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STUDIES ON PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY OF MONGOLIAN TRADITIONAL MEDICINAL PLANTS; *OXYTROPIS*, *BRACHANTHEMUM*, *CALLIGONUM*, AND *APOCYNUM* SPECIES

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Table of contents

Studies on phytochemical constituents and biological activity of Mongolian traditional medicinal plants; *Oxytropis*, *Brachanthemum*, *Calligonum*, and *Apocynum* species

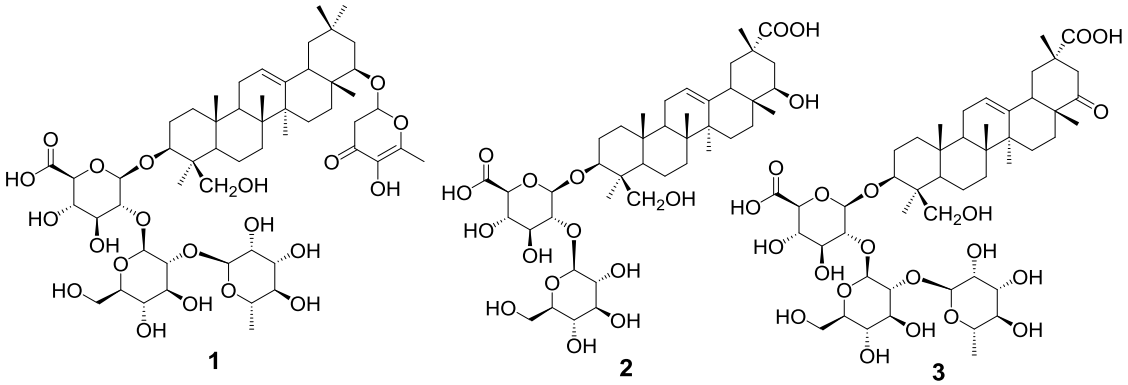
For many thousands of years, plants have been considered to be one of the major sources of food and medicines that are usually used as primary health care for humans. In this contemporary era, many researchers are paying their attention on the investigations of the medicinal plants for they might reach to success in the field of drug discoveries and developments in the future. Moreover, up to date, chemical constituents and bioactivities of numerous plant species which grow not only in Mongolia but also worldwide have not been investigated yet.

In addition, zoonotic diseases, including trypanosomosis, have been posing one of the big issues happening in livestock husbandry of Mongolia. Due to the consequences of the zoonotic diseases, financial losses have been widely observed in the animal industry which is vital sector of the Mongolian economy. Hence, studying highly effective compounds from Mongolian native plants is a really essential way to combat zoonotic diseases, especially trypanosomosis.

In this study, we focused on studying chemical constituents and biological activities of four plant species, named *Oxytropis lanata*, *Brachanthemum gobicum*, *Calligonum mongolicum*, and *Apocynum pictum*. Typically, this diploma thesis was written with five chapters that will be briefly explained below.

Chapter one generally covers medicinal plants growing in Mongolia and Worldwide and their brief history. The conditions of animal husbandry and zoonotic diseases in Mongolia and the overview of some biological activities were written in this chapter.

Chapter two describes phytochemical and biological investigations on the aerial parts of *Oxytropis lanata*, which belongs to the family Fabaceae. As a result of the phytochemical analysis, three new oleanane-type saponins (**1**, **2**, and **3**) and 33 known compounds, namely saponins, flavonoids, oxazole alkaloids, and glycosides, were obtained from aerial parts of this plant. In terms of compounds **28**, **29**, **33**, and **34**, they were previously isolated from the roots of *O. lanata*, while other compounds were reported for the first time to aerial parts. For trypanocidal screening, 5,7,4′-trihydroxyisoflavone (**25**) exhibited inhibitory activity against *T. congolense* (IC_{50} = 10.5 μM), the causative agent of African trypanosomosis in animals; this activity was little weak to compare with 2-(2′,3′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)oxazole (IC_{50} = 1.0 μM) isolated from roots of this plant. Furthermore, anti-hyaluronidase experiments of some compounds were carried out based on the traditional usage of *O. lanata* as used for the treatment of inflammatory diseases. Saponins, 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1 → 2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-en-22-oxo-29-oic acid (**3**) and dehydroazukisaponin V (**9**) showed potent inhibitory activity.

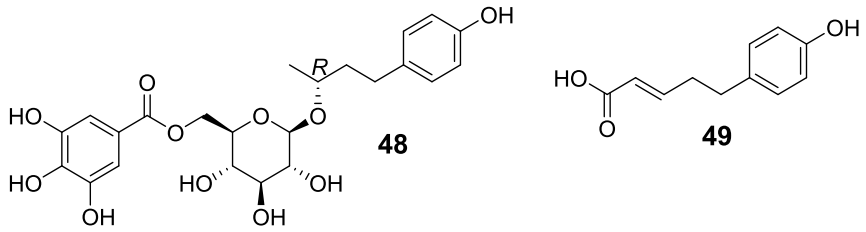


Chemical structures of previously unreported oleanane-type saponins isolated from the aerial parts of *O. lanata*

In **chapter three**, absolute configurations of compounds isolated from *Brachanthemum gobicum*, which included in the family Asteraceae, were discussed. Drs B. Odonbayar and T. Murata performed almost all of the experiments in this research work of *B. gobicum*. Consequently, 11 new and 40 known compounds were isolated from aerial parts of this plant. However, there were some compounds with the racemic mixture before purifying pure enantiomers by using chiral phase HPLC. After purification, absolute configurations of **37**, **38**, **39**, **41**, **44**, and **45** were elucidated by spectral data obtained from specific rotation and ECD spectra. For compounds **40**, **42**, **43**, **46**, and **47**, absolute configurations were not determined due to the inadequate yield of enantiomers. In the trypanocidal screening, compounds **37**, **38**, **39**, **40**, **44**, and **45** showed inhibitory activities. Among them, compounds **38** (IC_{50} = 2.8 μM) and **44** (IC_{50} = 2.4 μM) inhibited more efficiently than other compounds.

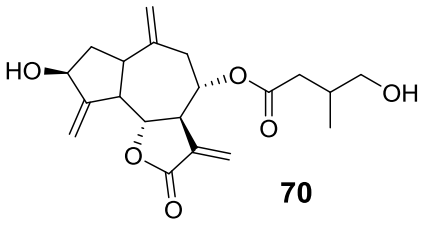
Chapter four presents phytochemical and biological investigations on the aerial parts of *Calligonum mongolicum*, which is one of the largest genera in the family Polygonaceae. As the result of the phytochemical studies, two new compounds, named *R*-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (**48**) and 5-(4-hydroxyphenyl) 2-pentenoic acid (**49**), and 20 known (**50**-**69**) compounds, including flavonols, flavanonols, flavanols, alkaloids, a monoterpenoid, a phenol, and glycosides, were isolated for the first time from this plant. Compound **48** was not reported previously to literature, while compounds **49** and **50** were newly obtained from a natural source. With regard to an examination of the trypanocidal activities, all of the isolated compounds did not show significant activity. In the anti-phenoloxidase screening, however, catechin (**54**) was roughly ten times higher phenoloxidase inhibitory activity (IC_{50} 9.1 μM) compared with epicatechin (**55**) (IC_{50} 148.3 μM). Generally, compounds **54** and **55** have

a similar molecular structure except for their stereochemistry. This result was supported by a reproducibility test using pure guaranteed authentic samples.



Chemical structures of new compounds isolated from the aerial parts of *C. mongolicum*

Chapter five illustrates phytochemical investigations of *Apocynum pictum*, which belongs to the family Apocynaceae. By doing phytochemical investigations on the aerial parts of *A. pictum*, one new (**70**) and 20 known compounds (**71**-**90**), including flavonoids, sesquiterpenoids, glycosides, and amino acids, were isolated. Therefore, these compounds were obtained from this species for the first time. Interestingly, no previous research on the genus *Apocynum* reported that quaiane-type sesquiterpenes are one of the chemical constituents in this genus.



Chemical structures of new compound isolated from the aerial parts of *A. pictum*

Lists of publications

1. **Buyankhishig B.,** Murata T., Suganuma K., Batkhuu J., Sasaki K., Hyaluronidase inhibitory saponins and a trypanocidal isoflavonoid from the aerial parts of *Oxytropis lanata*. *Fitoterapia* 145 (2020) 104608.
2. **Buyankhishig B.,** Murata T., Odonbayar B., Batkhuu J., Sasaki K., New compounds from the aerial parts of *Calligonum mongolicum*. *Phytochemistry Letters* 41. (2021) 147-151.
3. Odonbayar B., Murata T., Suganuma K., Ishikawa Y., **Buyankhishig B.,** Batkhuu J., Sasaki K., Acylated lignans isolated from *Brachanthemum gobicum* and their trypanocidal activity. *J. Nat. Prod*. 82 (2019) 774-784.
4. Banzragchgarav O., Murata T., Odontuya G., **Buyankhishig B.,** Suganuma K., Davapurev B., Inoue N., Batkhuu J., Sasaki K., Trypanocidal activity of 2,5-diphenoloxazoles isolated from the roots of *Oxytropis lanata*. *J. Nat. Prod*. 79 (2016) 2933-2940.

CHAPTER I. INTRODUCTION

1.1. Medicinal plants

From the earliest time, a number of plants are growing worldwide and play an essential role on the planet and all living things. For instance, they produce a wide variety of secondary metabolites that are considered to be important sources for pharmaceuticals, fine chemicals, flavors, and food additives (Pagare et al., 2015). Plant secondary metabolites are divided into several classes, including phenolics, alkaloids, saponins, terpenes, lipids, and carbohydrates, according to their biosynthesis origin and chemical structures (Hussein et al., 2019). They have been shown different kinds of biological activities, such as antibacterial, anticancer, anti-inflammatory, antioxidant, and anti-trypanosomal (Hussein et al., 2019). Artemisinin, which was isolated from *Artemisia annua* and used for the treatment of malaria, is one of the useful second metabolites (Woerdenbag et al., 1990).

