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Assessment of Chemical Composition of High Molecular Fractions from *Myosotis arvensis* and *Myosotis micrantha* (*Boraginaceae*) by Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Background: In our previous studies, we investigated water-soluble mucilaginous high molecular fractions (HMF) of medicinal plants *Symphytum asperum, S. caucasicum, S. grandiflorum, S. officinale, Anchusa italica, Cynoglossum officinale, Borago officinalis*, and *Paracynoglossum imeretinum* (*Boraginaceae* family). The water extracts of the aforementioned plants were fractionated using ultrafiltration with membrane filters having cut-off values of 1000 kDa or 500 kDa. This fractionation procedure allowed us to remove most polysaccharides and obtain water-soluble HMFs. The main chemical constituent of HMFs of plants described above was found to be biologically active unique caffeic acid-derived biomacromolecule poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] that is poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDHPGA).

Objectives: Within our ongoing search for biologically active biopolymers in plant species belonging to different genera of the *Boraginaceae* family, the present study aimed to isolate and investigate water-soluble high-molecular fractions (Mr>500 kDa) of roots for *Myosotis arvensis* (MA) and stems-roots for *Myosotis micrantha* (MM) (HMF-MA and HMF-MM, respectively) to study their main chemical constituents and carry out their structure elucidation.

Methods: As described in some earlier publications, HMF-MA and HMF-MM were isolated from water mucilage extracts using ultrafiltration with a membrane filter having a cut-off value of 500 kDa. Analyses of HMF-MA and HMF-MM were conducted using non-destructive physicochemical instrumental methods, including Fourier Transform Infrared Spectroscopy (FTIR), a method considered one of the most effective for identifying functional groups, and Nuclear Magnetic Resonance (NMR) techniques, which are powerful tools for elucidating the structure of chemical compounds.

Results: The NMR spectroscopy revealed that the main chemical constituent of HMF-MA and HMF-MM is a complex pectin-type polysaccharide.

Conclusions: Thus, the high-molecular fraction of *Myosotis arvensis* and *Myosotis micrantha* (Mr > 500 kDa) does not contain biologically active PDHPGA, and its main chemical component is a pectin-type polysaccharide, acetylated rhamno-arabino-galacto-galactopyranosyluronan, where carboxyl groups of galacturonic acid are partially methylated.

Keywords: Fourier Transform Infrared Spectroscopy (FTIR); Nuclear Magnetic Resonance (NMR); *Myosotis arvensis; Myosotis micrantha*; pectin; polysaccharide.

BACKGROUND

In our previous studies we investigated water-soluble mucilaginous high molecular fractions (HMFs) of medicinal plants *Symphytum asperum, S.caucasicum, S.grandiflorum, S. officinale, Anchusa italica, Cynoglssum officinale, Borago officinalis* and *Paracynoglossum imeretinum* (*Boraginaceae* family). The water extracts of these plants were fractionated using ultrafiltration with membrane filters having cut-off values of 1000 kDa or 500 kDa. This fractionation procedure enabled the removal of most polysaccharides, allowing for the isolation of water-soluble HMFs. The main chemical constituent of these HMFs was identified as a biologically active, unique caffeic acid-derived biomacromolecule poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], specifically poly[3-(3,4-dihydroxyphenyl) glyceric acid] (PDHPGA).[1-7] The structure of PDHPGA was elucidated through FTIR and various NMR spectroscopy techniques.[1-8]

PDHPGA exhibited a wide spectrum of biological activities due to numerous catechol groups covalently linked to the macromolecule's polyethylene glycol (PEG) backbone. PDHPGA consequently demonstrated immunomodulatory (anticomplementary), antioxidant, anti-inflammatory, wound-healing, antimicrobial, and anticancer properties.[1,2,8-13]

However, PDHPGA was not detected in certain species of the Boraginaceae family, including *Asperugo procumbens, Aegonichon purpurocaeruleum, Echium rubrum*, and *Lythospermum officinale*. Additionally, FTIR and NMR spectroscopy revealed it is necessary to emphasize that the main chemical constituent of HMF of Onosma sericea (Boraginaceae) was a novel poly[oxy-1-carboxy-2-(4-hydroxyphenyl)ethylene], specifically poly[3-(4-hydroxyphenyl)glyceric acid] (PHPGA). Another significant constituent of the HMF from *O.sericea* was found to be a complex pectin-type polysaccharide.[14]

