European Journal of Medicinal Plants
2(3): 230-241, 2012

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Pharmacological Activities of a Mongolian Medicinal Plant, *Malva mohileviensis* Down.

G. Odontuya[1], G. Enkhmaa[1], N. Batbayar[1], R. Naran[1], K. T. Inngjerdingen[2], T. E. Michaelsen[2] and B. S. Paulsen[2*]

[1]Natural Product Chemistry Laboratory, Institute of Chemistry and Chemical Technology, Mongolian Academy of Sciences, 13330 Peace ave., 4th Building, MAS, Ulaanbaatar, Mongolia.
[2]Department of Pharmacognosy, School of Pharmacy, P.O. Box 1068, Blindern, 0316 Oslo, Norway.

Authors' Contributions

Author GO designed part of the study and performed the assays related to anti-inflammatory and DPPH scavenging effects together with GE and they wrote parts of the manuscript. NB managed the chemical analyses on the polysaccharide from the seeds. RN was involved in the writing process of the manuscript. KTI and TEM were responsible for the complement assay. BSP had the overall design of the project and was responsible for the final part of the writing process of the manuscript and literature search. All authors read and approved the final manuscript.

Research Article

Received 7th March 2012
Accepted 30th April 2012
Online Ready 6th June 2012

ABSTRACT

Aims: To investigate the Mongolian plant *M. mohileviensis* Down. for the presence of possible bioactive products that could be related to the traditional use of the plant in Mongolia.
Methodology: Ethanolic and water extracts of both seeds and herb were tested for anti-inflammatory and DPPH scavenging activity. Polysaccharides were isolated from the seeds using sequence of chromatographic methods. The polysaccharide fraction from the cold water extract was then analyzed for the presence of monosaccharides and their type of linkages by GC and GC-MS. The effect of the polysaccharides on the complement system was then determined.
Results: The ethanolic and aqueous extracts of seeds and herbs, as well as crude polysaccharides from cold and hot water extracts exhibited a significant anti-inflammatory

**Corresponding author: Email: b.s.paulsen@farmasi.uio.no;*

activity in the model based on histamine-induced paw edema. Anti-inflammatory effects of all samples were high during the 3rd hour of inflammation. Moreover, the ethanolic extracts of seeds and herbs exhibited DPPH scavenging activity. Phytochemical studies of the cold water seed polysaccharide revealed the presence of an uncommon pectic type polysaccharide. Galacturonic acid (38%) and rhamnose (30%) were present as the main monosaccharides, and linkage analyses revealed that galacturonic acid was present as terminal, 1→4 and 1→3,4 linked units and rhamnose basically as 1→3 linked units. The complement fixation activity was appr.15 µg/ml, substantially lower than that of the standard used. The results also indicate strongly that the *M. mohileviensis* and *M. verticillata* are two different *Malva* species.

Keywords: *Malva mohileviensis*; *pectic polysaccharide; complement fixation activity; anti-edema activity; DPPH radical scavenging activity.*

1. INTRODUCTION

In Mongolia there is a long tradition for the use of medicinal plants as part of their health system. These plants may be of importance for potentially new drug as described by Kletter et al. (2008).

The genus *Malva* (Malvaceae) is commonly called mallow in English. *Ca.* 20 species of *Malva* being herbs or shrubs and small trees are widespread in different geographical regions of the world (Cronquist, 1981).

M. mohileviensis Down. from the Mongolian flora, used in traditional medicine, has not yet been studied for its chemical constituents and possible biological activities. *M. mohileviensis* is an annual herb growing up to 150 cm high. Mostly the seeds of this species are used as a drug with diuretic and anti-edema activities, and are also said “to stimulate the general well being of the body” (Ligaa, 1996). Infusion made from flowers and leaves are used for the release of chest pain and for the treatment of inflammation of the respiratory system (Sokolov, 1990). Previously, only the fatty acid composition of the seeds, oleanolic acid, linoleic acid and stearinic acid, has been determined (Tsevegsuren, 1999).