Approximately 391,000 species of vascular plants are still recorded to science. Of them, 28,187 species are estimated as being of medicinal use in the world (Willis, 2017). In Mongolia, as of 2019, about 3,191 species are registered as vascular plants. Of these, over 1,100 species are currently considered as medicinal plants (Urgamal et al., 2019).

In many regions of the world, humans are still based on traditional medicine, specifically medicinal plants, for their primary healthcare. Traditional medicine has been used for thousands of years. To cite an example, it was proven by fossil record with 60,000 years old they used the eight medicinal plants in Iraq, including *Ephedra sinica* (Andrew, 2016). According to the World Health Organization (WHO), traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences and in the native plant to multi-cultures, whether explicable or not, used in

the maintenance of health as well as in the prevention, diagnosis, improvement, and treatment of physical and mental illness (WHO, 2019). As of 2018, 170 or 88% of WHO member states officially avowed that they have their own traditional medical systems. Amongst, Ayurveda, Unani, Kampo, Chinese Traditional Medicine, and Tibetan Traditional Medicines are increasing popularly (WHO, 2019; Andrew, 2016). A number of publications, roughly 14,884 by 2018, have been reported over the last decades, focusing on various medicinal plants and their many investigations, such as phytochemicals and biological activities (Fitzgerald et al., 2020). However, there have still been hypothetical undiscovered species and biological activities that may be a source of valuable bioactive ingredients.

Mongolian Traditional Medicine originated from Mongolian Folk Medicine has a history of more than 2500 years, and it was influenced by indigenous people's lifestyle such as herdsmen or nomads who have conventional cultural heritage, and by the harsh climatic and geographic conditions (Wurchaih et al., 2019; Pitschmann et al., 2013). Moreover, Tibetan Traditional Medicine and Ayurveda have been considered potent effects to expand the Mongolian traditional medical system (Wurchaih et al., 2019). Historically, the management of Mongolian Traditional Medicines was once prohibited from the early 1900s until 1990 due to communism. After the fall of communism in 1990, all constraints on Mongolian Traditional Medicine were completely lifted, and it began to redevelop (Pitschmann et al., 2013). According to the Ministry of Health of Mongolia, the merchandise of traditional medicines in 2018 was about 198 million tugriks (MHM, 2019). Data from WHO's reports indicated that, at present, roughly 40-59% of the population in Mongolia uses indigenous traditional medicine (WHO, 2019). Currently, many research works on Mongolian plants were carried out successfully and said to

achieve academic results. However, it is extremely necessary to proceed with further studies on Mongolian plants. It might influence drug discoveries and developments. Another most important reason is to explain scientifically the usage of medicinal plants.

1.2. Mongolian animal husbandry and trypanosomosis

From ancient times to the present day, livestock husbandry is still the mainstay of the Mongolian economy and part of the herdsmen's way of life. As of 2019, according to the census, roughly 70.9 million livestock animals were counted in Mongolia. Among them, 472.4 thousand heads of camel, 4.2 million heads of horses, 4.7 million heads of cattle, 32.2 million heads of sheep, and 29.2 million heads of goats were registered (MFALIM, 2020). In the meantime, zoonotic diseases have become one of the major issues that often happen to herdsmen and consumers depending on livestock and their products such as milk, meat, and by-products. Therefore, there have been huge financial losses in the livestock industry and agriculture.

According to the Ministry of Food, Agriculture, and Light Industry of Mongolia, at the end of 2018, approximately 8,740 livestock were infected with a total of 20 kinds of epidemic diseases; of them, about 26.6% or 2,325 head of livestock died (MFALIM, 2019). For instance, trypanosomosis is an infectious disease that is especially infected to horses and camels in Mongolia, so do widely to human and animals in other countries, such as Democratic Republic of the Congo, Zambia, and Republic of Tanzania. Commonly, it is caused by several species of the genus *Trypanosoma*, such as *T. congolense*, *T. equiperdum*, *T. evansi*, and *T. brucei* (Büscher et al., 2019). Depending on the affected species, this disease is used different names, such as dourine (*T. equiperdium*), surra (*T. evansi*), and nagana (*T. congolense* and *T. brucei*) (Büscher et al., 2019). *T. congolense* is the most prevalent and pathogenic trypanosome in sub-Saharan livestock.

This parasite is biologically transmitted by tsetse flies (*Glossina* spp.), and causes the severe African trypanosomosis called nagana in animals, particularly cattle (Giordani et al., 2016). In addition, the types of non-tsetse-transmitted animal trypanosomosis caused by infection with *T. evansi* (surra) and *T. equiperdium* (dourine) have been reported in Mongolia (Suganuma et al., 2016). It was proven by the research work carried out mainly at Obihiro University of Agriculture and Veterinary Medicine. They investigated 3,641 samples of horse sera collected in 19 regions of Mongolia from July 2014 to December 2017. Among them, 173 samples were seropositive for horse dourine (Mizushima et al., 2020).

At present, only six trypanocidal drugs, including diminazene aceturate and isometamidium chloride, have been licensed for the treatment of animal trypanosomosis, but adverse effects and drug-resistant cases have been described with almost all of them (Giordani et al., 2016). Investigation of the trypanocidal activity related to the native plants of Mongolia is crucial for the development of new treatments for these diseases.

Studying trypanocidal activities for compounds isolated from Mongolian medicinal plants is one of the main purposes of our research work. In terms of plants chosen to investigate, some compounds obtained from the roots of *O. lanata* and aerial parts of *B. gobicum* showed high activity, while those of *C. mongolicum* did not exhibit significant activity against *T. congolense*. Hence, some enzyme experiments based on the structural features of compounds were carried out for compounds known as a non-trypanocidal activity. Following two sections (1.3. and 1.4.) discussed more comprehensively these enzyme experiments.

1.3. Hyaluronidase

The hyaluronidase inhibitory experiment is considered as the first screening used to study anti-inflammatory and anti-allergenic activities because hyaluronidase is involved with the degranulation mechanisms (Kakegawa et al., 1985). Hyaluronidases are classified into enzymes that degrade principally hyaluronic acid during the tissue regeneration process. Hyaluronic acid is an essential component of the extracellular matrix of connective tissues such as cartilage and the synovial membrane and synovial fluid of joints (Bralley et al., 2007). It is synthesized by hyaluronic acid synthases on the surface of the cellular membrane as a single long-chain carbohydrate with high molecular weight. In addition, hyaluronic acid is considered one of the main players in the regulation of all phases of tissue remodeling, including not only inflammation but also cellular migration and angiogenesis (Litwiniuk et al., 2016). Its biological and structural roles depend on its molecular size that can reach 10^7 kDa. For instance, high molecular weight hyaluronic acid displays anti-inflammatory and immunosuppressive properties (Bralley et al., 2007). According to the literature (Gebrelibanos et al., 2014), by hydrolysing the constituents of connective tissue, hyaluronidase promotes the spread of inflammatory mediators throughout these tissues, thereby contributing to the pathogenesis of inflammatory diseases. Thus, the anti-hyaluronidase effect may be a good target in the search for the prevention and treatment of disorders caused by the inflammatory process.

1.4. Phenoloxidase

Phenoloxidase is one of the oxidative enzymes mostly obtained in microorganisms, arthropods, and plants. This enzyme is an essential component in an insect's immune system, and it plays an important role in eliminating pathogens by producing melanin (Stączek et al., 2020). These enzymes are present as zymogens in insect hemolymph

activated physiologically by a serine protease (Stączek et al., 2020; Odonbayar et al., 2016). Activated phenoloxidase can catalyze the conversion of mono- and di-phenolic substrates to dopaquinones. Asides from that, the enzyme induces melanization against invading bacteria and parasites (Decker et al., 2000). Currently, kojic acid, quercetin, and 4-hexylresorcinol have been regarded as significant phenoloxidase inhibitors. In the present investigations, phenoloxidase isolated from an aphid *Acyrthosiphon pisum* was used. *A. pisum* feeds on several species of legumes and is considered a model organism for various biological studies.

1.5. The goal of the research

Studying the medicinal plants using for various purposes of humans, such as for medicines and food over thousands of years, is really essential to explain scientifically their traditional usage, and it might highly influence drug discoveries and developments against numerous diseases. During the present studies, we chosen four plant species, named *Oxytropis lanata*, *Brachanthemum gobicum*, *Calligonum mongolicum*, and *Apocynum pictum*. These plants were selected based on their specific descriptions, including usages of traditional medicine, research situations on them, and distributions due to the fact, there is still a significant lack of research data on those plants.

On the other hand, investigating biological activities, especially the trypanocidal activity of isolated compounds from the above four plants, is hugely important because it can be lead to point out their uses in medical applications. Thus, our team primarily focused on studying trypanocidal activities for isolates as this experiment was one of the major aims of our research. But, compounds isolated from aerial parts of *O. lanata* and *C. mongolicum* did not show potent activity against *T. congolense*. Hence, some enzyme

experiments, named anti-hyaluronidase and anti-phenoloxidase, were performed for compounds obtained from these plants.

In our previous studies on the roots of *O. lanata*, all 17 compounds were isolated, and some of them showed potent trypanocidal activity. After knowing these results of the roots of *O. lanata*, we decided to do experiments on the aerial parts of *O. lanata* in order to search for new compounds for structure and high inhibitory activity against trypanosome species and hyaluronidase. The results of this study were described in **chapter 2**.

For *B. gobicum*, Odonbayar et al. have already done almost all of the significant investigations, except for some compounds with the racemic mixture. **Chapter 3** exhibited how enantiomers of these compounds were isolated, and how their absolute configurations were determined, and how trypanocidal activities of them were evaluated. Furthermore, in **chapters 4** and **5**, phytochemical studies of *C. mongolicum* and *A. pictum*, especially isolation of main compounds and their chemical structure identification, were discussed. In terms of the isolated compounds from *C. mongolicum*, insect phenoloxidase inhibitory tests were performed on them.

