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Research Article

Microscopical characteristics, phytochemical investigation and biological activities of different *Lysimachia* species growing in Vietnam

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Abstract

Several species of the genus *Lysimachia* L. (*Primulaceae*) are well known in folk medicine, but very few studies have been conducted on them. The aim of our study was to investigate the microscopical characteristics and chemical composition of *Lysimachia insignis* Hemsl., *L. decurrens* G. Forst., and *L. congestiflora* Hemsl. as well as to evaluate some biological activities of their methanol extracts. The transverse sections of stem and powder characteristics of the three *Lysimachia* species shared some similarities. Also, some differences were indicated. The chemical investigation of three aforesaid species including the preliminary phytochemical screening, the quantification of the total polyphenol content and total flavonoid content, as well as the determination of metabolites. The phytochemical screening showed the presence of flavonoids, saponins, tannins and sterols in all the three species. On the other hand, coumarins were detected in *L. insignis* while anthranoids were present in *L. decurrens* and *L. congestiflora*. The total polyphenol content and the total flavonoid content of the aforesaid species were determined by the spectrometric method. The metabolite profiling approach of three species was performed by LC-Q-ToF-HRMS and HPTLC. The methanol extract of *L. insignis* showed a weak inhibitory effect on NO production (I% = 57.4 at the concentration of 100 μg/ml) while the extract of *L. decurrens* exhibited moderate anti-oxidant activity *in vitro* (SC% = 62.5 at the concentration of 100 μg/mL).

Keywords: biological activity; characteristics of stems and leaves; *Lysimachia*; metabolites; powder characteristics

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Introduction

The genus *Lysimachia* L., belonging to the family *Primulaceae* Vent., consists of about 180 species which are distributed in temperate and subtropical areas of the northern hemisphere, but represented with only a few species in Africa, Australia, and South America (Hu and Kelso, 1996). Among them, *Lysimachia vulgaris* L. was recorded in Kashmir, Kazakstan, Pakistan, Russia, NW Africa, SW Asia, Europe, and North America (Hu and Kelso, 1996) while *Lysimachia nutans* Nees is endemic to South Africa (Omar *et al.*, 2016) and the native range of *Lysimachia ruhmeriana* Vatke is Cameroon to Eritrea and S. Africa, Madagascar (Germishuizen and Meyer, 2003). In Vietnam, ca 20 species of this genus have been recorded. Among them, many species have been used in folk medicine: *L. insignis* Hemsl. for treating arthritis and high blood pressure; *L. congestiflora* Hemsl. was used as a cure for hepatitis and productive cough while *Lysimachia decurrens* G. Forst. served as a remedy to heal wounds (Vo, 2012). Thus, these plant species may have potential in the development of modern herbal medicines.

Regardless of these facts the literature survey shows lack of data about the chemical composition and biological activities of these species. Only five species – *L. clethroides*, *L. congestiflora*, *L. lobeloides*, *L. candida* and *L. fortunei* – have been chemically investigated. The phytochemical studies of the aforesaid species have led to the identification of flavonoids, saponins and triterpenes/sterols. 70 flavonoids are reported to be present in the species, with kaempferol, quercetin, myricetin and their glucosides being the main constituents (Zhang *et al.*, 1998; Huang *et al.*, 2007; Wang *et al.*, 2007; Liu *et al.*, 2010). Up to date, there is no information available about the chemical composition of *L. insignis* and *L. decurrens*. This is also the first time that some biological activities of these species were evaluated.

Moreover, the anatomical features of the aforenamed species were not described, which could have caused some difficulties in the identification of the plant material. These little-studied plant species may be promising as sources of biologically active substances and as natural remedies for the improvement of human health. The aim of this study has been the investigation of microscopical characteristics to identify three *Lysimachia* species including *L. insignis*, *L. decurrens*, and *L. congestiflora*, as well as the development of high-performance thin-layer chromatography (HPTLC) and LC-QToF-HRMS to identify some metabolites on the aforementioned species for the first time, along with studying their biological activities.

Materials and Methods

Plant material

The whole plants of *Lysimachia insignis* Hemsl. (1.0 kg), *L. decurrens* G.Forst (1.5 kg), and *L. congestiflora* Hemsl. (2.0 kg) were collected (Table 1). The plant species were identified by Dr. Nguyen Quang Hung, Department of Plant Resources, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The voucher specimens were deposited at the Department of Plant Resources, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The plant materials were dried in the shade and stored in sealed polyethylene bags prior to the analysis.

Table 1. Samples of *Lysimachia* species studied by the authors

Id	Scientific names	Collecting time & location	Geographical coordinates	Number of voucher specimens
Li	*Lysimachia insignis* Hemsl.	Ham Yen district, Tuyen Quang province; April 2019	22°17'04.6"N 104°57'30.8"E	(106.03-2018.06)-3
Ld	*Lysimachia decurrens* G.Forst.	Cuc Phuong National Park, Ninh Binh province; April 2019	20°15'45.3"N 105°42'05.8"E	(106.03-2018.06)-4

Lc	*Lysimachia congestiflora* Hemsl.	Phong Tho district, Lai Chau province; May 2019	22°23'27"N 103°19'49"E	(106.03-2018.06)-1

Chemical and reagents

Toluene, ethyl acetate, formic acid, glass acetic acid, acetonitrile and HPTLC silica gel 60 F254 plates were obtained from Merck (Darmstadt, Germany). The chemicals used for the preparation of NP/PEG reagent – 2-aminoethyl diphenylborinate and polyethylene glycol – were obtained from Sigma-Aldrich (St. Louis, USA). Gallic acid, kaempferol, quercetin and myricetin (purity 98%) were provided by Biopurify Phytochemicals Ltd. (Sichuan, China).

Investigation of microscopical characteristics

The transverse sections of the stems and leaves of *L. insignis*, *L. decurrens*, and *L. congestiflora* were made using a razor blade. The sections were then washed with chloramine B before being stained with 1% methylene blue (Merck, Darmstadt, Germany), and saturated solution of carmine (Merck, Darmstadt, Germany). Then the samples were put in mixture of glycerine and distilled water (1:1, v/v) on glass slides with a glass lamina. Observations and photos were done using a Light Microscope Leica (Wetzlar, Germany) connected to a digital camera. The transverse sections of three above-stated species were described in detail and illustrated with pictures (Figures 1-3). Besides, the plant materials were powdered and sifted through the sieve of 180 μm. The powder characteristics were investigated by using a Light Microscope Leica (Wetzlar, Germany).