It is also important to note that HMFs of the aforementioned plants, in addition to the significant component PDHPGA, contained minor amounts of residual complex polysaccharides, such as pectin-type acidic rhamno-arabino-galacto-galacturonan.[1-7]

Plant materials

The genus *Myosotis* belongs to the *Boraginaceae* family and includes about 100 species in Western Eurasia and New Zealand.[15]

The interest in herbal medicine has increased considerably in recent years, and the need to discover new sources of biologically active substances has prompted studies on previously unexplored objects. One such object is presented by plant species of genus *Myosotis L*. that do not find any application in official medicine, being at the same time used in folk medicine to treat epilepsy, respiratory apparatus diseases, malignant tumors of mouth and genitals, and tuberculosis.[15,16]

The medicinal properties of plants are determined by the presence of biologically active substances therein. According to the literature data, higher concentrations of fatty acids, alkaloids, saponins, anthocyanins, flavonoids, and essential oils have been reported in plant in plant species of the genus *Myosotis*.[15,16] However, detailed reports on the chemical constituents are scarce so far. It has already been demonstrated that essential oils can contribute to the therapeutic properties of plants, despite their small amounts. Among the significant biological activities of essential oils, antibacterial, antifungal, and anti-inflammatory properties are mentioned.[15]

Myosotis arvensis and *Myosotis micrantha* (*Boraginaceae*) were collected on 16.05.2023 in the surrounding area of Tbilisi (Georgia). The voucher specimens of *M. arvensis* (TBPH № 22357) and *M. micrantha* (TBPH № 22356) were deposited at the Tbilisi State Medical University I.Kutateladze Institute of Pharmacochemistry.

METHODS

Apparatus

The UV spectra were recorded using a UV/VIS spectrophotometer (Jasco V-730, Japan).

Fourier-transform infrared (FTIR) transmission spectra were obtained using an FTIR spectrophotometer (Jasco FT/IR-4600, Japan) with a KBr disc.

One-dimensional ^{1}H and ^{13}C NMR spectra were recorded for 1% solutions in D_2O at 353^0K with a Bruker Avance III 400 spectrometer (Uster, Switzerland) at operating frequencies of 400.13 MHz and 100.57 MHz, respectively. Acetone was used as internal standard ^{1}H ($C\underline{H}_3$) at δ_H 2.69 ppm and ^{13}C ($C\underline{H}_3$) at δ_C 31.45 ppm, relative to Me_4Si.

The ultrafiltration fractionation procedure was performed using a stirred ultrafiltration cell (Model 8200, Millipore Corporation, Billerica, MA, USA) with a Biomax-500 ultrafiltration disc (molecular weight cut-off, 500,000 NMWL).

Extraction and isolation of high-molecular fractions of *Myosotis arvensis* (HMF-MA) roots and *Myosotis micrantha* (HMF-MM) stems-root

23.45 g of air-dried and ground *M.arvensis* roots were preliminarily pretreated sequentially in a Soxhlet apparatus with chloroform, methanol, and acetone and afforded 19.77 g (84.3 %) roots. Quadruple hot water extraction of 10.59 g of preliminary pretreated roots yielded 800 mL of mucilage water extract, which was directly subjected to ultrafiltration and freeze-drying. The yield of HMF-MA was 0.06 g (0.48 %) based on air-dried biomass.

23.45 g of air-dried and ground *M.micrantha* stems-roots were preliminarily pretreated sequentially in a Soxhlet apparatus with chloroform, methanol, and acetone and afforded 18.76 g (81.3 %) stems-roots. Quadruple hot water extraction for 10.05 g of preliminary pretreated stems-roots afforded 800 ml of mucilage water extract, which was directly subjected to ultrafiltration and freeze-drying. The yield of HMF-MM was 0.11 g (0.89 %) based on air-dried biomass.

RESULTS

HMF-MA and HMF-MM did not exhibit any absorption peaks in the UV region. Based on our previous studies, the polyethylene glycol (PEG) chain serves as the backbone of the biomacromolecule PDHPGA, with catechol moieties as regular substituents along the PEG backbone.[1-8] Typically, catechol exhibits an absorbance peak at approximately 270–290 nm.[17] The absence of such absorption peaks in HMF-MA and HMF-MM suggests that these fractions do not contain the caffeic acid-derived biopolymer PDHPGA.