CHAPTER II. CHEMICAL CONSTITUENTS OF AERIAL PARTS OF *OXYTROPIS LANATA* AND THEIR HYALURONIDASE INHIBITORY ACTIVITY AND TRYPANOCIDAL ACTIVITY

2.1. Introduction

Figure 1. *Oxytropis lanata*

Scientific classification:
Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: *Oxytropis*
Species: *O. lanata*

Currently, according to the data, approximately 450 *Oxytropis* species are registered worldwide (Malyshev, 2008); of these, roughly 99 *Oxytropis* species are recorded in Mongolia (Urgamal et al., 2016). *Oxytropis* is considered to be one of the largest genera in the family Fabaceae, and it is distributed predominantly in Central Asia and Southern Siberia (Malyshev, 2008).

The reviews of literary sources have been described numerous phytochemical and biological investigations carried out successfully on various species of *Oxytropis* (Amirkhanova et al., 2018). For instance, it was demonstrated by several studies on *Oxytropis falcata*. At the present time, about 91 different flavonoids, namely isoflavone, flavanone, flavonol, dihydrochalcone, homoisoflavonoid, chalcone, chalcone dimers, and pterocarpans, have been obtained from this plant (Zhang et al., 2019). Therefore, some of these secondary metabolites have been showing a wide variety of bioactivities, such as

antioxidant, antibacterial, anti-inflammatory, anti-cardiovascular disease, hemostatic effects, analgesic, and antitumor. In addition, the *Oxytropis* genus contains not only flavonoids but also alkaloids, essential oils, terpenoids, saponins, and phenolic compounds found from this genus (Amirkhanova et al., 2018).

Oxytropis lanata is a herbaceous perennial plant with softly lanate, still 30 cm tall. They grow principally in the fine forest on sandy soil, sands in river valleys, and stony slopes (Ligaa et al., 2005). As in the literature (Ligaa et al., 2005), *O. lanata* has potential medicinal benefits in traditional Mongolian and Tibetan medicine due to the fact that it has been regularly used to treat bone fractures, fever from anthrax, bacterial fever, inflammation, bleeding, and wounds.

Looking back to ten years ago, Olennikov and Rokhin primarily investigated the seeds of this plant. They detected the polysaccharide, called galactomannan, which consists of mannose and galactose residues (Olennikov et al., 2010). Soon afterward, our research team began to study chemical constituents and their trypanocidal activity on the roots of *O. lanata*. After a while, it resulted in the isolation of eleven 2,5-diphenyloxazoles and six isoflavonoid derivatives. Among them, seven of the oxazole derivatives were reported as new. The oxazoles contained more than two hydroxy groups in their phenyl rings exhibited trypanocidal activity. From them, 2-(2′,3′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)oxazole has shown potent inhibitory activity against *T. congolense* (Banzragchgarav et al., 2016).

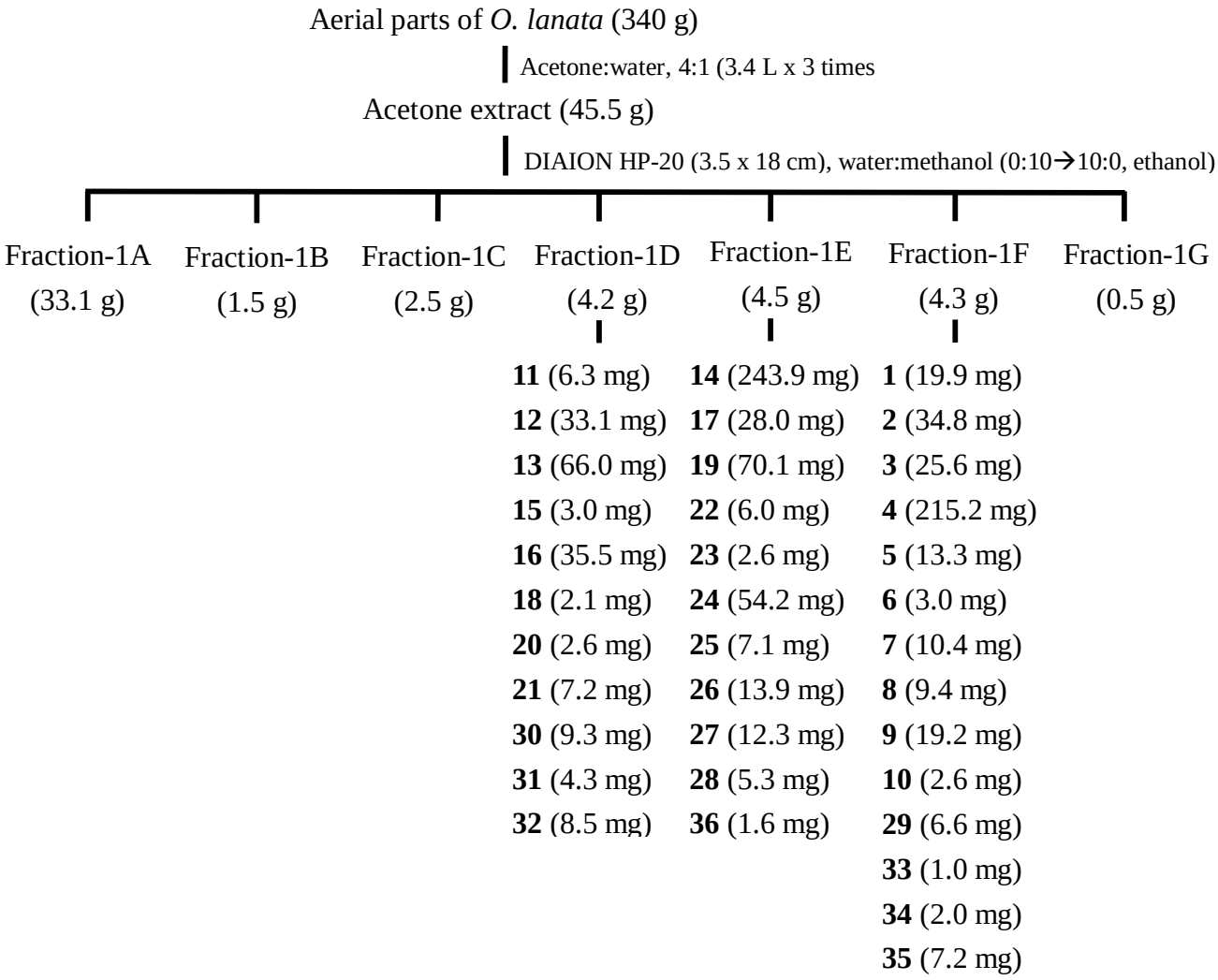
In the present report, we noted previously unreported three saponins and 33 known compounds obtained from the aerial parts of *O. lanata*. The spectroscopic data analyses played a really essential role in elucidating the structures of isolated compounds. Moreover, the trypanocidal and hyaluronidase inhibitory effects were estimated on these

isolated compounds.

2.2. Results and discussion

2.2.1. Extraction and isolation

Scheme 1. Extraction and isolation of aerial parts of *O. lanata*



Around six years ago today, we began to focus on the investigations of chemical characterization and biological activity, particularly trypanocidal screening, for roots of *O. lanata*. As a result of that study, 2,5-diphenyloxazoles and their derivatives with trypanocidal activity were isolated (Banzragchgarav et al., 2016). Thus in the present work, we were continued chemical investigations of aerial parts of *O. lanata* to find more

prospective trypanocidal compounds. Firstly, acetone–water (4:1) (46 g) extract obtained from the aerial parts of *O. lanata* was separated by column chromatography to isolate and identify previously unreported oleanane-type saponins and other known compounds. Afterward, further purifications were performed by preparative HPLC with various solution systems in order to obtain 36 pure compounds, including oleanane glycosides (**1**–**9**), flavonoids (**10**–**29**), and oxazoles (**33** and **34**).

2.2.2. Identification of known compounds

The compounds **1-9** were all isolated as colorless powders and elucidated oleanane-type triterpene glycosides and their derivatives based on their ^{1}H and ^{13}C NMR spectra. Totally, about 24 saponins that exhibited various biological activities, such as antibacterial (Sonfack et al., 2019), hepatoprotective (Udayama et al., 1998), and anti-inflammatory activities (Lee et al., 2010), have been reported as one of the constituents of *O. glabra*, *O. bicolor*, and *O. falcate* (Li et al., 2012). Moreover, many saponins have been reported to show hyaluronidase inhibitory activity, which is used for screening of anti-inflammatory activity as hyaluronic acid and hyaluronidase are involved in inflammation mechanisms.