Phytochemical screening evaluation

The phytochemical screening was performed for usual plant secondary metabolites such as flavonoids, coumarins, tannins, saponins, anthranoids, cardiac glycosides, triterpenes/sterols and alkaloids either. The tests followed the methods described in literature (Ngo and Tran, 2011; Pham and Nguyen, 2015). Color intensity or the formation of precipitate were used as analytical response to the relevant tests.

Determination of total polyphenol content and total flavonoid content

1 g of powdered air-dried herbs of each species was extracted with 80% aqueous methanol (10 mL x 30 minutes x 5 times) in a sonic bath. After filtration, the filtrates were transferred to a 50.0 mL volumetric flask and filled up to volume with the same solvent. After that the extracts were diluted with 80% aqueous methanol for the determination of the total polyphenol content and total flavonoid content.

The total polyphenol content of the three *Lysimachia* species was determined by applying the method described above (Singleton *et al.*, 1999). To a 10 mL volumetric flask, 1 mL of test sample and 2.5 mL of 10 % aqueous Folin-Ciocalteu reagent were added and left for 5 minutes at room temperature. Subsequently, 5.0 mL of 2% sodium carbonate was added and filled up to volume with distilled water. After incubation for 60 minutes, the absorbance was measured at 745.5 nm by means of a HITACHI U-1900 (Tokyo, Japan). The calibration curve was prepared by using gallic acid solutions at concentrations 2.0-10.0 μg/mL using Microsoft Excel software.

The total flavonoid content of the above introduced species was determined by the aluminum chloride colorimetric method with some modifications (Seyfi *et al.*, 2010). To a 10.0 mL volumetric flask, 1.00 mL of test sample, 0.2 mL of aluminum chloride (10 % w/v) and 0.4 mL of sodium acetate (0.5 M) were added and filled up to volume with methanol. The mixture was left at room temperature for 30 minutes, after which its absorbance was measured at 425 nm using a HITACHI U-1900 (Tokyo, Japan). The calibration curve was prepared, using quercetin solutions at different concentrations (2.94-8.82 μg/mL) thanks to the Microsoft Excel software.

Tentative identification of metabolites by LC-QToF-HRMS and HPTLC

For chromatographic analysis, five grams of powdered air-dried herbs of each species were extracted with 80% aqueous methanol (5 times x 10 mL x 30 minutes) in a sonic bath. After filtration, the filtrate was evaporated *in vacuo*. The extract was then suspended in water and successfully partitioned with diethyl ether (3 times x 10 mL) and ethyl acetate (5 times x 10 mL). The ethyl acetate fractions were evaporated *in vacuo*. The ethyl acetate extracts were then dissolved in methanol and transferred to a 5.0 mL volumetric flask, which was filled up to volume with methanol. The mixtures were then filtered through a 0.45 μm syringe filter membrane, and the filtrate were transferred into vials prior to LC-QToF-HRMS and HPTLC analysis.

The liquid chromatographic analysis of the solutions was carried out by using an Exion LC™ coupled to a X500R Q-TOF mass spectrometer (Sciex, USA). The separation of compounds was performed using a Hypersil GOLD Dim. column (150 mm x 2.1 mm; 3μm; Thermo Scientific, USA). The flow rate from the delivery system was set at 0.400 mL/min, the sample injection volume was 2.0 μL. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile (Merck, Darmstadt, Germany). A linear gradient elution program applied was as follows: 0–1.0 min (0% B), 1.0–20.0 min (2% B), 20.0–25.0 min (98% B). MS/MS detection was performed in negative ion mode in the m/z interval of 50-2000 amu. Phenolic compounds were identified by mass to charge ratio (m/z), retention time, and MS fragmentation patterns. The identification was confirmed with commercial standards of *p*-coumaric acid, trans caffeic acid, kaempferol, myricetin, quercetin.

High-performance thin-layer chromatography (HPTLC) was performed on HPTLC silica gel 60 plates (Merck, Germany). Reference compounds (kaempferol, quercetin and myricetin) were dissolved in methanol to the concentration of 1.0 mg/mL. Sample application on the plates was performed with the help of CAMAG Linomat 5 semi-automatic sample Spotter (CAMAG, Muttenz, Switzerland) and a 100 μL volumetric syringe. Three samples of the Lysimachia species as well as the reference solutions were applied onto the same HPTLC silica gel 60 F254 plate. After that, the plates were developed, using some different eluent systems: (I) Toluene – ethylacetate – formic acid (14:10:1, v/v/v), (II) Ethylacetate – formic acid – glass acetic acid – distilled water (100:11:11:26, v/v/v). The experiment was carried out in a twin chamber at the temperature of 25 ± 2 °C and relative humidity of 33%, using the Auto developing chamber (CAMAG, Switzerland). The plates were observed at the wavelengths of 254 nm and 366 nm by means of a TLC Visualizer (CAMAG, Switzerland) linked to visionCATs 2.5 software. The chromatographic evaluation was performed to calculate the number and retention factor values of bands based on the chromatogram observed at UV 366 nm after derivatization with NP/PEG agent thanks to the visionCATS software.

Biological assays

For the biological assays, 100 grams of each previously mentioned species were macerated with methanol (3 times x 24 hours). The total methanolic extracts were filtered, then the filtrates were evaporated *in vacuo*. The methanol residues of *L. insignis*, *L. decurrens* and *L. congestiflora* were prepared in DMSO at the concentrations of 30 and 100 μg/mL.

The inhibitory effect on the NO production

The inhibitory effect on the NO production of the extracts of the three *Lysimachia* species was evaluated by applying the previously described method (Dat *et al.*, 2012). RAW264.7 cell lines were cultured in RPMI 1640 or DMEM, supplemented with 10% (v/v) FBS, penicillin and streptomycin sulphate at 37 °C with 5% CO2. Cells were seeded in a 96-well plate at 2.5 x 10^5 cells/well. After that, the cells were treated with different concentrations of samples prepared in DMSO, followed by incubation for 24 h. The Griess method was utilized to estimate the nitrite concentration in the culture supernatant. The absorbance (OD) of the reaction solution was measured at 570 nm. Cardamonin was used as a positive control. The inhibitory activity (I %) was calculated based on the following formula:

