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CONFIRMATION OF LARGE LEAF GENTIAN (*GENTIANA MACROPHYLLA* PALL.) IDENTITY BY THIN-LAYER CHROMATOGRAPHY

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ABSTRACT: Large-leaf gentian herb is a promising source of biologically active substances, which is actively used in the traditional medicine of many countries. It contains flavonoids, iridoids, secoiridoids, polysaccharides, xanthones with a wide spectrum of pharmacological activity. The aim of the research was to establish a procedure for identification of large-leaved gentian (*Gentiana macrophylla* Pall.) using thin-layer chromatography (TLC). In order to identify both hydrophilic and hydrophobic compounds in extracts from the plant two mobile phases were proposed which allow distinguishing specific zones by their color and relative position. Validation of developed procedure was carried out in terms of specificity and chromatographic system suitability. The specificity of the procedure was assessed by matching of TLC profiles for all studied samples. It was found that the main zones on the chromatograms are matching. Thus, chromatographic characteristics of large leaf gentian aerial parts were established for the first time using validated TLC procedure. The results of the study can be used for standardization of large-leaved gentian herb.

INTRODUCTION: Recent rise of interest in herbal medicinal products serves as a promoter for search of novel sources of biologically active compounds. Current requirements for the herbal medicinal products quality standards prescribe obligatory inclusion of "Identification of main groups of biologically active substances" section into the pharmacopoeial monograph; this section should describe procedures of analysis, which utilize modern Physico-chemical methods, including thin-layer chromatography (TLC).

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Large leaf gentian (*Gentiana macrophylla* Pall.) (GM) is widely used for the treatment of various disorders in traditional medicine of China, Tibet, and Mongolia [1-5]. Usually, flowers, leaves, whole herb (aerial parts), or roots are utilized; the monograph for the latter is included in the Chinese Pharmacopoeia. However, it is considered troublesome to establish and preserve constant supply of raw material when using roots as a source of biologically active substances; therefore, use of aerial parts looks more promising [6]. Therefore, our work aimed to develop a procedure for determination of both hydrophilic and hydrophobic compounds in extracts from large leaf gentian.

MATERIALS AND METHODS: All reagents and solvents used in the study were of analytical grade and were used without further purification.

Rutin reference standard was purchased from Merck (Germany).

A total of five samples and one commercial lot of GM herb were analyzed:

- three samples were gathered in the Tuva Republic in August 2012, 2014 and 2016.
- two samples were gathered in Krasnoyarsk Krai in August 2016 and July 2017.
- One commercial lot of dry tea-like beverage "Gorechavka" (Gentian), manufactured by "Azbuka Trav" (Altai Krai) in 2016.

Taking available literature data on the chemical composition of large leaf gentian into account, the following extraction solvents were tries for extraction of biologically active substances from herbal raw material: 96% ethyl alcohol, 70% ethyl alcohol, 50% ethyl alcohol and 20% ethyl alcohol.

Optimal separation of chemical constituents is the key to proper TLC identification of herbal materials. Therefore, several mobile phases that can be utilized in phenolic compounds analysis were evaluated [6-9]:

- ✓ butanol - glacial acetic acid-water (4:1:1)
- ✓ butanol – water – glacial acetic acid (4:2:1)
- ✓ butanol – water – glacial acetic acid (5:2:1)
- ✓ ethyl acetate – water - glacial acetic acid – anhydrous formic acid (100:26:11:11)
- ✓ butanol – water – ethyl acetate (20:10:5)
- ✓ toluene – acetone - anhydrous formic acid (9:9:2)
- ✓ ethyl acetate - anhydrous formic acid – water – toluene (30:4:3:1,5)
- ✓ ethyl acetate - dichloromethane - water - anhydrous formic acid - glacial acetic acid (100:25:11: 10:10)
- ✓ *n*-propanol- glacial acetic acid-water (16:6:1)
- ✓ *n*- propanol - glacial acetic acid-water (18:6:2)
- ✓ *n*-propanol – anhydrous formic acid - water (5:5:5)
- ✓ *n*-propanol - anhydrous formic acid - glacial acetic acid-water (16:5:1:1)

A solution of the rutin reference standard (RS) in 96% ethyl alcohol was used as the standard solution. A 1% diphenylboryloxyethylamine solution in 96% ethyl alcohol and 5% polyethylene glycol (PEG) solution in 96% ethyl alcohol were used as spray reagents.