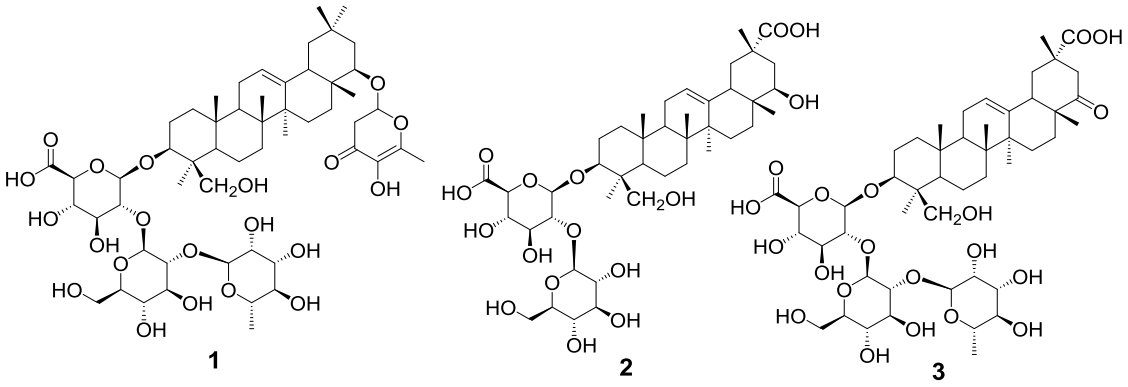


Figure 2. Chemical structures of previously unreported oleanane-type saponins

The structures of known compounds were identified as azukisaponin V (**4**) (Tsunoda et al., 2008), robinoside B (**5**) (Cui et al., 1992), 3-*O*-[β-D-glucopyranosyl-(1′′-2′)-β-D-

glucuronopyranosyl] soyasapogenol B (**6**) (Kitagawa et al., 1983), robinoside F (**7**) (Cui et al., 1993), caraganin B (**8**) (Zheng et al., 2013), dehydroazukisaponin V (**9**) (Tsunoda et al., 2008), kaempferol (**10**) (Chang et al., 2000), kaempferol 3-glucopyranoside (**11**), quercetin 3-glucopyranoside (**12**) (Kazuma et al., 2003), quercetin 3-glucuronide (**13**), isorhamnetin 3-glucuronide (**14**) (Needs et al., 2006), quercetin 3-*O*-β-D-glucuronide-6′′-methyl ester (**15**) (Cho et al., 2012), isorhamnetin 3-*O*-β-D-glucuronide-6′′-methyl ester (**16**) (Mezache et al., 2009), kaempferol 3-*O*-(6′′-*O*-malonyl)-β-glucopyranoside (**17**), quercetin 3-*O*-(6′′-*O*-malonyl)-β-glucopyranoside (**18**), isorhamnetin 3-*O*-(6′′-*O*-malonyl)-β-glucopyranoside (**19**) (Wald et al., 1989), kaempferol 3-rutinoside (**20**), quercetin 3-rutinoside (**21**), isorhamnetin 3-rutinoside (**22**) (Kazuma et al., 2003), naringenin 4′-*O*-β-D-glucopyranoside (**23**) (Yamamoto et al., 2004), dihydrokaempferol 4′-*O*-β-glucopyranoside (**24)** (Matlawska et al., 1999), 5,7,4′-trihydroxy isoflavone (**25**) (Murthy et al., 1986), genistein-7-yl β-glucopyranoside (**26**) (Al-Maharik et al., 2008), 6′′-*O*-malonylgenistin (**27**) (Yerramsetty et al., 2011), arizonicanol A (**28**) (Lambert et al., 2005), 3,9-dimethyl-10-hydroxy-pterocarpan (**29**) (Lotti et al., 2010), isophentyl β-D-glucopyranoside (**30**) (Kurashima et al., 2004), benzyl alcohol glucopyranoside (**31**) (Seigler et al., 2002), 2-phenylethyl β-glucopyranoside (**32**) (Yoneda et al., 2008), 2-(3′-hydroxyphenyl)-5-(2′′-hydroxyphenyl)-oxazole (**33**), 2-(2′,5′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)-oxazole (**34**) (Banzragchgarav et al., 2016), (+)-(9*Z*,11*E*,13*S*,15*Z*)-octadeca-9,11,15-trien-13-olide (**35**) (Schulz et al., 2007), and L-tryptophan (**36**) (Yan et al., 1999) by comparing their MS, ^{1}H and ^{13}C NMR data with those in the literature.

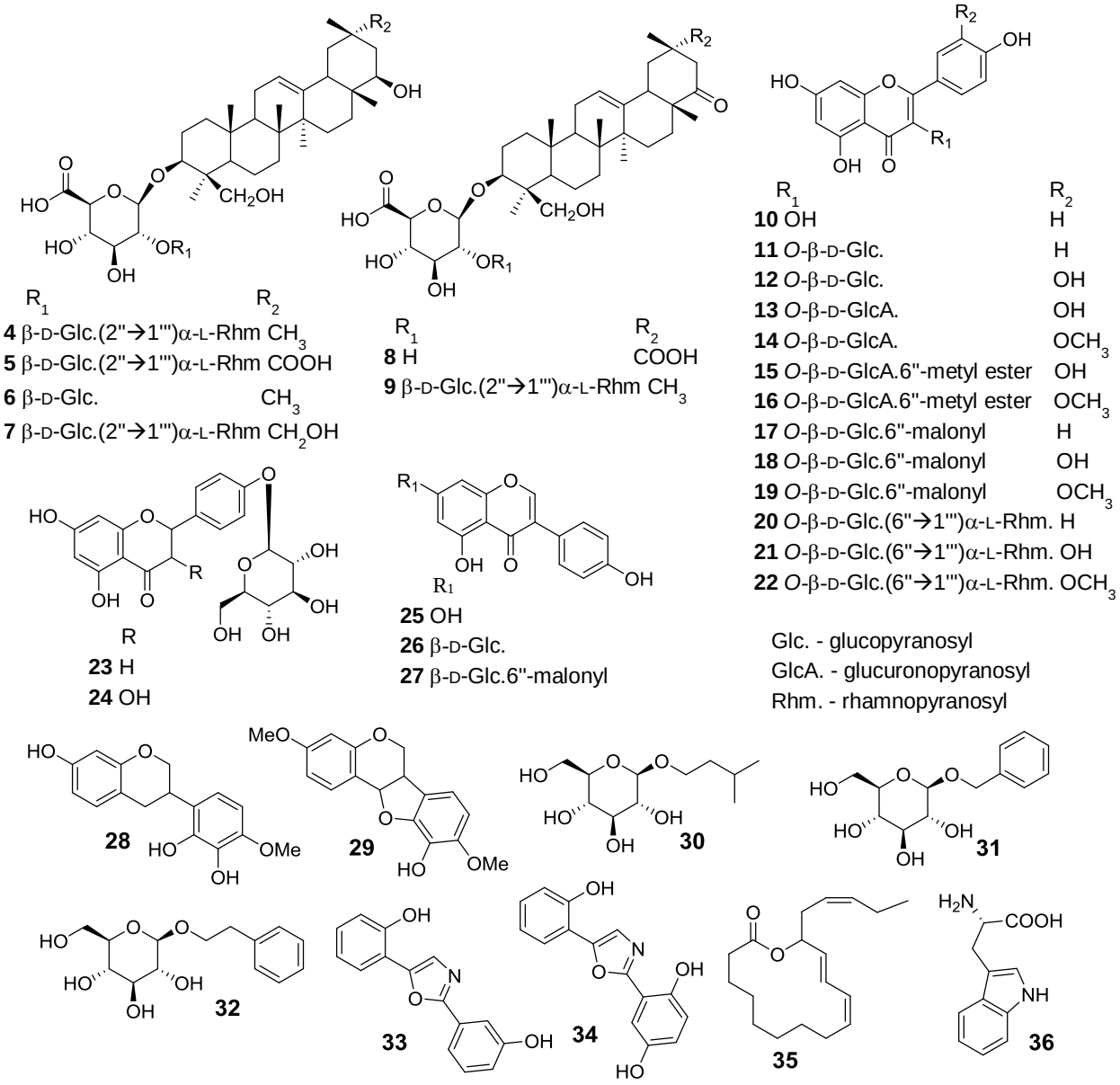


Figure 3. Chemical structures of known compounds isolated from *O. lanata*

2.2.3. Structural elucidation of new compounds 1, 2, and 3

The previously unreported below compounds contained oleanane-type triterpene aglycone and β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl(1→) sugar moiety as NMR spectral features in their structures. The most NMR spectra, especially ^{1}H and ^{13}C, were recorded for them in methanol-d_4 (Table 1) and pyridine-d_5 (Table 2) as solvents.

Compound **1** showed the molecular formula $C_{54}H_{84}O_{21}$ based on the ion $[M–H]^-$ at

m/*z* 1067.5401, calcd for $C_{54}H_{83}O_{21}$ (1067.5428), in its HRFABMS. The ^{1}H NMR spectrum showed an olefinic hydrogen resonance at δ_H 5.20 (H-12) and seven singlet methyl resonances at δ_H 1.23 (H_3-23), 1.16 (H_3-27), 0.98 (H_3-26), 0.97 (H_3-28), 0.90 (H_3-29), 0.88 (H_3-25), and 0.82 (H_3-30), which indicated the characteristic of an oleanane-type skeleton. According to the long-range correlations spectrum between H_3-23 and C-3 (δ_C 93.0), C-4 (δ_C 44.6), C-5 (δ_C 57.4), C-24 (δ_C 64.2); H_3-25 and C-5 (δ_C 57.4); H_3-26 and C-7 (δ_C 34.0), C-8 (δ_C 42.4); H_3-27 and C-8 (δ_C 42.4) C-13 (δ_C 145.3); H_3-28 and C-19 (δ_C 47.2), C-20 (δ_C 31.4), C-21 (δ_C 37.3), C-29 (δ_C 33.9); H_3-29 and C-19 (δ_C 47.2), C-20 (δ_C 31.4), C-21 (δ_C 37.3), C-28 (δ_C 28.3); H_3-30 and C-16 (δ_C 28.8), C-17 (δ_C 38.1), C-18 (δ_C 45.9), C-22 (δ_C 83.2) observed in the HMBC, it was supported the presence of the oleanane-type triterpene aglycone (Figure 4). Moreover, the ^{13}C NMR spectra for aglycone of **1** were in good agreement with those of the aglycone moiety of chromosaponin I (Tsurumi et al., 1992), which suggested that the aglycone of **1** is [3′-hydroxy-2′-methyl-5′,6′-dihydro-4′-pyrone(6′→)]-3β,22β,24-trihydroxyolean-12-ene.