I% = [OD (sample) – OD (blank)]/[OD (control) – OD (blank)] × 100

The DPPH free radical scavenging activity

The DPPH free radical scavenging activity of the extracts of the three *Lysimachia* species was determined spectrophotometrically according to the above reported method with some minor modification (Brand-Williams *et al.*, 1995). 10 µL of the DMSO solution of the extract was added to a 96 well plate, followed by the addition of 190 µL of 150 µM DPPH solution. The samples were incubated for 20 min at room temperature. The absorbance of the solution was measured at 517 nm. Ascorbic acid was utilized as a positive control. The DPPH scavenging effect (SC%) was calculated by the following equation:

$$SC\% = [OD\ (sample) - OD\ (blank)]/[OD\ (control) - OD\ (blank)] \times 100$$

Results

Microscopical characteristics

Figures 1-3 showed the transverse sections of stems and leaves, as well as the powder characteristics of three *Lysimachia* species. The stem transverse sections of these species shared some similarities: narrow phloem, xylem arranged in a ring; pericyclic fibres arranged in an interrupted ring or a nearly continuous ring (Figure 1A, 2A, 3A). The leaf transverse sections of three aforesaid species have some differences: midvein of *L. insignis* consist of pericyclic fibres while they were absent in leaf transverse section of *L. decurrens* and *L. congestiflora*. On the other hand, glandular hairs and covering trichomes were observed only in leaf transverse section of *L. congestiflora* (Figure 1B, 2B, 3B). Some powder characteristics of these *Lysimachia* species were also similar, such as long fibre, spiral vessel, epidermis containing stomata, and pollen grains (Figure 1C, 2C, 3C). Besides, pollen grains of these species have some differences: the pollen grains of *L. congestiflora* were sub-spherical to spherical while the pollen grains of *L. insignis* and *L. decurrens* were oval. Besides, glandular hair and multi-cellular covering trichome could be observed in the stem transverse section and powder characteristics of *L. congestiflora* but they were absent in the stem transverse section and powder characteristics of other species. These features could be useful in the identification and distinction of these species. Full interpretation of the Figures 1-3 has been given in the end of Results.

Phytochemical screening evaluation

The phytochemical screening evaluation of the aforesaid species is presented in Table 2. Phytochemical screening revealed the presence of flavonoids, saponins, tannins and sterols in all the samples of the three species. Furthermore, coumarins were detected in *L. insignis* but were absent in *L. decurrens* and *L. congestiflora*. Additionally, the screening evaluation revealed the presence of anthranoids in *L. decurrens* and *L. congestiflora* while these metabolites were absent in *L. insignis*.

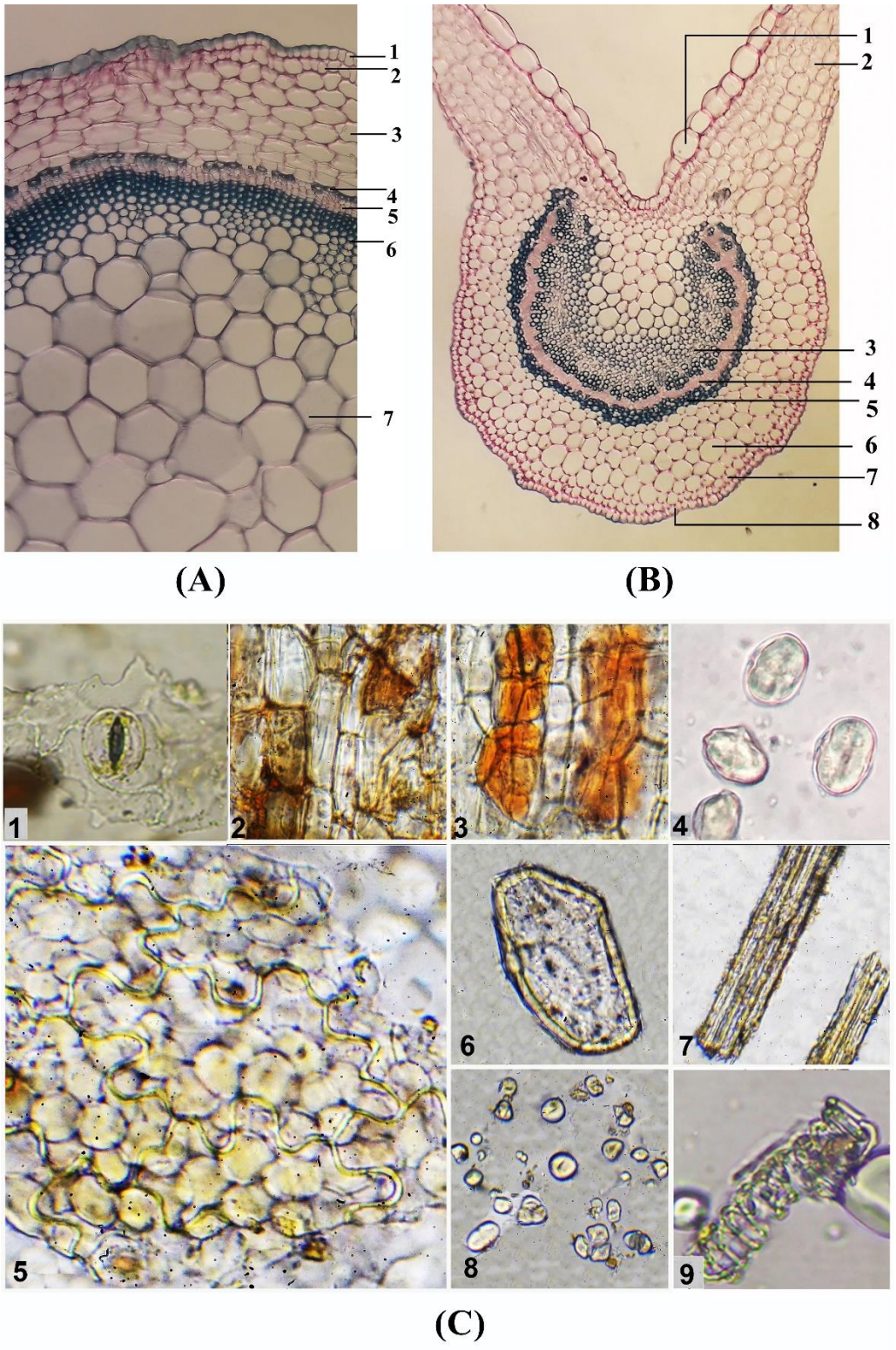

Figure 1. Microscopical characteristics of *L. insignis*: (A) Stem transverse section (1. Epidermis, 2. Thick tissue, 3. Cortex, 4. Pericyclic fibres, 5. Phloem, 6. Xylem, 7. Parenchymatous cells); (B) Leaf transverse section (1. Upper epidermis, 2. Spongy mesophyll, 3. Xylem, 4. Phloem, 5. Pericyclic fibres, 6. Parenchyma, 7. Thick tissue, 8. Lower epidermis); (C) Powder characteristics (1. Epidermis containing stomata; 2,3. Epidermis of stem; 4. Pollen grains; 5. Epidermis of leaf; 6. Sclereid; 7. Bundle of fibre; 8. Starch granules; 9. Spiral vessel)