The following mobile phases that can be used in the analysis of lipophilic compounds were assessed for optimal separation of gentian chemical constituents [6-9]:

- toluene-ethyl acetate (95:5)
- hexane-ethyl acetate – glacial acetic acid (65:35:0,5)
- ethyl acetate-hexane - toluene - glacial acetic acid (65:35:20:0,5)
- toluene - hexane (80:20)
- chloroform – ethyl alcohol - glacial acetic acid (94:5:1)
- hexane-ethyl acetate (93:7)

In this case, sudan III solution in 96% ethyl alcohol was used as a standard solution. A solution of anisaldehyde in 96% ethyl alcohol was used as spray agent. The solutions were prepared according to the following procedures [7, 8].

Rutin RS Solution: About 0.005 g of rutine (rutine trihydrate) were dissolved in 10 ml of 96% ethyl alcohol. The solution should be stored in a cool place, protected from light, for not more than 3 months.

Sudan III RS solution: About 0.005 g of sudan III RS were dissolved in 10 ml of 96% ethyl alcohol. The solution should be stored in a cool place, protected from light, for not more than 3 months.

Diphenylboryloxyethylamine Solution in 96% thyl-alcohol: 1.0g of diphenylboryloxyethylamine (diphenyl boric acid-β-ethyl amino ester) were dissolved in 100 ml of 96% ethyl alcohol. The solution should be stored in a cool place, protected from light, for not more than 3 months.

Polyethylene Glycol Solution in 96% Ethyl Alcohol: Five milliliters of PEG 400 were mixed with 100 ml of 96% ethyl alcohol. The solution should be stored in cool place, protected from light, for not more than 6 months.

Anisaldehyde Alcoholic Solution: The following reagents were consequently mixed together: 0.5 ml of anisaldehyde, 10 ml of glacial acetic acid, 85 ml of 96% ethyl alcohol, and 5 ml of concentrated sulfuric acid. The solution should be stored in cool place, protected from light, for not more than 30 days

RESULTS AND DISCUSSION: The results of our study suggest that optimal extraction agent for GM is 96% ethyl alcohol, and optimal separation of phenolic constituents of the herb on 100 × 100 TLC Silica gel 60 F254 aluminum plates (MERCK, Germany) plates is achieved using ethyl acetate – water - glacial acetic acid – anhydrous formic acid (100:26:11:11) mobile phase. The samples were applied to the plate as bands with an automatic TLC applicator Linomat 5 (CAMAG, Switzerland)

The plates were placed in chromatographic chamber; after the mobile phase has passed about 80-90% of the length of the plate it was removed from the chamber and dried at room temperature until evaporation of mobile phase residues. The detection and photography were performed using CAMAG REPROSTAR 3 (CAMAG, Switzerland) under UV light (254 nm).

The chromatogram of rutin RS shows a single dark adsorption zone.

The chromatograms of tested GM extracts shows four dark adsorption zones below zone due to rutin RS, and three dark adsorption zones above zone due to rutin RS. Other adsorption zones may also be observed in **Fig. 1**.