On the other hand, the ^{13}C NMR data for the sugar moiety were almost the same as those of 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl(1→)] (Arao et al., 1997). In addition, the ^{1}H NMR [δ_H 5.39 (1H, dd, *J* = 4.0, 3.5 Hz), 2.92 (1H, dd, *J* = 17.0, 4.0 Hz), and 2.51 (1H, dd, *J* = 17.0, 3.5 Hz)] and ^{13}C NMR (δc 187.8, 155.9, 134.5, 98.5, 41.1, and 15.6) spectral data indicated the presence of 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety (Massiot et al., 1992). Generally, its ^{1}H and ^{13}C NMR shifts were closely analogous to those of **4,** except for DDMP's resonances. Compound **4** was assigned as azukisaponin V by comparison of its optical rotation and ^{1}H and ^{13}C NMR data with the literature data (Udayama, 1998; Tsunoda, 2008; Arao, 1997). The ROE correlation was detected

between H-24 and H_3-25, indicating a β-orientation of CH_2OH at C-24, and the other correlations suggested the relative configurations of **1** shown in Figure 4 (Schmid et al., 2018). The sugar analysis result indicated that **1** presented D-glucuronic acid (GlcA), D-glucose (Glc), and L-rhamnose (Rha). The coupling constants of GlcA and Glc (J = 7.0~7.5 Hz) and of Rha (J = 1.5 Hz) exhibited the β and α configurations, respectively. The HMBC correlations observed from H-GlcA-1 (δ_H 4.46) to C-3 (δ_C 93.0), from H-Glc-1 (δ_H 4.92) to C-GlcA-2 (δ_C 78.4), and from H-Rha-1 (δ_H 5.22) to C-Glc-2 (δ_C 78.7) proven that the sugar moiety is 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl(1→)]. Based on the above evidence and comparisons, the structure of **1** was identified as shown in Figure 4.

The HRFABMS analysis of **2** afforded a molecular formula of $C_{42}H_{66}O_{16}$, based on a deprotonated molecular ion peak $[M–H]^-$ at m/z 825.4283, calcd for $C_{42}H_{65}O_{16}$ at m/z 825.4274. The ^{1}H NMR spectrum (methanol-d_4) showed an olefinic hydrogen resonance at δ_H 5.29 (H-12) and six singlet H_3 resonances at δ_H 1.30 (H_3-28), 1.20 (H_3-23), 1.13 (H_3-27), 0.97 (H_3-26), 0.89 (H_3-25), and 0.83 (H_3-30). Compared to the ^{1}H NMR data in aglycone of **2** with those of **1**, it clearly appeared that the H_3-29 proton resonance was not observed. Moreover, in the HMBC spectrum, long-range correlations were observed from H_3-28 to the carbonyl carbon atom at δ_C 182.4 (C-29) and from H_3-30 to C-22 (δ_C 76.5), indicating the presence of a 3β,22β,24-trihydroxyolean-12-en-29-oic acid moiety as the aglycone. For the sugar portion, compound **2** exhibited resonances corresponding to two anomeric protons at δ_H 4.51 (1H, d, J = 7.0 Hz) and 4.77 (1H, d, J = 8.0 Hz). On the basis of ^{1}H–^{1}H COSY, HMQC, and HMBC data, sugar units were determined as β-D-glucuronopyranosyl and β-D-glucopyranosyl, and these units were suggested by HPLC sugar analysis. The determination of the sequence and linkage sites was obtained from

HMBC correlations between the proton resonance at δ_H 4.51 (GlcA-1) and carbon resonance at δ_C 92.5 (C-3), and proton resonance at 4.77 (Glc-1) with carbon resonance at δ_C 80.7 (GlcA-2). Furthermore, the ^{13}C NMR spectra of **2** (pyridine-d_5) were in good agreement with those of the previously reported aglycone (Cui et al., 1992) and sugar (Udayama et al., 1998) moieties of literature data. Compound **2** was a derivative of robinioside B (**5**), and it differed from that of **5** only for the absence of a rhamnopyranosyl moiety (Cui et al., 1992). The ROE correlations were detected between H-18 (δ_H 2.07, 1H, m) and H_3-28 and H_3-30, indicated the 17*R*, 18*S*, and 20*S* configurations. Besides, the presence of ROE correlations from H-3 to H-5 and from H_3-23 to H-3 and H-5 confirmed the 3*S*, 4*S*, and 5*R* configurations (Schmid et al., 2018). A comparison with the data described for the 20*S* and 20*R* compounds ((Takeshita et al., 1991) demonstrated its 20*S* configuration. Consequently, **2** was established as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3β,22β,24-trihydroxyolean-12-en-29-oic acid as shown in Figure 4.

HRFABMS data of **3** revealed a molecular formula of $C_{48}H_{74}O_{20}$, as determined from its $[M+H]^+$ peak at *m*/*z* 993.4669, calcd for $C_{48}H_{74}O_{20}Na$ (933.4671). In the proton NMR data, an olefinic hydrogen resonance at δ_H 5.37 (H-12) and six singlet H_3 resonances at δ_H 1.26 (H_3-27), 1.24 (H_3-23), 1.13 (H_3-28), 0.99 (H_3-30), 0.98 (H_3-26), and 0.89 (H_3-25) were observed. One of the main features in the ^{13}C NMR spectrum of **3** (methanol-d_4, Table 1) was a carbonyl carbon atom at δ_C 217.7 as it exhibited instead of the oxygenated carbon atoms of **1**, **2**, **4**, and **5**. The HMBC correlation from methyl proton at δ_H 0.99 (H_3-30) to carbonyl carbon at δ_C 217.7 suggested the location of the keto group at C-22 in **3**. Interestingly, in literature data (Gülcemal et al., 2013), **3** was assigned as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3β,24-

dihydroxyolean-12-en-22-oxo-29-oic acid. Nevertheless, the data in literature material (Gülcemal et al., 2013) were dissimilar to the optical rotation value of $[\alpha]_D^{25}$ –20 (*c* = 0.29, MeOH) and the assigned ^{1}H and ^{13}C NMR data (Table 1) in **3**. Specifically, the olefinic carbon atoms and carbonyl carbon atoms were mismatched. For instance, compound **3** showed the resonances of the carbonyl carbon atoms at δ_C 179.3 (C-29) and 172.5 (C-GlcA-6), which were shifted an up-field, in contrast to reported data (δ_C 184.0 (C-29) and 176.4 (C-GlcA-6), respectively). Adding a few drops of NaOH to **3**, a sodium salt (**3a**) was obtained. As a result, ^{13}C NMR chemical shifts in **3a**, including δ_C 220.4 (C-22), 184.6 (C-29), and 176.6 (C-GlcA-6) greatly resembled the reported values (Table 1). Although, the optical rotation value and the resonances of the olefinic carbon atoms at δ_C 125.1 and 142.8 were non-identical with previously reported values. The optical rotation of the reported compound was a positive value, but that of **3** was a negative value, which points out that they are distinct compounds. The correlations from H-18 (δ_H 2.41, 1H, m) and H-28 (δ_H 1.13, 3H, s) to H_3-30 in the ROESY spectrum of **3** (Figure 4) suggested 20*S* configuration (Zheng, 2013; Gülcemal, 2013). The other ROE correlations established that H-3, H-4, and H-5 are *S, S, and R*-oriented, respectively. For the sugar moiety, it was assigned as α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl(1→), based on its HPLC sugar analysis, the coupling constants of the sugar units, and the HMBC correlations. On the basis of these data, the structure of **3** was determined to be 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-en-22-oxo-29-oic acid as shown in Figure 4.

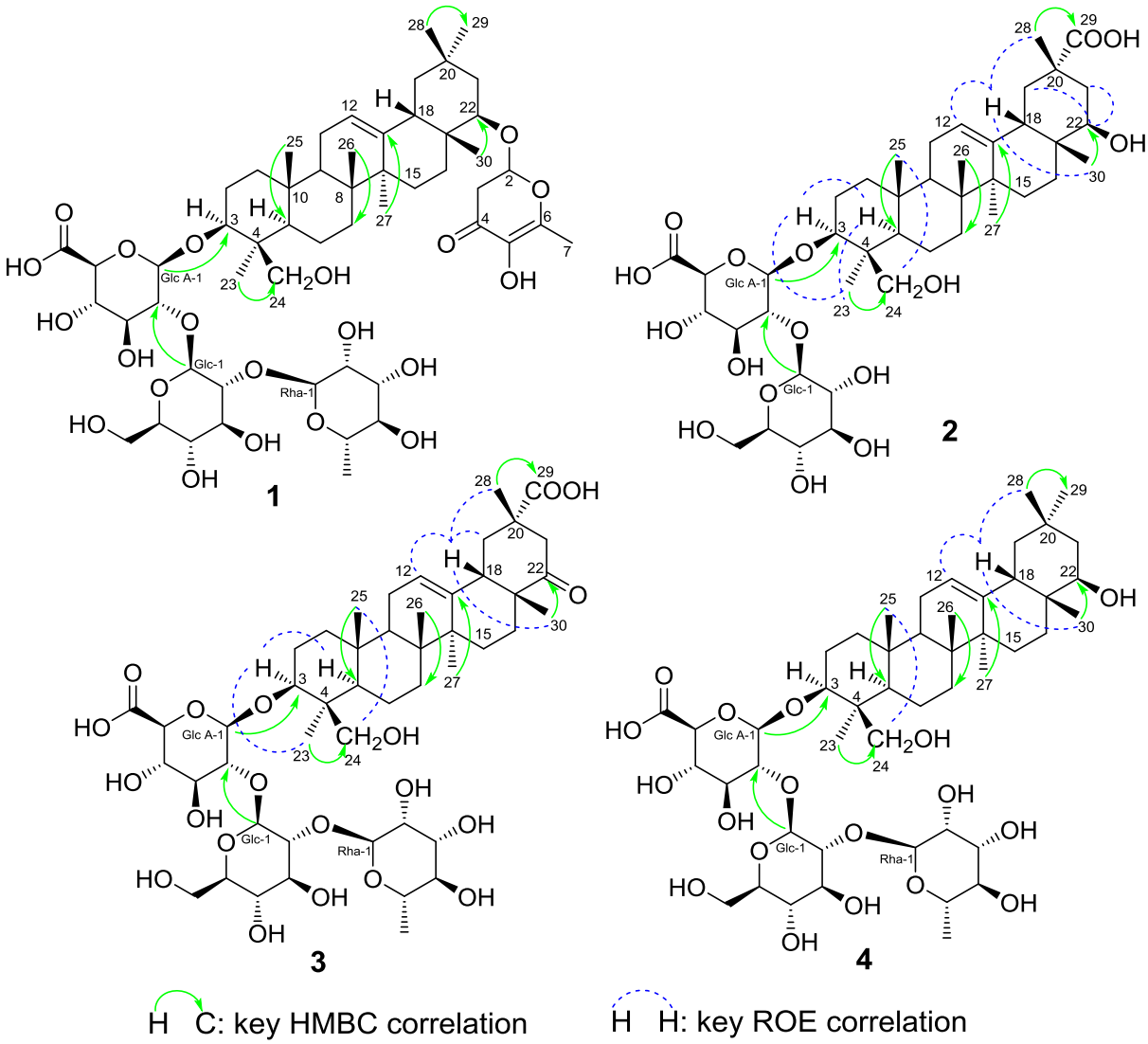


Figure. 4. Key HMBC and ROE correlations of **1, 2**, **3**, and **4**

Table 1. ^{13}C NMR spectroscopic data (100 MHz, methanol-d_4) of compounds **1**-**3** and **3a**.