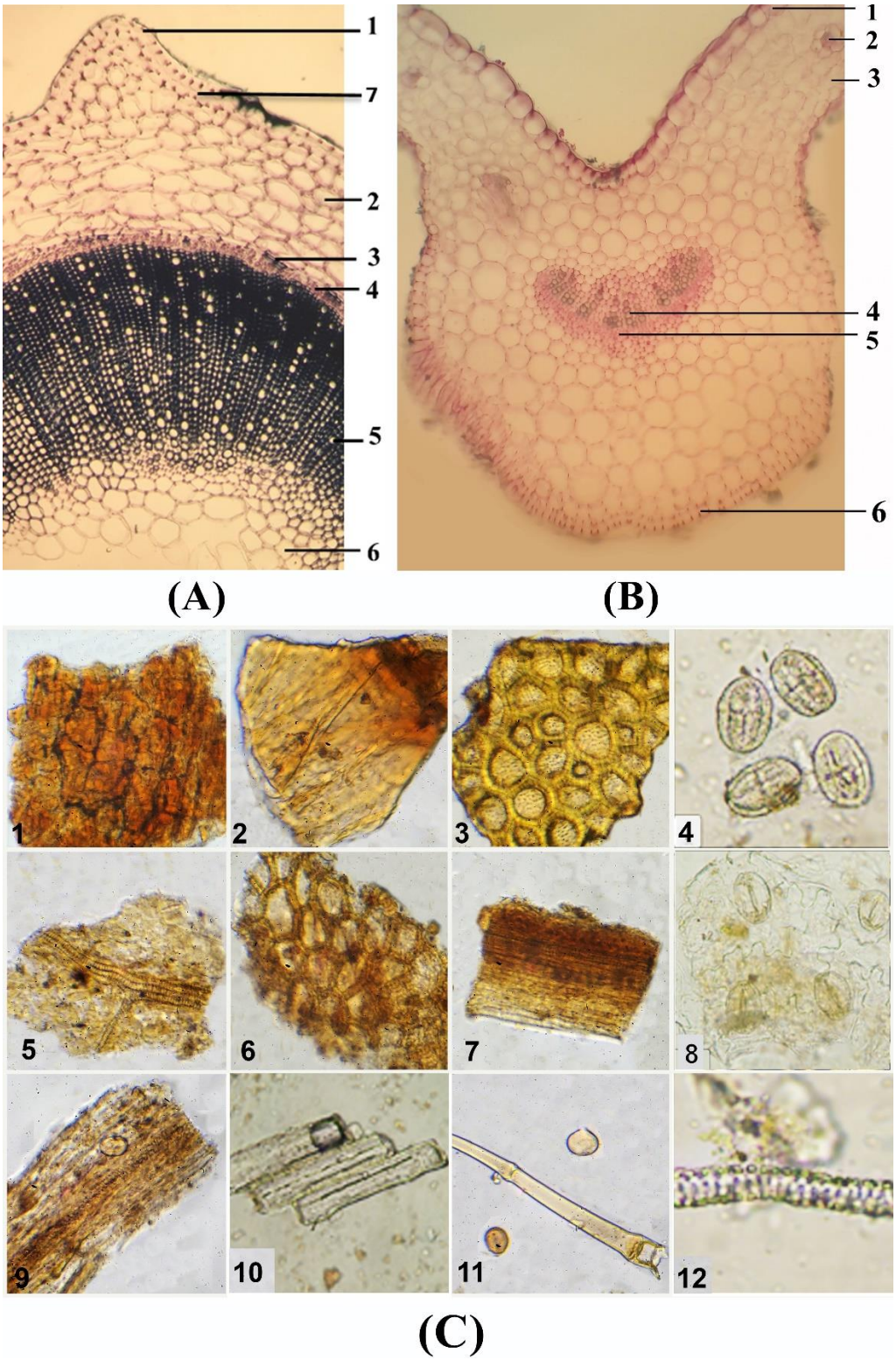

Figure 2. Microscopical characteristics of *L. decurrens* (A) Stem transverse section (1. Epidermis, 2. Thick tissue, 3. Pericyclic fibre, 4. Phloem, 5. Xylem, 6. Parenchyma); (B) Leaf transverse section (1. Upper epidermis, 2. Bundle of xylem-phloem, 3. Spongy mesophyll, 4. Xylem, 5. Phloem, 6. Lower epidermis); (C) Powder characteristics (1,3,6. Epidermis of stem; 2. Fragment of petal; 4. Pollen grain; 5. Fragment of lamina containing vessels;7, 9. Parenchymatous cells containing vessel; 8. Epidermis containing stomata; 10. Fibres; 11. Covering trichome; 12. Spiral vessels)