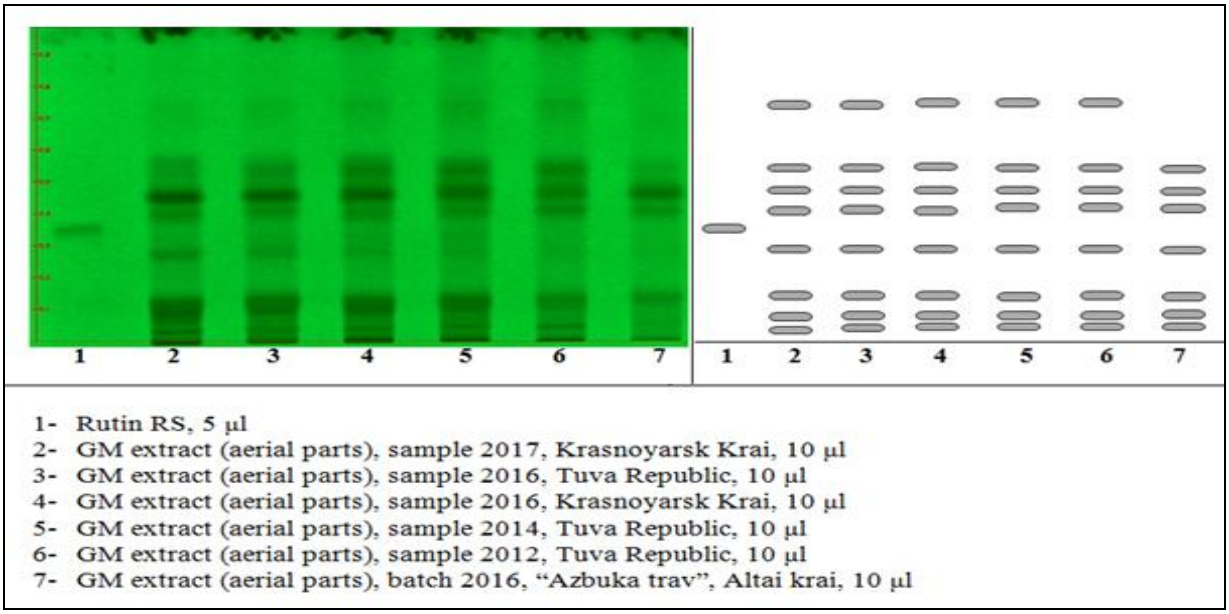

FIG. 1: THE CHROMATOGRAM OF PHENOLIC COMPOUNDS FROM GM EXTRACT IN UV LIGHT (254 nm)

After consequent spraying of the plate with 1% diphenylboryloxyethylamine solution, then with 5% PEG solution, it was placed in a temperature chamber and dried at 105-110 ºC for 2-3 min. The plate was detected and photographed in UV light (365 nm).

The rutin RS chromatogram reveals orange fluorescent adsorption zone.

The GM extracts chromatogram shows several zones below zone due to rutin RS: light blue to the blue zone, above that – grayish-green to dark green zone, then green to dark green zone, and light blue to blue zone.

There are also zones above that due to rutin RS: very intensively colored yellowish-green to green zone, above that – yellowish-green to the green zone, then light blue to green zone, another yellowish-green to green zone, and two zones with fluorescence from light blue to blue color. Other adsorption zones may also be observed **Fig. 2**.

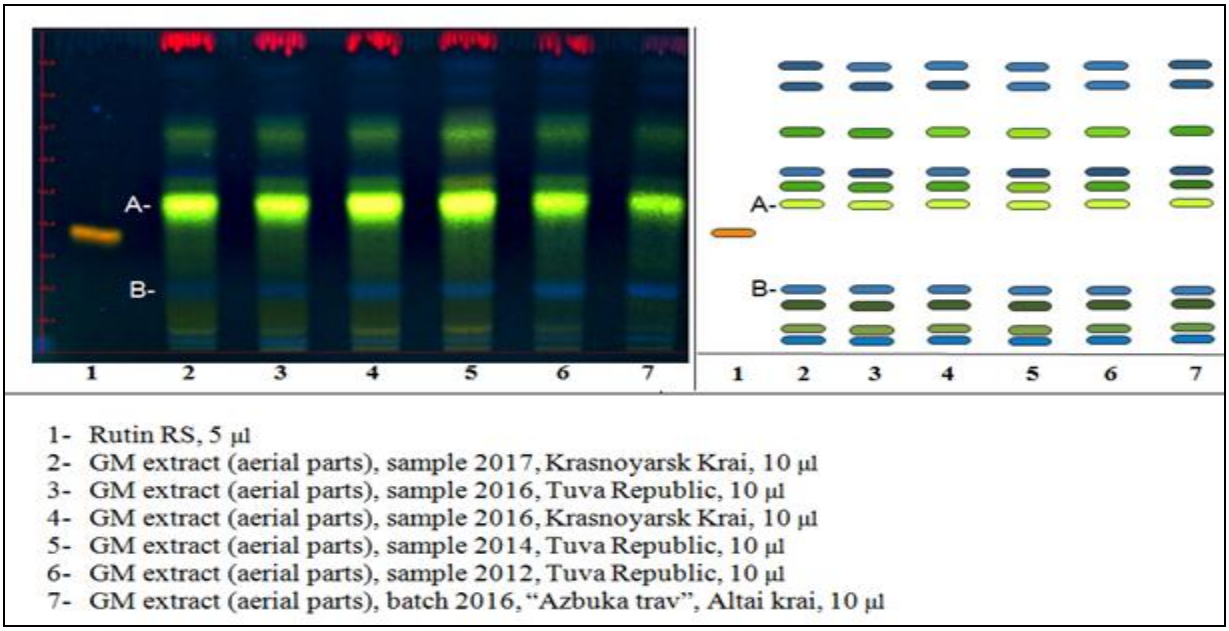

FIG. 2: THE CHROMATOGRAM OF PHENOLIC COMPOUNDS OF GM ALCOHOLIC EXTRACT IN UV LIGHT (365 nm) AFTER SPRAYING WITH 1% DIPHENYLBORYLOXYETHYLAMINE SOLUTION AND 5% PEG SOLUTION