Seed polysaccharides have not been studied extensively for their biological effect. It is interesting to note that the acidic polysaccharide fraction isolated from the seeds of *Sterculia lychnophorae* showed a potent dose-dependent anti-inflammatory activity when tested on ear edema induced by dimethyl benzene as well as on cotton pellet-induced granuloma tissue in murine models (Wu et al., 2007). Recently seed polysaccharides from *Camellia sinensis* were shown to inhibit the growth of K562 cells (Wei et al., 2011). None of these two investigations included structural aspects of the polysaccharides. The importance of pectic like polysaccharides as bioactive compounds have been described in a review by Yamada’s group (Yamada and Kiyohara, 2007) where they focus on the effect on the immune system.

Studies on *Malva* spp. have shown the presence of compounds, mainly polysaccharides that may influence the immune system (Drodzdova and Bubenchikov, 2005; Gonda et al., 1990b; Nosalòva et al., 1994; Tomoda et al., 1992; Yamada and Kiyohara, 2007).These studies include polysaccharides from the species *Malva verticillata*, a species that in some

databases indicate that *M. mohileviensis* and *M. verticillata* are the same plant, but a firm conclusion has not been reached yet (http//www.theplantlist.org/tpl/record/kew-2504078, accessed 05032012). Aspects related to what should be the correct name of the plant under study will be presented in this paper.

The DPPH-test is an anti-oxidative test, and can be related to the "well-being" of people and is chosen for possible effect of the low molecular weight extracts of the plant. The rat-paw test is relevant for the anti-oedema test of high molecular weight compounds, and if positive, the effect of the purified polysaccharide will be tested in the complement system in order to find out if this is involved in the anti-oedema effect. Partial structural elucidation of the possible bioactive polysaccharide will also take place in order to establish what type of polysaccharide that carries the activity.

2. MATERIALS AND METHODS

2.1 Plant Materials

Aerial parts and seeds of *M. mohileviensis* Down. were collected from Botanical garden, Ulaanbaatar, Mongolia, in July and middle of September 2001.

The plant was identified at the Department of the Plant Systematic in the Botany Research Institute of Mongolia and a voucher specimen is deposited at the herbarium of this Institute. Plant materials were dried at room temperature in the well-ventilated place.

2.2 Preparation of Ethanolic and Aqueous Extracts from Seeds and Herbal Material

Both ethanolic extract and water extract from both seeds and herbs of *M. mohileviensis* were prepared by the maceration method and dried by the rotary evaporator and followed by freeze drying. At the time of use each extract was resuspended in distilled water at the desired concentrations. All prepared extracts were tested for anti-edema and DPPH scavenging activity.

2.3 Anti-edema Effect on Histamine Induced Edema on Mice Paw

Anti-inflammatory activity of ethanolic and aqueous extracts of seeds and herbs as well as crude polysaccharides from seeds of *M. mohileviensis* has been studied on 160 experimental white mice, male, weighing 20.0-32.0g.

Edema in the left hind paw of mice was induced by injecting 0.1 ml of 1% histamine in saline into the footpad, subcutaneously (Zakirov and Karshiev, 2000; Winter et al., 1962). The paw volume of the mouse was measured before histamine injection and then after 30 minutes and 1, 2, 3, 4 and 5 hours, with a Plethysmometer-7140 (UGO, Basil, Italy).

Experimental mice were divided into 3 groups. The test group received orally ethanolic and aqueous extracts of seeds and herbs with doses 200 and 400 mg/kg, respectively, as well as cold and hot water crude polysaccharides from seeds at 20 mg/kg, 30 min before injection of histamine. The control group received distilled water, while the standard group was administered a solution of 0.3% amidopyrine.

The anti-edema effect was expressed by percentage,

$$Percentinhibition(\%) = \frac{A - B}{A} x\ 100;$$

Where, *A* - mean for the control group, *B* - mean for the treated group

2.4 DPPH Scavenging Activity

Anti-oxidative activity of ethanolic and aqueous extracts of seeds and herbs with different concentration was determined by scavenging of the DPPH radical. Four ml of a methanol solution of samples was added to 1ml $1.5x10^{-4}$ M DPPH in methanol. After standing at room temperature for 30 min, the absorbance was determined at 515 nm on the spectrophotometer Bio-Tek, Power Wave XS. The remaining DPPH was calculated compared to a standard ascorbic acid. The method is a modification of the one described by Brand-Williams, et al. (1995).