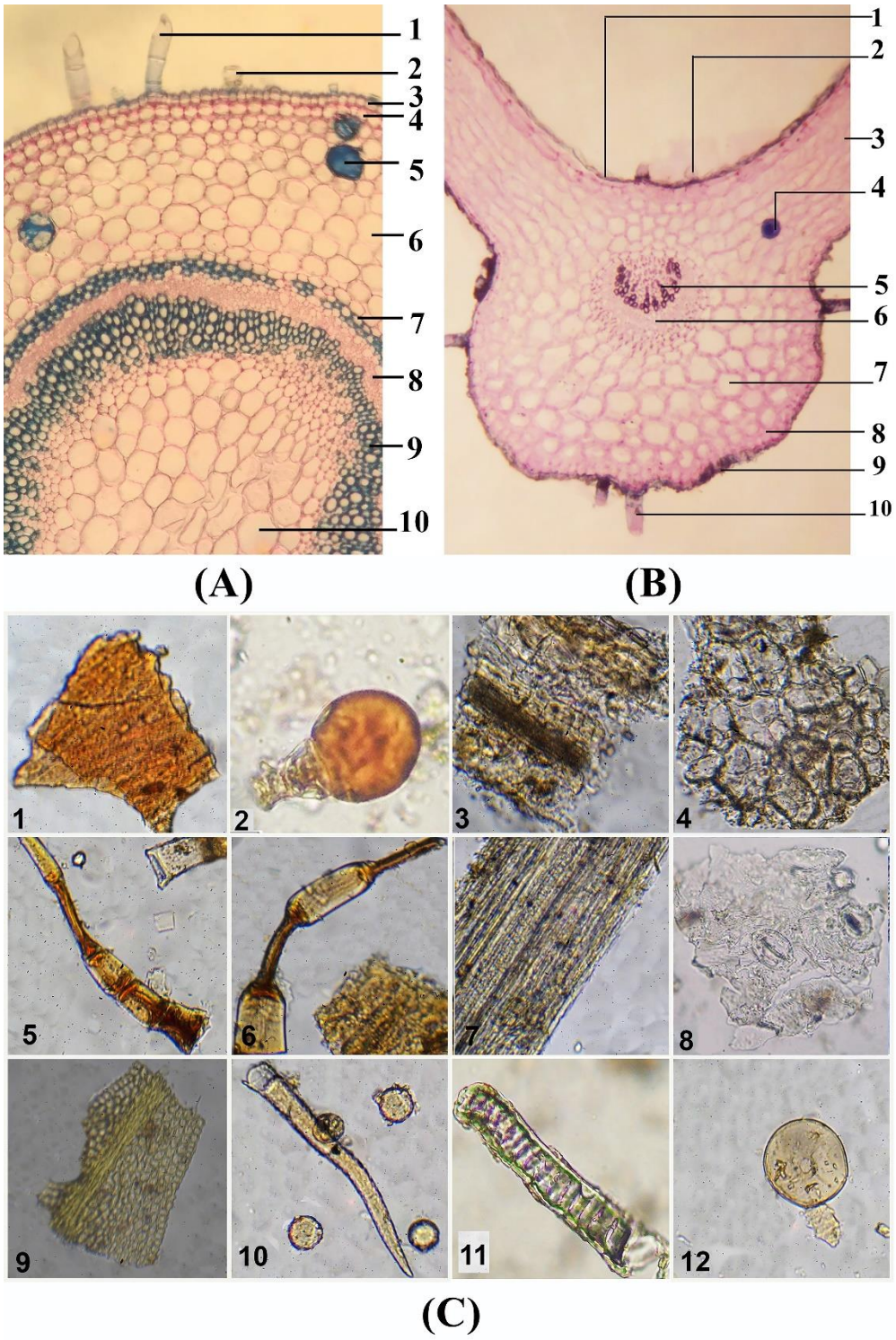

Figure 3. Microscopical characteristics of *L. congestiflora*: (A) Stem transverse section (1. Covering trichome, 2. Glandular hair, 3. Epidermis, 4. Thick tissue, 5. Secretory cell, 6. Cortex, 7. Pericyclic fibre, 8. Phloem, 9. Xylem, 10. Parenchyma); (B) Leaf transverse section (1. Upper epidermis, 2. Glandular trichome, 3. Spongy mesophyll, 4. Secretory cell, 5. Xylem, 6. Phloem, 7. Parenchyma, 8. Thick tissue, 9. Lower epidermis, 10. Covering trichome); (C) Powder characteristics (1. Fragment of petal; 2. Glandular hair; 3,4. Parenchymatous cells; 5,6. Covering trichome; 7. Fibrous bundle with vessel; 8. Epidermis containing stomata; 9. Fragment of lamina; 10. Covering trichome;11. Vessels; 12. Pollen grain)

Table 2. Phytochemical screening evaluation of three *Lysimachia* species

	Lysimachia insignis	*Lysimachia decurrens*	*Lysimachia congestiflora*
Flavonoids	+	+	+
Saponins	+	+	+
Coumarins	+	-	-
Tannins	+	+	+
Alkaloids	-	-	-
Anthranoids	-	+	+
Cardiac glycoside	-	-	-
Sterols	+	+	+

Note: (+): present, (-): absent

Total polyphenol content and total flavonoid content

L. congestiflora showed the highest total polyphenol content (57.33 mg GAE/g), which was four times higher than the total polyphenol content of *L. insignis* (11.20 mg GAE/g) and nine times higher than that of *L. decurrens* (6.63 mg GAE/g). The total flavonoid content of these species was quite similar (1.54 – 3.40 mg QE/g) (Table 3).

Table 3. Total polyphenol content and total flavonoid content of three *Lysimachia* species

Content	Linear equation ($y = mx + b$)	*R*	*L. insignis*	*L. decurrens*	*L. congestiflora*
TPC (mg GAE/ g)	$y = 0.0923x + 0.0027$	0.9989	11.20	6.63	57.33
TFC (mg QE/g)	$y = 0.0799x - 0.0081$	0.9985	2.84	1.54	3.40

TPC: total polyphenol content, TFC: total flavonoid content

LC-QToF-HRMS analysis

Forty-two compounds were identified in the ethyl acetate fractions of the three *Lysimachia* species (Table 4). Trans-caffeic acid, *p*-coumaric acid and kaempferol were detected in chromatogram of all three samples. Besides, salicylic acid, mellein, quercetin-hesoxyl-desoxyhexoside, quercetin, anthraflavic acid were identified in both chromatograms of *L. decurrens* and *L. congestiflora*. DL-malic acid, 3-Hydroxy-4-methoxycinnamic acid were two identified compounds in chromatograms of *L. insignis* and *L. decurrens* while myricitrin, kaempferitrin, quercitrin, rosmarinic acid, phlorizin and valerenic acid were among compounds which were observed in both chromatograms of *L. insignis* and *L. congestiflora*.

On the other hand, chromatogram of each *Lysimachia* species possessed some compounds which could not be observed in chromatogram of other species. 3-hydroxybenzoic, 3,5-dimethoxy-4-hydroxycinnamic acid, flavanone, citrinin and some derivates of kaempferol including kaempferol-hexosyl-di(desoxyhexoside) and kaempferol-hexosyl-desoxyhexoside] could be identified only in chromatogram of *L. insignis*. Hyperoside, luteolin-4'-O-glucoside were some compounds which were identified in chromatomagram of *L. decurrens*. Chromatogram of *L. congestiflora* was characterized by the presence of pyrocatechol, 1,2,4-benzene tricarboxylic acid, flavanomarein, salvianolic acid A, scutellarin, 3,5,7,3',4'-pentahydroxyflavanone, narirutin, salvianolic acid B, quercetin-pentoside, quercetin 3-O-β-D-glucose-6'-acetate, baicalin, 5,7,3',4',5'-Pentahydroxyflavone, poncirin, naringenin, 4-hydroxy benzophenone, myricetin and its derivates (myricetin-hexoside, 3'-O-Methyl myricetin).