As for lipophilic compounds of GM, our study revealed that their optimal separation in 96% ethanolic extract on 100 × 100 TLC Silica gel 60 F254 Aluminium plates (MERCK, Germany) plate is achieved using chloroform – ethyl alcohol - glacial acetic acid (94:5:1) mobile phase. The samples were applied to the plate as bands by an automatic TLC applicator Linomat 5 (CAMAG, Switzerland). After the mobile phase has passed about 80-90% of the plate length it was removed from the chamber, air-dried. Then detected and photographed by CAMAG REPROSTAR 3 (CAMAG, Switzerland) under UV light (365 nm).

The sudan III RS chromatogram shows single dark violet adsorption zone.

The chromatogram of GM extracts showed several zones below zone due to sudan III RS: light blue to blue, above that – grayish-blue zone, then 2 adsorption zones with red to dark red fluorescence, above it – light blue to the blue zone, then bright red zone, and light blue to blue zone. There is also a single bright red zone above that due to sudan III RS. Other adsorption zones may also be observed **Fig. 3**.

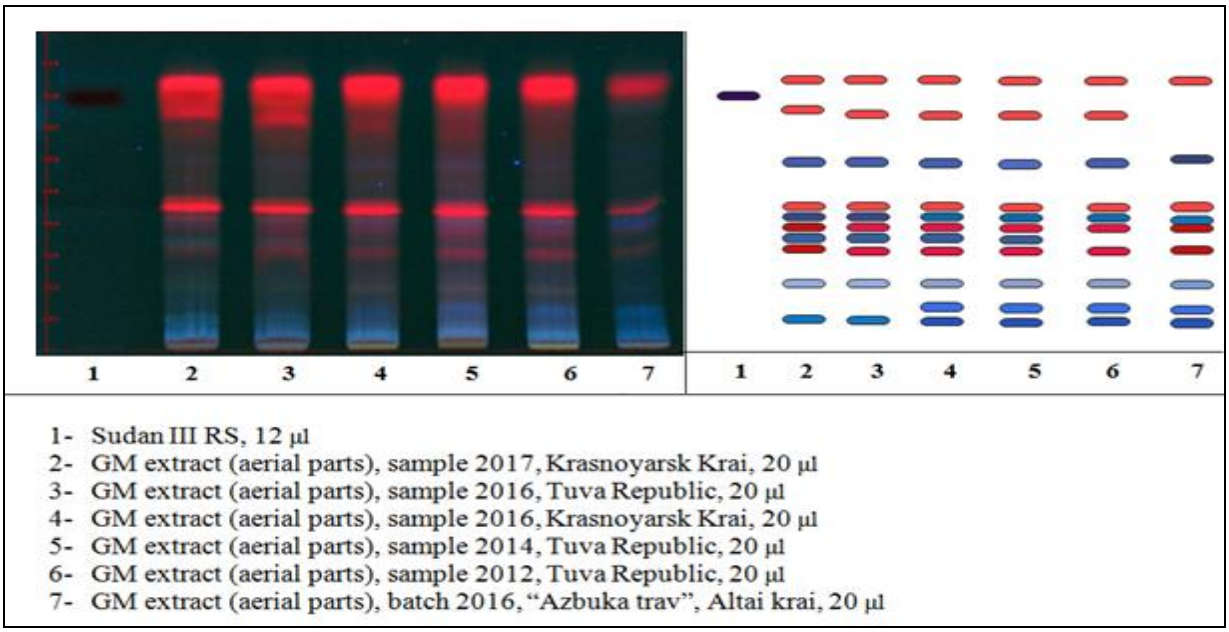

FIG. 3: THE CHROMATOGRAM OF LIPOPHILIC COMPOUNDS FROM GM EXTRACT UNDER UV LIGHT (365 nm)

After spraying with anisaldehyde, the plate was dried under fume hood, then heated in temperature chamber at 105-110 ºC for 2-3 min, detected and photographed in daylight. A blue adsorption zone due to sudan III RS can be observed on the chromatogram after spraying.

The chromatogram of GM extracts shows several adsorption zones below zone due to sudan III RS: a grayish-brown zone, above that – light yellow to yellow zone, then greenish-brown zone, violet zone, and light violet to violet zone. A violet to brown adsorption zone can be observed at the level of the zone due to sudan III RS. There is also a single pinkish-red zone above that due to sudan III RS. Other adsorption zones may also be observed in **Fig. 4**.