2.5 Extraction and Isolation of Polysaccharides from the Seeds

Air dried and powdered seeds (319g) were exhaustively extracted with of ethanol at room temperature and the residue of seeds dried. The dry residue (290g) was extracted twice with cold water (1.5 w/v) for 2 hours with stirring. The combined water extracts were filtered through glass fiber filter and concentrated using a rotary evaporator at a temperature not more than 40ºC and dialysed for 24 hours against distilled water. After dialysis the extract was precipitated by absolute ethanol obtaining a crude polysaccharide weighing 280 mg.

The seed residue after the cold water extraction was washed several times with 96% ethanol and dried again. Approximately 285g of the residue were extracted twice using hot water (1.5 w/v) at 100ºC for 2 hours with continuous stirring. The combined hot water extracts were treated as above. The hot water crude extract weighed 3.8 g after freeze drying.

280 mg of the cold water extract was purified by anion exchange chromatography. The crude extract was dissolved in 10 ml water and filtered through a Millipore 0.8 µm filter. This filtrate was applied onto a Pharmacia Biotech XK50 column containing DEAE- Sepharose Fast-flow anion-exchange material. At first the column was eluted with 1 L water followed by elution with NaCl of 0.5 M, 1 M and 2 M stepwise to get the acidic fractions. The profile of the polysaccharide was determined by the phenol-sulphuric acid test and the relevant fractions pooled (Dubois et al., 1956). The purified polysaccharide was used for phytochemical and complements fixating ability studies.

2.6 Determination of the Monosaccharide Composition

The monosaccharide composition of the cold water extracted polysaccharide was determined by GC. The polysaccharide sample (1-2 mg) was subjected to methanolysis with 4 M HCl in anhydrous methanol for 24 h at 80ºC. Mannitol was used as an internal standard (Reinhold, 1972; Barsett et al., 1992).

2.7 Determination of Linkages Present in the Polysaccharide

Prior to methylation, the galacturonic acids present in the sample were reduced to the corresponding neutral sugars essentially as described by Sims and Bacic, (1995).

Methylation of the polymer was carried out after the method of Kim and Carpita, (1992), followed by GC-MS analysis of the derived partially methylated alditol acetates (Barsett et al., 1992; Samuelsen et al., 1995).

2.8 Molecular Weight Determination

The approximate size of the isolated polysaccharide was determined by gel filtration on a Superose 6 column fitted into FPLC system of Pharmacia. The column was eluted with water at a flow rate of 30 ml/h. Fractions of 0.5 ml were collected with a Frac-100 Pharmacia fraction collector and the eluate was automatically monitored with a Shimadzu Refractive Index Detector 10A. The phenol sulphuric acid test was used to determine the profile of the polysaccharide eluted from the column. Dextran polymers (Pharmacia) of molecular weight 223, 98.4, 19 and 5.6 kDa were used as standards.

2.9 The Complement Fixation Test

The complement fixation test is an *in vitro* test for the ability of the extracts to interact with the complement cascade reaction (Michaelsen et al., 2000). Sheep erythrocytes were washed twice with 9 mg/ml NaCl and once with veronal buffer pH 7.2 containing 2 mg/ml Bovine Serum Albumin (BSA) and 0.02% sodium azide (VB/BSA) and sensitized (Virion amboceptor 9020, Ruschlikon, Switzerland). After incubation at 37ºC for 30 min on a shaker, the cells were washed as described above and a 12% cell suspension in VB/BSA was prepared. The human serum with intact complement proteins was pre-treated for removal of antibodies against sheep erythrocytes as described by Michaelsen et al. (2000). The serum was diluted by VB/BSA to a concentration that gave approximately 50% haemolysis.