Table 4. Characterization of LC chromatograms of the three *Lysimachia* species

No.	Rt	Tentative assignment	$[M-H]^-$	Fragment ions	Li	Ld	Lc
1	5.86	Pyrocatechol	109.0312				X
2	6.83	Salicylic acid	137.0259	108, 93, 92, 91, 81, 53		X	X
3	6.92	3-Hydroxybenzoic acid	137.0252	93, 92, 91, 75, 65	X		
4	7.04	1,2,4-Benzene tricarboxylic acid	209.047	165, 147, 121, 119, 106, 93, 65, 59			X
5	7.35	Mellein	177.021	149, 133, 107, 105, 89, 79, 77		X	X
6	7.52	Trans-caffeic acid[a]	179.0361	135, 134, 107, 89	X	X	X
7	7.83	Flavanomarein	449.1096	342, 287, 269, 259, 179, 125			X
8	7.9	DL-Malic acid	133.0154	100, 71	X	X	
9	8.15	Myricetin-hexoside	479.0842	317, 316, 287, 271, 151			X
10	8.32	Quercetin-hesoxyl-desoxyhexoside	609.1448	343, 301, 300, 271, 179		X	X
11	8.34	Salvianolic acid A	493.1146	197			X
12	8.4	*p*-Coumaric acid[a]	163.0414	119, 117, 93	X	X	X
13	8.41	Kaempferol-hexosyl-di(desoxyhexoside)	739.2069	593, 431, 285	X		
14	8.46	Myricitrin	463.0871	317, 316, 287, 270, 179, 151	X		X
15	8.55	Hyperoside	463.0873	301, 300, 299, 271, 255, 151		X	
16	8.57	Kaempferitrin	577.1536	431, 430, 285, 283, 255	X		X
17	8.76	3,5-Dimethoxy-4-hydroxycinnamic acid	223.0616	149, 121, 93	X		
18	8.76	Scutellarin	461.0735	357, 285, 151, 85			X
19	8.81	3-Hydroxy-4-methoxycinnamic acid	193.0511	134, 133, 89	X	X	
20	8.85	3,5,7,3',4'-Pentahydroxyflavanone	303.0523	285, 189, 175, 125, 83, 57			X
21	8.95	Narirutin	579.1726	463, 316, 300, 271, 151			X
22	8.98	Luteolin-4'-O-glucoside	447.0931	301, 285, 284, 255, 227, 175, 151		X	
23	8.99	Salvianolic acid B	717.1476	519, 475, 365, 339, 229			X
24	9.03	Quercetin-pentoside	433.0781	301, 300, 271, 255, 243, 151, 61			X
25	9.04	Quercitrin	447.0921	301, 300, 271, 255, 121, 179	X		X
26	9.1	Quercetin 3-O-β-D-glucose-6'-acetate	505.0989	447, 317, 316, 301, 271			X
27	9.34	Baicalin	445.0788	269, 225, 113, 85			X
28	9.35	Kaempferol-hexosyl-desoxyhexoside	593.1535	285, 284	X		
29	9.39	Rosmarinic acid	359.0778	197, 179, 161, 135, 73	X		X
30	9.5	Phlorizin	435.1288	273, 229, 179, 167, 123, 93	X		X
31	9.54	Myricetin[a]	317.0308	271, 192, 179, 151, 137, 107			X
32	9.69	5,7,3',4',5'-Pentahydroxyflavone	301.0365	255, 151, 149, 107			X
33	10.41	Poncirin	593.1879	314, 285			X
34	10.42	Quercetin[a]	301.0361	229, 179, 151, 121, 107, 83		X	X
35	10.51	3'-O-Methyl myricetin	331.0471	316, 151			X

36	11.32	Naringenin	271.0625	177, 151, 119, 107, 65			X
37	11.35	Kaempferol[a]	285.0408	285, 239, 229, 211, 187, 185	X	X	X
38	11.98	4-Hydroxy benzophenone	197.0623	169, 151, 120, 92, 91, 77			X
39	12.79	Flavanone	223.1344	195, 166, 151, 138, 123	X		
40	13.95	Anthraflavic acid	239.1302	195, 179, 154, 139,		X	X
41	14.37	Citrinin	249.1865	234, 217	X		
42	16.02	Valerenic acid	233.1548	218, 217, 203, 190	X		X

Note: (X): compound was tentatively detected in sample

[a]The presence of compound was confirmed by reference substance

HPTLC fingerprinting of three Lysimachia species

The composition of the eluent system used for HPTLC was optimized by testing a combination of solvents of different polarity. Originally, the solvent system that was utilized consisted of ethyl acetate, formic acid, glass acetic acid, and distilled water in different ratios. The eluent system ethyl acetate – formic acid – glass acetic acid – distilled water (100:11:11:26, v/v/v/v) was chosen (Figure 4A). The summary of the results obtained with HPTLC using this eluent system is presented in Table 5. There were clear differences between chromatogram of above-mentioned *Lysimachia* species about the number and fluorescent colour of the band which demonstrated the different phytochemical composition of five species.

Table 5. Summary of the results obtained with HPTLC using the eluent system ethyl acetate – formic acid – glass acetic acid – distilled water (100:11:11:26)

Samples	Number of tracks	R_f[a] (Colour[b])
L. insignis (Li)	5	0.30 (Gr), 0.37 (Gr), 0.49 (Gr), 0.68 (Gr), 0.87 (Be)
L. decurrens (Ld)	7	0.27 (Yel), 0.32 (Gr), 0.38 (B), 0.44 (Yel), 0.58 (BBe), 0.65 (Be), 0.85 (BBe)
L. congestiflora (Lc)	9	0.35 (Gr), 0.46 (Yel), 0.54 (Yel), 0.64 (Be), 0.72 (Org), 0.79 (Org), 0.85 (Gr), 0.88 (Bbe), 0.93 (BBe)

[a] Data are presented in the order of appearance on the chromatographic plate, from bottom to top

[b] Color of tracks observed at λ = 366 nm: Yel: yellow; Be: blue; BBe: bright blue; Gr: green; Org: orange

Next, toluene, ethyl acetate and formic acid in varying ratios were tried. Finally, the mobile phase toluene – ethyl acetate – formic acid (14:10:1) was selected for HPTLC fingerprinting using some reference compounds. This mobile phase afforded distinguishable bands, good separation and symmetrical peak shapes of kaempferol and myricetin. The chromatographic evaluation was performed at the wavelength of 366 nm after derivatization with NP/PEG reagent. At this wavelength, kaempferol (Rf 0.56) could be observed as a bright blue band after derivatization with NP/PEG reagent and was clearly detectable in the chromatograms of *L. insignis*. By contrast, myricetin (Rf 0.37) and quercetin (Rf 0.47) were observed as yellow bands and could be detected in the chromatogram of *L. congestiflora* (Figure 4B). The summary of the results obtained with HPTLC using this eluent system is presented in Table 6.

