan; especially it provides valuable information on the natural distribution of the medicinally important lead compound lagochilin. Noteworthy, the endemic species *L. inebrians* has the highest lagochilin content among the investigated species. The presented HPTLC method can be used for preliminary screening and quantification of lagochilin in *Lagochilus* plant species. In the tested samples, 5-hydroxy-7,4'-dimethoxyflavone exhibited the strongest inhibitory effects on tyrosinase, glucosidase, AChE and BChE. Further chemical and pharmacological investigations will complete the information about this important genus of Central Asian flora.

Supplementary material

The original spectral data of the compounds are available online (Figures S1-S60).

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Nilufar Mamadalieva https://orcid.org/0000-0003-1756-3638, Davlat Kh. Akramov https://orcid.org/0000-0002-5653-1738; Gokhan Zengin http://orcid.org/0000-0002-5165-6013;

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