	P.	**1**	**2**	**3**	**3a**
		δ_C	δ_C	δ_C	δ_C
Aglycon	1	39.6	39.6	39.6	39.5
	2	27.1	27.1	28.3	30.3
	3	93.0	92.5	92.9	92.7
	4	44.6	44.5	44.6	44.5
	5	57.4	57.3	57.3	57.3
	6	19.5	19.4	19.4	19.4
	7	34.0	34.3	34.0	33.9
	8	42.4	40.8	40.9	40.9
	9	47.2	45.7	48.7	48.6
	10	37.5	37.5	37.5	37.4
	11	24.9	24.8	24.9	24.9
	12	123.6	124.4	125.8	125.1
	13	145.3	144.5	142.2	142.8
	14	43.0	43.3	43.0	43.8
	15	27.1	26.7	26.1	26.2
	16	28.8	29.8	28.3	28.6
	17	38.1	38.5	48.7	48.6
	18	45.9	45.7	48.0	47.9
	19	47.2	41.8	43.0	42.9
	20	31.4	42.9	46.7	47.2
	21	37.3	37.5	48.0	48.0
	22	83.2	76.5	217.7	220.4
	23	23.2	22.8	23.1	23.1
	24	64.2	64.2	64.2	64.2
	25	16.2	16.2	16.2	16.2
	26	17.4	17.5	17.4	17.3
	27	26.5	25.4	25.9	25.8
	28	28.3	20.3	21.1	21.0
	29	33.9	182.4	179.7	184.6
	30	21.5	24.8	21.6	22.1
22-*O*-DDMP	2	98.5			
	3	41.1			
	4	187.8			
	5	134.5			
	6	155.9			
	7	15.6			
Glc A	1	105.4	105.0	105.4	105.3
	2	78.5	80.7	78.5	78.7
	3	78.3	78.0	78.3	78.7
	4	74.2	72.8	74.2	74.2
	5	76.5	76.5	76.5	76.7
	6	172.4	172.3	172.5	176.6
Glc	1	102.3	104.2	102.3	102.2
	2	78.8	75.5	78.7	78.8
	3	79.2	78.0	79.1	79.2
	4	70.3	70.3	70.3	70.3
	5	77.9	78.2	77.9	77.8
	6	61.7	61.8	61.7	61.7
Rha	1	102.0		102.0	102.0
	2	72.2		72.2	72.2
	3	72.3		72.2	72.2
	4	73.7		73.7	74.2
	5	69.6		69.6	69.7
	6	18.3		18.3	18.2

P. - position

Table 2. ^{13}C NMR spectroscopic data (100 MHz, pyridine-d_5) of compounds **1**-**3**.

	P.	**1**	**2**	**3**
		δ_C	δ_C	δ_C
Aglycon	1	38.5	38.6	38.4
	2	26.6	26.7	27.4
	3	91.5	90.7	91.5
	4	43.7	43.8	39.8
	5	56.1	56.1	56.1
	6	18.5	18.6	18.5
	7	33.0	33.2	32.9
	8	40.0	39.9	39.8
	9	47.6	47.7	48.3
	10	36.4	36.5	36.4
	11	24.0	24.1	24.0
	12	122.3	123.0	124.6
	13	144.1	144.3	141.4
	14	42.0	42.4	43.7
	15	26.6	26.4	25.4
	16	28.6	28.9	26.6
	17	37.2	38.0	48.3
	18	44.0	44.6	47.0
	19	miss.	41.5	41.6
	20	30.5	42.6	44.6
	21	36.3	37.8	46.5
	22	81.8	75.3	214.9
	23	22.8	22.6	22.8
	24	63.4	63.4	63.4
	25	15.6	15.7	15.6
	26	16.7	17.0	16.7
	27	26.2	25.5	25.4
	28	27.7	21.0	20.9
	29	34.1	181.5	179.0
	30	21.2	25.0	21.7
22-*O*-DDMP	2	97.4		
	3	42.3		
	4	185.9		
	5	135.0		
	6	152.8		
	7	15.6		

	P.	**1**	**2**	**3**
Glc A	1	105.2	105.1	105.2
	2	78.8	81.8	78.7
	3	77.2	75.3	77.3
	4	73.8	73.0	73.8
	5	77.7	78.3	77.8
	6	172.8	172.7	172.8
Glc	1	102.1	104.8	102.1
	2	79.2	75.8	79.2
	3	78.0	78.5	78.1
	4	69.7	69.9	69.7
	5	78.5	78.3	78.5
	6	61.3	61.6	61.4
Rha	1	102.0		102.0
	2	72.4		72.4
	3	72.8		72.8
	4	74.4		74.4
	5	69.5		69.5
	6	19.0		19.0

P. - position

miss. - missing

2.2.4. Anti-trypanosoma activity of isolated compounds

Previously, we isolated oxazoles and isoflavonoid derivatives from the roots of *O. lanata*, and some of them displayed potent inhibitory activity against *T. congolense* (Banzragchgarav et al., 2016). In the present work, trypanocidal screenings were carried out on some of the isolated compounds (**1**–**5**, **7**, **9**, **12**, **14**–**27**, **30**–**32**, and **36**) in order to find active chemicals (Table 3). Among them, the 5,7,4′-trihydroxyisoflavone (**25**) showed inhibitory activity against *T. congolense* (IC_{50} = 10.5 μM). Other compounds did not show significant activity. One of the main features observed in the active compound was three hydroxyl groups in its phenyl rings. It seems that hydroxyl groups at 5, 7, and 4′ positions may influence the inhibition activity. The earlier publications have shown that 5,7,4′-trihydroxyisoflavone is an efficient inhibitor against protein tyrosine kinase in *Trypanosoma brucei* (Gale et al., 1994). In addition, some flavonoids demonstrated a trypanocidal activity in previous studies (Cockram et al., 2018). This example of an active isoflavonoid will provide information for consideration of structure-activity relationships.

Table 3. Evaluation of inhibitory activity against *T. congolense* for compounds isolated from aerial parts of *O. lanata*

Compound	IC_{50} (μM)
25	10.5
28 (3*R*)-(–)-Arizonicanol A[a]	4.1
34[a]	12.2
Pentamidine [a]	0.169
Diminazene [a]	0.109

Compounds **1-5**, **7**, **9**, **12**, **14-24**, **26**, **27**, **30-32**, and **36** were inactive at 50 μg/mL. The treatment was replicated two or three times for each concentration.

[a]Banzragchgarav et al., 2016

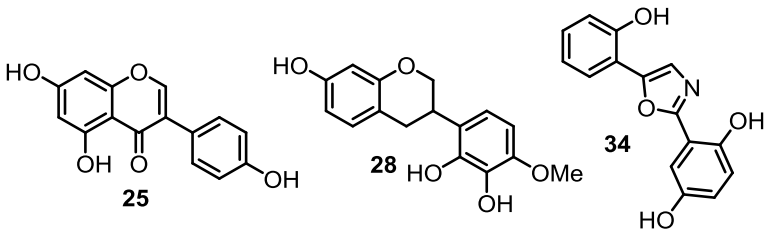


Figure. 5. Chemical structures of compounds **25**, **28**, and **34** with trypanocidal activity

2.2.5. Hyaluronidase inhibitory activity of isolated compounds

The isolated compounds **1**–**5, 7**–**9**, **13**, **16**, **19**, **25**, and **28**–**35** were screened for the hyaluronidase inhibitory activity tests, based on the traditional usage of *Oxytropis* species for the treatment of inflammatory diseases. Some of the saponins displayed potent inhibitory activities to compare with sodium cromoglicate, which was used as a positive control. As shown in Table 4, the IC_{50} values were indicated for those saponins that exhibited significant activities (IC_{50} = 150~220 μM). From these tested compounds, 3-*O*-[α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-en-22-oxo-29-oic acid (**3**) and dehydroazukisaponin V (**9**) imparted more potentially effect.

A 3-*O*-β-D-glucuronopyranoside moiety was contained in all of the isolated saponins. As reported by citation (Murata et al., 2013), a 3-*O*-β-D-glucuronopyranoside moiety in the sugar part exerted a potent inhibitory effect against hyaluronidase, which is in agreement with our results. For saponins **3**, **8**, and **9,** the keto group at C-22 was one of the main features which varied from the other ones. Curiously enough, the saponins bearing 3-*O*-β-D-glucuronopyranoside moiety and a keto group showed higher activity than those derivatives lacking the keto group. So, a keto group at C-22 of the aglycone seems that it may affect the enzyme inhibition activity. As far as we see, there are Fabaceae saponins, especially soyasaponins, which are similar to the saponins isolated in this study and known as potent anti-inflammatory constituents (Lee et al., 2010). The isolated saponins

from this plant have potential as anti-inflammatory compounds, and they may be the main components in explaining the anti-inflammatory properties of *O. lanata*.