Table 6. Summary of the results obtained with HPTLC using the eluent system toluene – ethyl acetate – formic acid (14:10:1)

Samples	Number of tracks	R_f[a] (Colour[b])	Standards assigned
L. insignis (Li)	5	0.01, 0.17, 0.36, 0.56 (BBe), 0.88	K
L. decurrens (Ld)	7	0.03, 0.13, 0.26, 0.37, 0.43, 0.52, 0.87	
L. congestiflora (Lc)	10	0.03, 0.06, 0.10, 0.13, 0.19, 0.24, 0.37 (Yel), 0.43, 0.47 (Yel), 0.52	Q, M
Kaempferol (K)		0.56 (BBe)	
Quercetin (Q)		0.47 (Yel)	
Myricetin (M)		0.37 (Yel)	

[a] Data are presented in the order of appearance on the chromatographic plate, from bottom to top

[b] Color of tracks observed at λ = 366 nm: Yel: yellow; Be: blue; BBe: bright blue

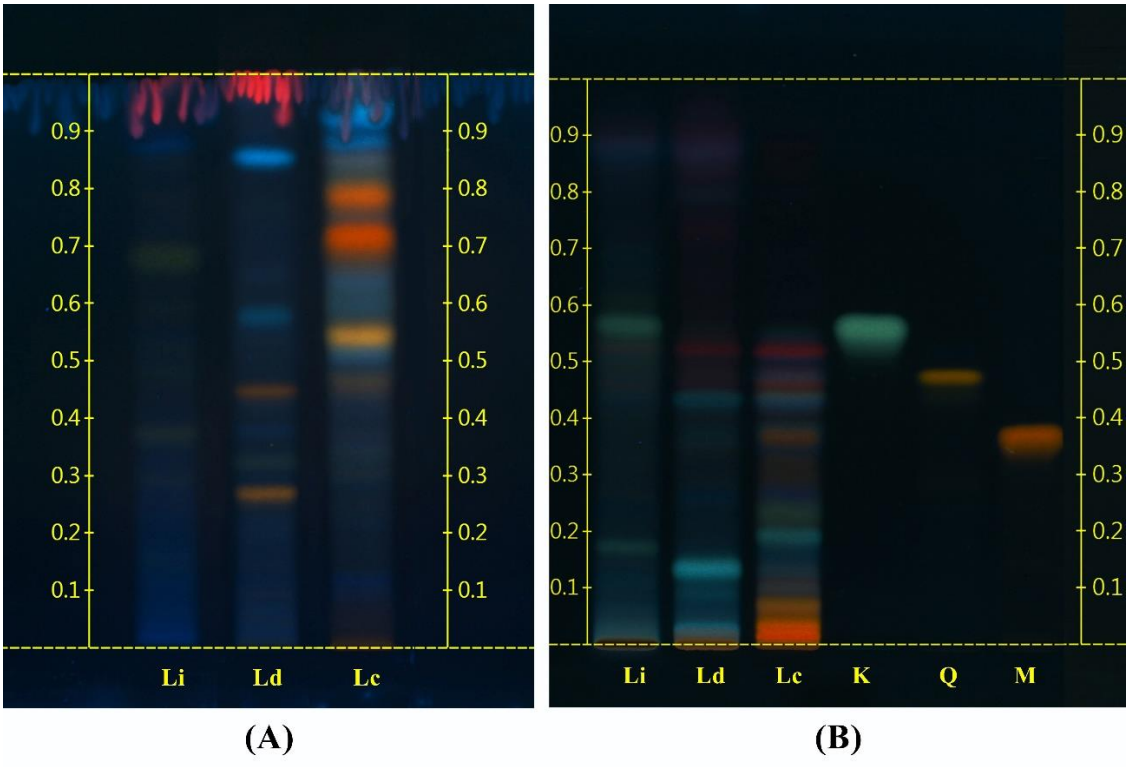

Figure 4. Thin layer chromatogram of three *Lysimachia* species: (A) Chromatogram developed using the eluent system ethyl acetate – formic acid – glass acetic acid – distilled water (100:11:11:26) observed at 366 nm after derivatization with NP/PEG reagent; (B) Chromatogram developed using the eluent system toluene – ethyl acetate – formic acid (14:10:1) observed at 366 nm after derivatization with NP/PEG reagent

Biological assays

The inhibitory effect on NO production and *in vitro* anti-oxidant activity of the methanol extracts of *L. insignis*, *L. decurrens* and *L. congestiflora* are presented in Table 7. The extract of *L. insignis* showed a moderate inhibitory effect on NO production, inhibiting 57.4% of NO production at the concentration of 100 µg/mL, whereas *L. decurrens* and *L. congestiflora* exhibited a weak effect. On the other hand, the methanol extract of *L. decurrens* showed a moderate effect on DPPH radical scavenging activity (SC% = 62.5 at the concentration of 100 µg/mL).

Table 7. Inhibitory effect on NO production and DPPH scavenging effect of *Lysimachia* species

Sample	Concentration (µg/mL)	Inhibitory effect of NO production (I%)	DPPH scavenging effect (SC%)
L. insignis	30	38.2	18.6
	100	**57.4**	25.2
L. congestiflora	30	33.5	25.0
	100	47.3	37.3
L. decurrens	30	15.7	41.8
	100	22.3	**62.5**

Interpretation of the Figures 1-3

Microscopic identification of *Lysimachia insignis*

Transverse section of stem: Epidermal cells covered with cuticle (1). Thick tissue consisting of 1-2 layers of cells (2). Cortex, broad (3). Endodermis, distinct. Pericyclic fibres arranged in an interrupted ring (4). Phloem, narrow (5). Xylem arranged in a ring (6). Parenchymatous cells in polygonal shape, lignified (7) (Figure 1A).

Transverse section of leaf: Upper epidermal cells subsquare (1). Spongy tissue consists of 4-6 layers of cells (2). Midvein consists of xylem (3), phloem (4) and pericyclic fibres (5). Lower epidermis cell (6) is smaller than the upper epidermis cell. (Figure 1B).

Powder: Color, dark green. Epidermis of lamina containing stomata (1). Fragments of stem epidermis (2,3). Pollen grains, scattered, oval (4). Fragment of leaf epidermis with underlying palisade (5). Sclereid (6). Fibres, long, lignified (7). Starch granules (8), simple or sometimes paired, subrounded. Vessels (9), mainly spiral. (Figure 1C).

Microscopic identification of *Lysimachia decurrens*

Transverse section of stem: Epidermis covered with cuticle (1). Thick tissue consisting of 1-2 layers of cells (2). Cortex, broad (2). Endodermis, distinct. Pericyclic fibres arranged in an interrupted ring (3). Phloem, narrow (4). Xylem arranged in a ring, broad (5). Parenchyma containing some voids (6) (Figure 2A).