FTIR of HMF-MA and HMF-MM

The FTIR spectra of HMF-MA and HMF-MM samples were interpreted according to the literature data, and the assignments of their infrared absorption bands are reported below.[18-20] The strong and broad absorption peak at around 3400 cm^{-1} was attributed to O-H stretching vibration due to the galacturonic acid backbone's intra- and intermolecular hydrogen bonding. The band at 2925.5 cm^{-1} indicated a characteristic of C-H stretching vibration from -CH, -CH_2, and -CH_3, methyl esters of galacturonic acid in polysaccharide components. The strong band at 1732.7 cm^{-1} corresponded to the carbonyl (C=O) in the methyl-esterified group (-$COOCH_3$). Meanwhile, the strong bands at 1611 cm^{-1} and 1365.4 cm^{-1} represented the asymmetrical and symmetric stretching vibration of the carboxylate ion (COO^-), respectively. Thus, two peaks at 1732.7 cm^{-1} and 1365.4 cm^{-1} have been ascribed to the methyl esterified carbonyl groups (–$COOCH_3$) and the ionic carboxyl groups (COO^-) of galacturonic acid in the pectin, respectively. The intensities of the 1732.7 cm^{-1} and 1611 cm^{-1} absorption bands strongly suggested the high degree of esterification in pectin. In agreement with the above finding, an absorbance peak around 1200–1237 cm^{-1} has been assigned to the stretching and bending characteristic for -$COOCH_3$ of galacturonic acid, and another peak at about 1421.3 cm^{-1} has been associated with the carboxylate groups. The characteristic bending asymmetric vibration of $\delta as(CH_3)$ methyl group appears in the region of 1438.6 cm^{-1}, and the symmetric vibration of $\delta s(CH_3)$ acetyl group in the region of 1366 cm^{-1}. A combination of peaks at 1231 cm^{-1} and 1086.7 cm^{-1} can be assigned to the presence of rhamnogalacturonan

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(RG-I). The absorption bands in the fingerprinted region, overlapped bands observed in the 1211-900 cm^{-1} characteristics for stretching asymmetric vibration of pyranose C-O-C and are characteristic of the pectin backbone and side groups. The band at 1143.6 cm^{-1} is assigned to the C-O-C stretching vibrations of the α-1,4-D-glycosidic bonds in the homogalacturonan (HG) chains. The two bands, at 1086.7 and 1074 cm^{-1} in the IR spectra, result from neutral sugars in the side chains of RG-I and are assigned to the same stretching modes of L-arabinosyl and D-galactosyl units, respectively. The spectra observed between 1300 and 900 cm^{-1} corresponded to the ether R-O-R and cyclic C-C ring linkages of the pectin structure. Several peaks around 1143.6 to 1000 cm^{-1} have been associated with the glycosidic bond vibrations involving C-O-C, C-C (C-O), and O=C-H bending in the pyranose ring in polysaccharides. The presence of a peak at 829 cm^{-1} suggested the existence of α-glycosidic linkages, and a weak band near 745 cm^{-1} can be assigned to ring stretching and ring deformation of α-D-(1–4) linkages. In addition, a weak band near 665 cm^{-1} implied the presence of the galacturonan structures. The spectral region ranging from 800 to 1200 cm^{-1} has been generally recognized as the "fingerprint" region for different pectins.[18-20]

NMR of HMF-MA and HMF-MM

NMR spectroscopy is a powerful analytical technique for investigating synthetic and natural compounds in solution and solid state. It provides molecular-level insights by analyzing the behavior of the atomic nuclei in a magnetic field. Modern pulsed NMR methods facilitate the structural assignment and authentication of low-molecular-weight compounds while also enabling the interpretation of polymer spectra. This approach has significant potential for elucidating molecular fragments within complex mixtures. In the present study, the interpretation of NMR spectra and the present data were primarily based on previously published works.[21,22]

The chemical structures of the constituents in the HMF-MA and HMF-MM samples were identified by comparing their ^{1}H NMR and ^{13}C NMR spectra with those literature data.[23-27]

The ^{1}H and ^{13}C NMR spectra of HMF-MA and HMF-MM did not display signals characteristic of aromatic nuclei in the regions 6-7 ppm (^{1}H NMR) and 110-150 ppm (^{13}C NMR), respectively. Consequently, based on the NMR spectral data, the presence of poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDHPGA) was ruled out in these fractions. The principal signals observed in the ^{1}H NMR spectra of the main chemical component of HMF-MA and HMF-MM were 5.32, 5.20, 4.98, and 4.91 ppm, corresponding to the anomeric H1 centers of various sugars, including arabinose, rhamnose, galactose, and galacturonic acid fragments:

- →4)-α-GalpA-(1→ - (H1) 5.20 ppm;
- →2)-α-Rhap-(1→ - (H1) 5.32 ppm;
- α-GalpA-(1→(2) - (H1) 4.98 ppm;
- →2,4)-α-Rhap-(1→ - (H1) 5.20 ppm;
- →3)-β-Galp-(1→ - (H1) 4.91 ppm;
- Araf-(1→ - (H1) 5.32 ppm.