Table 4. Hyaluronidase inhibitory activities for compounds isolated from aerial parts of *O. lanata*

Compound	IC_{50} (μM)
3	150
5	210
8	220
9	150
Sodium cromoglicate	370

Compounds **1, 2, 4, 7**, **13**, **16**, **19**, **25**, and **28-35** were inactive at 0.5 mM. The treatment was replicated two or three times for each concentration

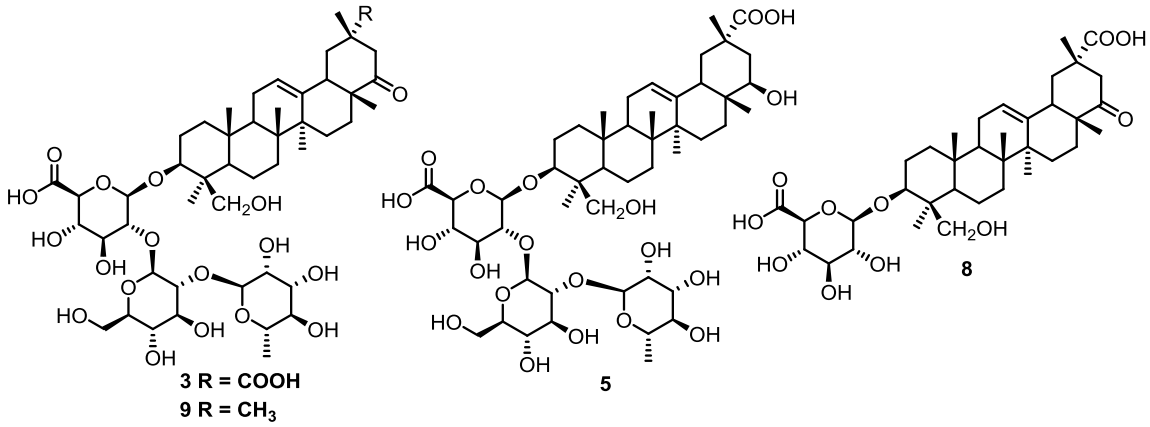


Figure. 6. Chemical structures of compounds **3**, **5**, **8**, and **9** with anti-hyaluronidase activity

2.3. Conclusion

To sum up, phytochemical analysis of *O. lanata* demonstrated that three new oleanane-type saponins and 33 known compounds, namely saponins, flavonoids, oxazole alkaloids, and glycosides, were presented in the aerial parts of this plant. Compounds **28**, **29**, **33**, and **34** were isolated from not only roots but also aerial parts of this plant species.

For the other compounds, this is the first report to mention them. Furthermore, trypanocidal activity of compounds **1, 2, 4, 7**, **13**, **16**, **19**, **25**, and **28**-**35** were investigated. Most of them did not show inhibitory activity, except for active compound 5,7,4′-trihydroxyisoflavone, but which was a little weak to compare with 2-(2′,3′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)oxazole isolated from roots of this plant and exhibited significant activity. When the investigations of hyaluronidase inhibition activity were carried out on the isolated compounds, 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1 → 2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-en-22-oxo-29-oic acid (**3**) and dehydroazukisaponin V (**9**) showed potent inhibitory activity. The main feature of these active compounds was a 3-*O*-β-D-glucuronopyranoside moiety and a keto group at C-22 may influence the enzyme inhibition activity. Moreover, the results of the current study allow suggesting that the above saponins may be beneficial in explaining the anti-inflammatory properties of *O. lanata*.

2.4. Experimental section

2.4.1. General experimental procedures

Optical rotations were recorded on a JASCO P-2300 polarimeter. A JEOL JNM-AL400 spectrometer was used to record ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra, and chemical shifts are given as δ_H and δ_C values with TMS as an internal standard at 25°C. Inverse-detected heteronuclear correlations were measured using HMQC (optimized for $^{1}J_{C\text{-}H}$ = 145 Hz) and HMBC (optimized for $^{n}J_{C\text{-}H}$ = 8 Hz) pulse sequences with a pulsed field gradient. A JEOL JNM-ECZ600R/S1 spectrometer was used to record ROESY (600 MHz). HRFABMS data were obtained on a JEOL JMS700 mass spectrometer by using *m*-nitrobenzyl alcohol or glycerol matrix. Preparative HPLC was performed on a JASCO 2089 instrument with UVdetection at 210 nm (JASCO),

using the following columns: TSKgel ODS-120T (Tosoh, Tokyo, Japan, 21.5 x 300 mm, flow rate: 8 ml/min), Develosil C_{30}-UG-5 (Nomura Chemical, Aichi, Japan, 20 x 250 mm, flow rate: 6 ml/min), Cosmosil 5C_{18}-AR-II (Nacalai Tesque, Kyoto, Japan, 20 x 250 mm, flow rate: 6 ml/min), and Mightysil RP-18 GP (Kanto Chemical, Tokyo, Japan, 20 x 250 mm, flow rate: 3 ml/min).

2.4.2. Plant materials

The aerial parts of *O. lanata* were collected from Multsug els in the territory of Altanbulag soum, Tuv province, Mongolia, at 1371 m above the sea level, in July 2016. Dr. Shagdar Dariimaa, Mongolian State University of Education, identified the plant species. A voucher specimen (No.49.15.25.16A) was deposited at the Laboratory of Bioorganic Chemistry and Pharmacognosy, National University of Mongolia.

2.4.3. Extraction and isolation

The powdered and air-dried aerial parts of *O. lanata* (340 g) were extracted with 9 L of acetone-water (4:1) for seven days, around 20℃. After filtration, the extract was concentrated under reduced pressure to provide 46 g of dry extract. This crude extract was suspended in H_2O and subjected to the DIAION HP-20 resin column (3.5 x 18 cm) with water/methanol as the eluent (gradient from 1:0 to 0:1, v/v) to give seven fractions (1A–1G). Fraction 1F (4.32 g) was loaded on a reverse-phase ODS-SM-50C-M column and eluted with water/methanol (gradient of 2:3 to 0:1, v/v) to give subfractions 2A–2Y. Subfractions 2H (154.8 mg), 2I (456.9 mg), 2J (197.4 mg), 2K (405.1 mg), 2L (299.6 mg), 2M (145.3 mg), and 2N (137.5 mg) were separated by preparative HPLC [column: ODS-120T; mobile phase: CH_3CN/H_2O (3:17 to 3:7, v/v) containing 0.2% TFA; column: C_{30}-UG-5; mobile phase: CH_3CN/H_2O (3:17 to 3:7, v/v) containing 0.2% TFA] to afford 14 compounds; **1** (19.9 mg), **2** (34.8 mg), **3** (25.6 mg), **4** (215.2 mg), **5** (13.3 mg), **6** (3.0 mg),

7 (10.4 mg), **8** (9.4 mg), **9** (19.2 mg), **10** (2.6 mg), **29** (6.6 mg), **33** (1.0 mg), **34** (2.0 mg), and **35** (7.2 mg). Fraction 1E (4.52 g) was chromatographed over a reverse-phase ODS-SM-50C-M column with water/methanol as the eluent (gradient from 3:2 to 0:1, v/v) to yield subfractions 3A–3N. Compounds **14** (243.9 mg), **17** (28.0 mg), **19** (70.1 mg), **22** (6.0 mg), **27** (12.3 mg), and **36** (1.6 mg) were isolated from subfractions 3H (294.4 g) and 3I (276.2 g) using preparative HPLC [column: ODS-120T; mobile phase: CH_3CN/H_2O (3:17 to 3:7, v/v) containing 0.2% TFA; C_{30}-UG-5, CH_3CN/H_2O (3:17 to 3:7, v/v) containing 0.2% TFA; RP-18 GP, CH_3CN/H_2O (3:17 to 3:7, v/v) containing 0.2% TFA]. Compounds **23** (2.6 mg), **24** (54.2 mg), **25** (7.1 mg), **26** (13.9 mg), and **28** (5.3 mg) were purified from subfractions 3C (43.7 mg), 3D (224.3 mg), 3E (260.3 mg), 3J (641.4 mg), and 3K (464.3 mg) using preparative HPLC [column: ODS-120T, mobile phase: CH_3CN/H_2O (gradient of 3:17 to 3:7, v/v) containing 0.2% TFA; column: C_{30}-UG-5, mobile phase: CH_3CN/H_2O (gradient of 3:17 to 3:7, v/v) containing 0.2% TFA; column: RP-18 GP, mobile phase: CH_3CN/H_2O (gradient of 3:17 to 3:7, v/v) containing 0.2% TFA], respectively. Separation of fraction 1D (4.21 g) over reverse-phase ODS-SM-50C-M column with water/methanol as the eluent (gradient from 4:1 to 0:1, v/v) yielded subfractions 4A–4J. Subfractions 4C (579 mg), 4D (290.7 mg), 4E (511.9 mg), 4G (438.3 mg), 4H (336.1 mg), and 4I (446.1 mg) were separated by preparative HPLC [column: ODS-120T, mobile phase: $CH_3CN–H_2O$ (gradient of 9:1 to 3:1, v/v) containing 0.2% TFA; column: C_{30}-UG-5, mobile phase: $CH_3CN–H_2O$ (gradient of 3:17 to 3:7, v/v) containing 0.2% TFA] to obtain 11 compounds, **11** (6.3 mg), **12** (33.1 mg), **13** (66.0 mg), **15** (3.0 mg), **16** (35.5 mg), **18** (2.1 mg), **20** (2.6 mg), **21** (7.2 mg), **30** (9.3 mg), **31** (4.3 mg), and **32** (8.5 mg).