Transverse section of leaves: Upper epidermal cells subsquare (1). Spongy tissue consists of 4-6 layers of cells (3). Midvein consists of xylem (4), phloem (5). Lateral veins small, undeveloped (2). Lower epidermis cell (6) is smaller than the upper epidermis cell. (Figure 2B).

Powder: Color, dark green. Epidermis (1,3,6). Fragment of petal (2). Pollen grains, scattered, oval (4). Fragments of lamina containing vessels (5). Parenchymatous cells containing vessels (7,9). Epidermis of lamina containing stomata (8). Fibres, gathered together, lignified, rectangular (10). Covering trichomes (11). Vessels (12), mainly spiral. (Figure 2C).

Microscopic identification of *Lysimachia congestiflora*

Transverse section of stem: Covering trichomes (1) and glandular hairs (2), present occasionally. Epidermis covered with cuticle (3). Thick tissue consisting of 1-2 layers of cells (4). Cortex, quite narrow (6), containing scattered secretory cells (5). Endodermis, distinct. Pericyclic fibres arranged in a nearly continuous ring (7). Phloem, quite narrow (8). Xylem arranged in a ring (9). Parenchymatous cells, void (10) (Figure 3A).

Transverse section of leaves: Upper epidermal cells were covered with cuticula (1). Spongy tissue consists of 6-8 layers of cells (3). Secretory cell, scattered (4). Midvein consists of xylem (5), phloem (6). Glandular hairs present occasionally (2). Covering trichomes scattered in both upper and lower epidermis (10) (Figure 3B).

Powder: Colour, pale brown to brown. Fragments of petals (1). Glandular hairs, reddish brown, consisting of unicellular head (2). Parenchymatous cells (3,4). Covering trichomes, multi-cellular (5,6) or sometimes one-cellular (10). Fibres, long, lignified and gathered in a bundle (7). Epidermis of lamina

containing stomata (8). Fragment of lamina (9). Vessels (11), mainly spiral. Pollen grains, scattered, spheric, light-yellow (12) (Figure 3C).

Discussion

Principally, research on medicinal plants deals with many different aspects. In our study, microscopical characteristics of the studied *Lysimachia* species have been described for the first time which could help identify these species correctly. The database on the microscopical characteristics also allows for phytochemical characterization of the plant materials to standardize their identification methods on the level of the state pharmacopoeia.

Among the *Lysimachia* species, the dried herb of *Lysimachia christinae* Hance has been used to treat some diseases such as dampness-heat, jaundice, gallbladder distention and hypochondriac pain, stone strangury, heat strangury, slow and painful urination, swelling abscess, deep-rooted boil and sore, bite wound of insect, worm or snake (Chinese Pharmacopoeia Commission, 2020). These indications could be related to kaempferol and quercetin which are biomarkers for the identification of *L. christinae*. Of these two compounds, kaempferol was isolated previously from some other *Lysimachia* species, including *L. clethroides* (Liu *et al.*, 2010) and *L. fortunei* (Huang *et al.*, 2007). Furthermore, myricetin had been isolated previously from *L. congestiflora* (Wang *et al.*, 2007). Some flavonoids had been detected in methanol extracts of *L. vulgaris*, *L. nummularia* and *L. punctata* using LC-MS/MS (Toth *et al.*, 2014). However, this is the first time that some phenyl propanoids (trans caffeic acid, *p*-coumaric acid) as well as some flavonoid aglycones including kaempferol, quercetin, myricetin, luteolin and their derivates were tentatively identified in the three species *L. insignis*, *L. decurrens* and *L. congestiflora*. The HPTLC profiling of the three *Lysimachia* species displayed some clear differences: kaempferol could be clearly observed in the HPTLC chromatogram of *L. insignis*, whereas quercetin and myricetin could be detected in the chromatogram of *L. congestiflora*. Kaempferol could be presence in ethyl acetate fractions of all three species but maybe in low contents in *L. decurrens* and *L. congesiflora* so this substance was not detected using HPTLC.

Because each medicinal plant accomodated a complex mixture of natural compounds, it is nearly impossible to isolated all the substances that are present in a plant material. In this case, the phytochemical screening of three *Lysimachia* species suggested us the presence of main metabolites. Moreover, the chromatographic analysis could help tentatively identify some compounds in the studied species, as well as provide the chromatography fingerprinting of the plant material.

The total polyphenol content and total flavonoid content of the three *Lysimachia* species were also different, so that the differences in the chemical compositions of these species could lead to differences in the biological activities of the aforesaid species. This is also the first time the TPC and TFC values of these species were investigated.

In addition, some biological activities of the above-stated *Lysimachia* species were investigated. The methanol extract of L. *insignis* showed a weak inhibitory effect on NO production, and the extract of *L. decurrens* exhibited moderate anti-oxidant activity. Some flavonoid aglycone including kaempferol, quercetin and myricetin had some contribution to the DPPH scavenging activity of hydrolysed extracts of six *Lysimachia* species (Toth *et al.*, 2017). The nitrite concentration, as an indicator for the presence of NO in the culture medium, helps to show the possible anti-inflammatory activity of plant extracts. The previous studies on the biological activities of Estonian and Vietnamese medicinal plants show that the experience of folk medicine is often confirmed by research on their biological activities (Raal *et al.*, 2015; Nguyen *et al.*, 2018; Tran *et al.*, 2019). Therefore, it is important to direct attention to these plants that have so far been scientifically studied only little but are known in folk medicine.

It is necessary to conduct further studies on other biological activities of *Lysimachia* species, which may be related to the experience of folk medicine and prove promising in the light of the development of modern herbal remedies.

Conclusions

The microscopical characterisics of *Lysimachia insignis* Hemsl., *L. decurrens* G. Forst., and *L. congestiflora* Hemsl. were described. The stem and leaf transverse sections and powder characteristics of the three *Lysimachia* species shared some similarities. Besides, some differences were indicated. The chemical investigation of three above-stated species were carried out, including the preliminary phytochemical screening, the chromatography analysis and the quantification of TPC and TFC. Forty-two compounds had been tentatively identifying in three species using LC-QToF-HRMS. The inhibitory effect on NO production and anti-oxidant activity of these species was also evaluated.

Authors' Contributions

QHN had contributed to the study conception, design and Project administration. Plant was collected by QHN. Materials were prepared by QHN and TTN. Data collection and analysis were performed by QHN and VTN. The data was interpreted by MHL, TTN, and AR. The first draft of the manuscript was written by TTN and AR. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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