Additionally, the 1H NMR spectra of HMF-MA and HMF-MM exhibited signals corresponding to O-methyl protons of carboxylic acid methyl ester (COOC**H**$_3$) for D-galacturonic acid with a chemical shift of 3.8 ppm. The signals at 2.5 ppm and 2.67 ppm were attributed to the methyl protons of the O-acetyl groups (OCC**H**$_3$) attached to D-galacturonic acid. The former was assigned to 3-*O*-acetyl groups and the latter to 2-O-acetyl groups. The signals at 1.56 ppm and 1.77 ppm were respectively assigned to the methyl protons of rhamnose (Rha-C**H**$_3$) linked only at O-2 and to the rhamnose (Rha-C**H**$_3$) linked both at O-2 and O-4.

The ^{13}C NMR spectra of HMF-MA and HMF-MM revealed signals attributed to the anomeric C1 centers of various sugar fragments, including arabinose, rhamnose, galactose, and galacturonic acid, as well as the pyranoid rings of their derivatives (C2, C3, C4, C5) and C6:

- →4)-α-GalpA-(1→ - (C1) 101 ppm; (C 2,3,5) 71.6 ppm; (C4) 78 ppm;
- →3)-β-Galp-(1→ - (C1) 104.8 ppm; (C3) 82 ppm; (C6) 62 ppm;
- Araf-(1→ - (C1) 109 ppm; (C5) 62 ppm;
- →2)-α-Rhap-(1→ - (C1) 95 ppm; (C2) 78 ppm;
- →2,4)-α-Rhap-(1→ - (C1) 95 ppm; (C2) 78 ppm; (C4) 82 ppm.

Furthermore, the ^{13}C NMR spectra of HMF-MA and HMF-MM contained signals corresponding to the methyl carbon atoms of the *O*-acetyl groups (OC**C**H$_3$) attached to D-galacturonic acid at 20 ppm and 24 ppm. The former was assigned to 3-*O*-acetyl groups and the latter to 2-*O*-acetyl groups. The signals at 17 ppm and 19 ppm were respectively assigned to the methyl carbon atoms of rhamnose (Rha-C**H**$_3$) (C6) linked only at O-2 and to the methyl carbon atoms of rhamnose (Rha-C**H**$_3$) (C6) linked both at O-2 and O-4.[21-27]

DISCUSSION

The high-molecular fractions of *Myosotis arvensis* and *Myosotis micrantha* (Mr > 500 kDa) do not contain biologically active PDHPGA. Their primary chemical component is a pectin-type polysaccharide, acetylated rhamno-arabino-galacto-galactopyranosyluronan, in which the carboxyl groups of galacturonic acid are partially methylated. Pectin is a complex polysaccharide composed of a backbone of α-1,4-linked D-galacturonic acid and α-1,2-L-rhamnose, along with a significant proportion of neutral sugars, including arabinose, galactose, and smaller amounts of other monosaccharides. The structural classification of pectin includes homogalacturonan (HG), rhamnogalacturonan I (RG-I), and substituted galacturonans such as rhamnogalacturonan II (RG-II). It consists of up to 17 different monosaccharides and more than 20 distinct glycosidic linkages.[28,29]

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CONCLUSIONS

The main chemical constituent of high-molecular fractions of *Myosotis arvensis* and *Myosotis micrantha* (Mr> 500 kDa) represents the complex pectin-type polysaccharide, acetylated rhamno-arabino-galacto-galactopyranosyluronan. The carboxylic groups of galacturonic acid are partially methylated. This pectin-type polysaccharide consisted of a disaccharide repeating unit [→α-D-GalpA-1,2-α-L-Rhap-1,4→] backbone, with side chains containing branched arabinan and linear galactan.

AUTHOR AFFILIATION

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