Samples dissolved in VB/VSA (500, 250, 125, 62.5, 31.3, 15.0 µg/ml) (50 µl) and serum (50 µl) were added in duplicate to wells on a micro plate and incubated on a shaker at 37ºC for 30 min. Sensitized sheep erythrocytes (50 µl) were added and the micro titer plate was incubated as described above. After centrifugation at 1500 rpm for 50 min, 100 µl of the supernatants was transferred to a flat bottom micro titer plate for reading the absorbance (A) at 405 nm.100% lysis was obtained with distilled water and sensitized sheep erythrocytes (=A_{water}). The control of the medium was VB/VSA, serum and sensitized sheep erythrocytes (=$A_{control}$). The pectin fraction PMII from the leaves of *Plantago major* L. (Samuelsen et al., 1995) was used as a positive control.

The degree of lysis is given by the formula,

$$[Acontrol/Awater]\ x100\%;$$

The % inhibition of lysis is calculated by the formula,

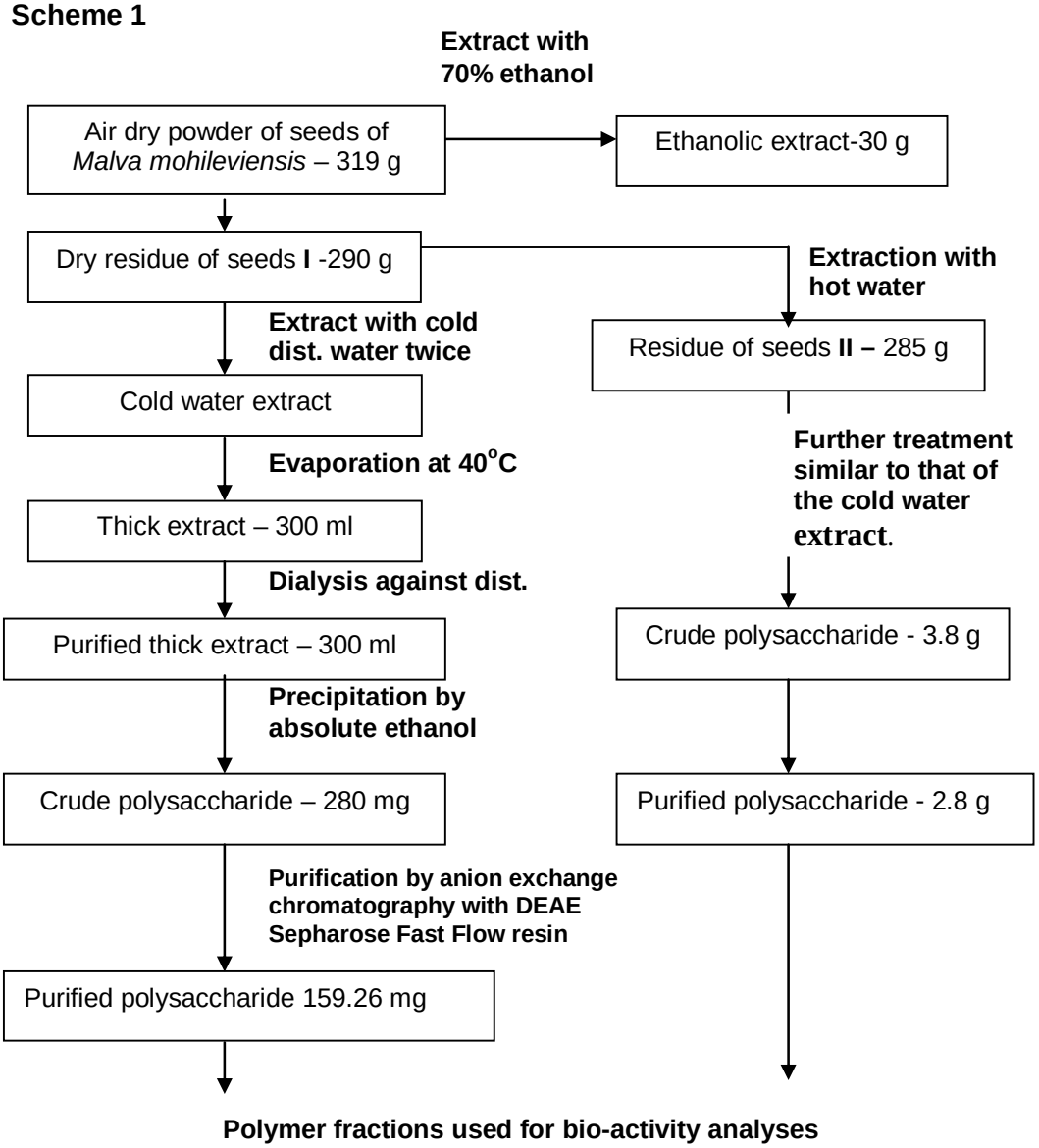
$$[(Acontrol - Asample)/Acontrol];$$

3. RESULTS AND DISCUSSION

3.1 Isolation of the Polysaccharides Present in the Seeds of *M. mohileviensis*

Air dried and powdered seeds (319 g) previously exhaustively extracted with ethanol, in which the low molecular weight substances were concentrated, were successively extracted with cold and hot water (100ºC) (Scheme 1) and gave respectively crude polysaccharides representing 0.1% and 1.2% of the total dry drug. Both polysaccharide fractions were further purified by anion exchange chromatography on DEAE Sepharose Fast-flow material obtaining one acidic fraction which was both used for bioassays.

Scheme 1



3.2 DPPH Scavenging Effect

The reactive oxygen species or free radicals can cause inflammation process, when produced in excess in living organisms.The results of the DPPH radical scavenging activity assay of ethanolic extracts of seeds and herbs are presented in the Table 1.