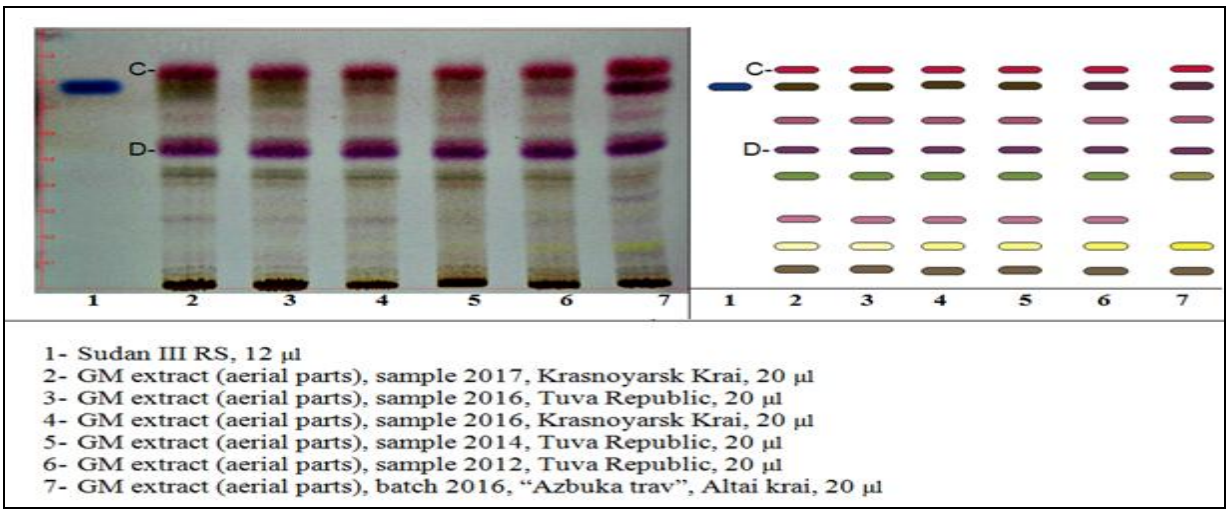

FIG. 4: THE CHROMATOGRAM OF LIPOPHILIC CONSTITUENTS OF GM ALCOHOLIC EXTRACT AFTER SPRAYING WITH ANISALDEHYDE (UNDER DAYLIGHT)

Validation of the developed procedure was carried out in terms of specificity and chromatographic system suitability. The specificity of the procedure was assessed by matching of TLC profiles of all studied samples (main zones on the chromatograms should match). All 6 objects of the study had similar TLC profiles, *i.e.*, main zones on the chromatograms were matched, as described in the procedure.

The resolution between characteristic zones A and B on the chromatogram of phenolic compounds **Fig. 2** was chosen as a suitability parameter for the chromatographic system. For lipophilic compounds, the resolution between zones C and D **Fig. 4** served as the suitability parameter.

Resolution between abovementioned zones was calculated according to the following formula:

$$R = 2(t_{R2} - t_{R1}) / W_{b1} + W_{b2}$$

Where:

t_{R1} – distance between start line and middle of zone B/D, mm; t_{R2} -distance between start line and middle of zone A/C, mm; W_{b1}, - distance between upper and lower margin of zone B/D (zone width), mm; W_{b2} - distance between upper and lower margin of zone A/C (zone width), mm.

Resolution values between zones A and B for phenolic compounds, and between C and D for lipophilic compounds, was not less than 1.5 **Table 1**.

The developed chromatographic procedure allows distinguishing specific zones in GM extracts by their color and relative position.

TABLE 1: CHROMATOGRAPHIC SYSTEMS SUITABILITY

Chromatographic System	Value				
	t_{R1}, (mm)	t_{R2}, (mm)	W_{b1}, (mm)	W_{b2}, (mm)	R
ethyl acetate – water – acetic acid – formic acid (100:26:11:11)	16	37	3	6	6,9
chloroform – ethanol – acetic acid (94:5:1)	44	68	3	4	4,7

CONCLUSION: A novel analytical procedure for the determination of lipophilic and hydrophilic compounds in large leaf gentian by TLC has been proposed and validated. For the first time, chromatographic characteristics of large leaf gentian aerial parts were established.

Obtained results can be included in the project of pharmacopoeial monograph for large leaf gentian aerial parts.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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