The different extracts showed a radical scavenging activity in a dose dependant manner. Ascorbic acid was used as a standard. Thus, it appears that *M. mohileviensis* has a good anti-oxidative effect.

Table 1. Comparative DPPH radical scavenging ability of *M. mohileviensis*

Test sample	% scavenging activity			
	200 «g/ml	100 «g/ml	20 «g/ml	2 «g/ml
Ascorbic acid	95±0.6	95±0.6	93±0.6	31±0.5
70% ethanolic extract of seeds	60±1.3	42±1.2	14±0.4	7±0.7
70% ethanolic extract of herbs	56±0.8	38±0.7	11±0.9	9±1.2

3.3 Anti-inflammatory Effect

Anti-inflammatory activities of the ethanolic and aqueous extracts of seeds and herbs of *M. mohileviensis,* as well as cold and hot water crude polysaccharides were studied by testing the inhibition of mice hind paw edema induced by histamine (Zakirov and Karshiev, 2000; Winter et al., 1962). The inhibition of edema gave different results depending on the type of sample analysed (Fig.1).

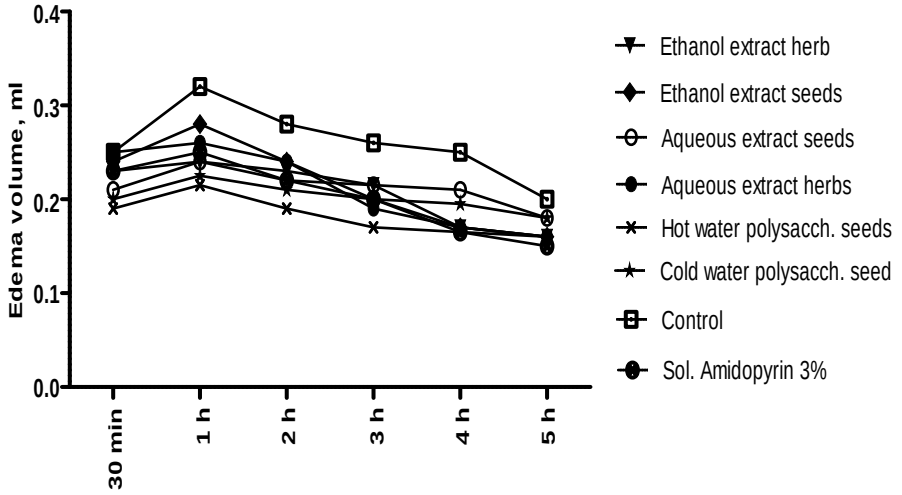


Fig. 1. Size decreasing effect of different extracts from *M. mohileviensis* on histamine induced paw edema by time

The ethanolic extracts of herbs and seeds at the dose 200 mg/kg, reduced the edema 35.3% and 33.3%, respectively, compared to control when estimated during the 3rd hour after start of the experiment. The aqueous extracts of the herbs and seeds at the dose 400 mg/kg also

demonstrated maximum reduction activity of 35.8% and 37.8%, respectively. Crude polysaccharides from cold and hot water extracts at the dose 20 mg/kg showed the best anti-edematogenic activity as 46.4% and 46.9%, respectively. These results indicate that the all investigated samples have anti-edema properties in the model using histamine-induced edema.

3.4 Chemical Characterization of the Polysaccharide from the Cold Water Extract of the Seeds of *M. mohileviensis*

As the cold water extract is the type of extract primarily used traditionally, it was decided to study only this fraction further. The monosaccharide composition of the cold water extracted polysaccharide fraction was determined by GC analysis (Table 2). Rhamnose (30 mol %), galacturonic acid (37.6 mol %), galactose (16 mol %) and arabinose (7 mol %) were the main compositional monosaccharides, which appear to be typical of acidic pectic type polysaccharides (Yamada and Kiyohara, 2007)). The other monosaccharides were present in negligible amounts.

Table 2. Monosaccharide composition (mol%) of the acidic polysaccharide isolated from the20ºC water extract of *M. mohileviensis* after purification on DEAE-Sepharose Fast flow anion exchange

Structural monosaccharides	Content, mol %
Arabinose	7.0
Rhamnose	30.0
Fucose	1.4
Xylose	1.6
Mannose	1.1
Galactose	16.0
Glucose	5.3
Galacturonic acid	37.6

The molecular weight of this polysaccharide was determined by the gel-filtration and estimated to be appr. 63 kDa.

The nature of the glycosidic linkages was determined after a sequence of chemical methods including reduction, methylation and hydrolysis followed by the preparation of partially methylated alditol acetates. These were analysed by GC-MS as described above and the results are given in Table 3.

Arabinose is mainly present as 1→5 linked (3.3%), rhamnose as 1→3 linked (22.5%), galactose as 1→6 (5%) and 1→3, 6 linked (5.5%), glucose as 1→4 linked (4.5%) and galacturonic acid as 1→4 linked (31.8%) units. Galacturonic acid is also responsible for parts of the branch points present in the pectic polymer, this being on position 3 of the otherwise 1,4 linked main chain unit. Mannose, fucose and xylose are present only as terminal sugars in minor amounts.

It is interesting to note that the polysaccharide isolated from the seeds of *M. mohileviensis* has rhamnose present with only 1→3 linkages. This is not a common type of linkage in pectins although it has been reported a few times before. One of the recent papers from our group focusing on Malian medicinal plants describe a pectic like polymer with equal amounts

of galacturonic acid 1→4 linked and rhamnose 1→3 linked (Austarheim et al., 2012). The discussion on the real name of *M. mohileviensis* is ongoing. It is anticipated that the plant name of *M. mohileviensis is* a synonym for the plant *M. verticillata*. Two papers refer to studies of the polysaccharide present in *M. verticillata* (Gonda et al., 1988 and 1990a) where they describe the polysaccharide as containing rhamnose linked 1→2 and 1→2,4. Since these studies also use GS-MS for the identification of the linkages in the polymer (comparable method to what is used in the present paper) the authors will suggest that the plant *M. mohileviensis* is a different species than *M. verticillata*.

Table 3. Relative amounts of linkages in the acidic polysaccharide from the 20ºC water extract of *M. mohileviensis* purified on DEAE Sepharose Fast Flow

Monosaccharides	Type of linkages	Compostion, mol%
	T	1.2
Arabinose	1→5	3.3
	1→2,5	1.2
	1→3,5	1.0
	T	7.5
Rhamnose	1→3	22.5
Fucose	T	1.4
Xylose	T	1.6
	T	1.9
Galactose	1→4	3.8
	1→6	5.0
	1→3,6	5.5
Mannose	T	1.0
Glucose	T	1.0
	1→4	4.5
	T	1.9
Galacturonic acid	1→4	31.8
	1→3,4	3.9

T = terminal non-reducing units

3.5 Complement Fixing Ability of the Acidic Polysaccharide Isolated with Cold Water

Since the effect of the water extracts on the rat paw model showed interesting results we wanted to investigate the polysaccharides present in these extracts on the complement system (Michaelsen et al., 2000).

Pectins are polysaccharides commonly present in the cell wall of plants, and some of these involve in the human immune system and other wound healing properties. Several of these from various plants have been shown to exhibit complement-fixating activity (Yamada and Kiyohara, 1989; Zhao et al., 1991; Paulsen and Barsett, 2005). The results of our studies are given in the Fig.2. The pectic polysaccharide from the cold-water extract had a complement fixation inhibition ability which was 5.3 times greater than the standard sample PMII, isolated from *Plantago major* (Michaelsen et al., 2000). The pectin PMII is used as a standard in the complement system as this is comparable in activity to the pectin polysaccharide used by professor Yamada´s group at the Kitasato Institute in Tokyo (Yamada and Kiyohara, 1989; Zhao et al., 1991).

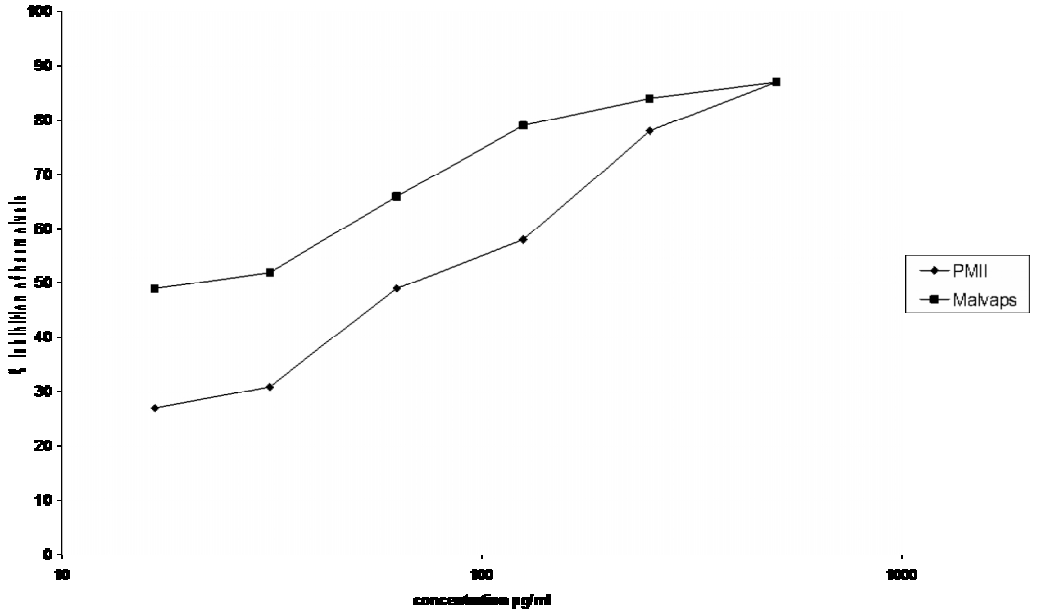


Fig. 2. A typical result of the complement fixation assay of the polysaccharide isolated from *M. mohileviensis* compared with the standard polysaccharide PMII. The concentration is in log scale.

4. CONCLUSION

According to our investigation the main substance of the cold water extracts of the seeds of *M. mohileviensis* is an unusual type pectic type acidic polysaccharide. It is composed of galacturonic acid and the neutral sugars rhamnose, arabinose, galactose, glucose, mannose, fucose and xylose and it exhibits a complement fixating activity. The 1→3 linked rhamnose units are not commonly found in pectic type polymers. Hence, it can be concluded that polysaccharides from seeds of *M. mohileviensis* are the main bio-active substances and their effects can substantiate the traditional use of this plant.

Moreover, ethanolic and aqueous extracts of seeds and herbs, in particular, cold and hot water crude polysaccharides, exhibited anti-inflammatory activity, and ethanol extracts contained antioxidants.

The results also indicate strongly that the *M. mohileviensis* and *M. verticillata* are two different Malva species.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Torun Aslaksen for valuable technical assistance, to Thomas Rundberget, The Veterinary Institute, Oslo, Norway, for assistance with the GC-MS experiments, and to the Norwegian Research Council project numbers 12948/730 and 149282/730 for financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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