2.4.3.1. 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucurono

pyranosyl]-[3′-hydroxy-2′-methyl-5′,6′-dihydro-4′-pyrone(6′→)]-3β,22β,24-trihydr oxy olean-12-ene (**1**)

Colorless powder; $[\alpha]^{26}_{D}$ –34 (*c* 0.41, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz): δ_H 1.02 (overlapping, H-1), 2.08 (1H, m, H-2), 1.94 (overlapping, H-2), 3.37 (overlapping, H-3), 0.94 (overlapping, H-5), 1.38 (overlapping, H-6), 1.86 (overlapping, H-11), 5.20 (1H, brs, H-12), 1.23 (3H, s, H-23), 3.20 (1H, d, *J* = 11.5 Hz, H-24), 4.11 (1H, d, *J* = 11.5 Hz, H-24), 0.88 (3H, s, H-25), 0.98 (3H, s, H-26), 1.16 (3H, s, H-27), 0.97 (3H, s, H-28), 0.90 (3H, s, H-29), 0.82 (3H, s, H-30), 4.46 (1H, d, *J* = 7.0 Hz, GlcA-1), 3.62 (1H, dd, *J* = 9.0, 7.0 Hz, GlcA-2), 3.62 (overlapping, GlcA-3), 3.48 (overlapping, GlcA-4), 3.75 (overlapping, GlcA-5), 4.92 (1H, d, *J* = 7.0 Hz, Glc-1), 3.40-3.45 (overlapping, Glc-2), 3.40-3.45 (overlapping, Glc-3), 3.48 (overlapping, Glc-4), 3.15 (1H, m, Glc-5), 3.70-3.80 (2H, m, Glc-6), 5.21 (1H, d, *J* = 1.5 Hz, Rha-1), 3.91 (1H, dd, *J* = 4.0, 1.5 Hz, Rha-2), 3.73 (overlapping, Rha-3), 3.43 (overlapping, Rha-4), 4.11 (1H, m, Rha-5), 1.26 (1H, d, *J* = 6.0 Hz, Rha-6), 5.39 (1H, dd, *J* = 4.0, 3.5 Hz, DDMP-2), 2.92 (1H, dd, *J* = 17.0, 4.0 Hz, DDMP-3), 2.51 (1H, dd, *J* = 17.0, 3.5 Hz, DDMP-3), 2.01 (3H, s, DDMP-7); ^{13}C NMR (methanol-d_4, 100 MHz): see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz): see Table 2; HRFABMS (negative) *m*/*z* 1067.5401 $[M–H]^-$ (Calcd for $C_{54}H_{83}O_{21}$: 1067.5428).

2.4.3.2. 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3β,22β,24-trihyd roxyolean-12-en-29-oic acid (2)

Colorless powder; $[\alpha]^{25}_{D}$ 0 (*c* 0.49, MeOH) ; ^{1}H NMR (methanol-d_4, 400 MHz): δ_H 1.62 (1H, m, H-1), 1.03 (1H, m, H-1), 1.95 (overlapping, H-2), 1.88 (overlapping, H-2), 3.37 (overlapping, H-3), 0.94 (overlapping, H-5), 1.39 (overlapping, H-7), 1.90 (overlapping, H-11), 5.29 (1H, brs, H-12), 1.30 (overlapping, H-16), 2.07 (1H, m, H-18), 2.30 (1H, brt, *J* = 13.0 Hz, H-19), 1.16 (overlapping, H-19), 1.98 (overlapping, H-21),

1.62 (overlapping, H-21), 3.46 (1H, dd, *J* = 9.0, 3.0 Hz, H-22), 1.20 (3H, s, H-23), 4.11 (1H, brd, *J* = 11.0 Hz, H-24), 3.19 (overlapping, H-24), 0.89 (3H, s, H-25), 0.97 (3H, s, H-26), 1.13 (3H, s, H-27), 1.30 (3H, s, H-28), 0.83 (3H, s, H-30), 4.51 (1H, d, *J* = 7.0 Hz, GlcA-1), 3.62 (1H, dd, *J* = 9.0, 7.0 Hz, GlcA-2), 3.61 (overlapping, GlcA-3), 3.51 (overlapping, GlcA-4), 3.77 (overlapping, GlcA-5), 4.77 (1H, d, *J* = 8.0 Hz, Glc-1), 3.19 (overlapping, Glc-2), 3.34 (overlapping, Glc-3), 3.41 (overlapping, Glc-4), 3.19 (overlapping, Glc-5), 3.71 (1H, dd, *J* =12.0, 4.0 Hz, Glc-6), 3.77 (overlapping, Glc-6); ^{13}C NMR (methanol-d_4, 100 MHz): see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz): see Table 2; HRFABMS (negative) *m*/*z* 825.4283 $[M–H]^-$ (Calcd for $C_{42}H_{65}O_{16}$: 825.4274).

2.4.3.3. 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-en-22-oxo-29-oic acid (3)

Colorless powder; $[\alpha]^{25}_D$ –20 (*c* 0.29, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz): δ_H 1.66 (1H, m, H-1), 2.14 (1H, m, H-2), 1.87 (overlapping, H-2), 3.37 (overlapping, H-3), 0.97 (overlapping, H-5), 1.30 (overlapping, H-6), 1.65 (overlapping, H-7), 1.39 (1H, d, *J* = 11.0 Hz, H-7), 1.93 (1H, m, H-11), 5.37 (1H, brs, H-12), 2.41 (1H, m, H-18), 2.57 (1H, brt, *J* = 14.0 Hz, H-19), 1.64 (overlapping, H-19), 3.01 (1H, d, *J* = 14.5 Hz, H-21), 2.23 (1H, d, *J* = 14.5 Hz, H-21), 1.24 (3H, s, H-23), 4.11 (1H, d, *J* = 11.0 Hz, H-24), 3.21 (1H, d, *J* = 11.0 Hz, H-24), 0.89 (3H, s, H-25), 0.98 (3H, s, H-26), 1.26 (3H, s, H-27), 1.13 (3H, s, H-28), 0.99 (3H, s, H-30), 4.46 (1H, d, *J* = 7.0 Hz, GlcA-1), 3.61 (overlapping, GlcA-2), 3.62 (overlapping, GlcA-3), 3.48 (overlapping, GlcA-4), 3.75 (overlapping, GlcA-5), 4.92 (1H, d, *J* = 7.5 Hz, Glc-1), 3.40-3.45 (overlapping, Glc-2), 3.40-3.45 (overlapping, Glc-3), 3.47 (overlapping, Glc-4), 3.18 (1H, m, Glc-5), 3.76 (1H, brd, *J* = 10.5 Hz, Glc-6), 3.73 (1H, dd, *J* = 10.5, 3.5 Hz, Glc-6), 5.22 (1H, brs, Rha-1), 3.92 (1H, brd, *J* = 3.5 Hz, Rha-2), 3.73 (overlapping, Rha-3), 3.46 (overlapping, Rha-4), 4.13 (1H,

m, Rha-5), 1.26 (1H, d, J = 6.0 Hz, Rha-6); ^{13}C NMR (methanol-d_4, 100 MHz): see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz): see Table 2; HRFABMS (positive) m/z 993.4669 $[M+H]^+$ (Calcd for $C_{48}H_{74}O_{20}Na$: 933.4671).

2.4.4. Evaluation of trypanocidal activity

The bloodstream form (BSF) of *T. congolense* IL3000 strain was used to evaluate trypanocidal activities of isolated compounds. In Brief, BSF was cultured in Hirumi's modified Iscove's medium by following the previously reported method (Hirumi et al., 1991). The trypanocidal activities of compounds **1**–**5**, **7**, **9**, **12**, **14**–**27**, **30**–**32**, and **36** were evaluated in the assay with concentrations ranging from 25 µg/mL to 1.6 ng/mL after fivefold serial dilution. The IC_{50} values of compounds **28**, **29**, **33**, and **34** were discussed in the Results and Discussion section. Pentamidine and diminazene were used as a positive control.

2.4.5. Hyaluronidase inhibition assay

The inhibitory activity toward hyaluronidase was determined by the Morgan–Elson method, which was modified by Davidson and Aronson. The assay was carried out following the procedure reported previously (Murata et al., 2013). Samples (**1**–**5**, **7**–**9, 13**, **16**, **19**, **25**, **28**–**35**) dissolved in 0.1 M acetate buffer (0.2 mL) were mixed with hyaluronidase (Type IV-S, from bovine testes; Sigma Chemical Co., St. Louis, USA) in buffer (final concentration: 400 U/mL, 0.1 mL), and the mixture was incubated at 37°C for 20 min. Compound 48/80 (Sigma Chemical Co.) in buffer (final concentration: 0.3 mg/mL, 0.2 mL) was then added, and incubation was continued at 37°C for 20 min. After hyaluronic acid potassium salt, from rooster comb (Sigma Chemical Co.), in buffer (final concentration: 0.4 mg/mL, 0.5 mL) had been added, the mixture was incubated at 37°C for 40 min. The reaction was then stopped by adding 0.4 M NaOH and borate solution

and subsequently boiling the mixture in a water bath for 3 min. An acetic acid solution of dimethylaminobenzaldehyde (6 mL; Wako Pure Chemical Industries Ltd., Osaka, Japan) was then added, and the mixture was incubated at 37°C for 20 min. Acetate buffer was used in place of the sample as a control, and buffer was added in place of hyaluronidase in buffer as a blank. The enzyme inhibitory activity (%) was calculated from the absorbance at 590 nm ($Abs_{600\ nm}$) as follows: Inhibitory activity (%) = [(Control $Abs_{590\ nm}$ – Control $_{blank}$ $Abs_{590\ nm}$) – (Sample $Abs_{590\ nm}$ – Sample $_{blank}$ $Abs_{590\ nm}$)]/ (Control $Abs_{590\ nm}$ – Control $_{blank}$ $Abs_{590\ nm}$) × 100. Sodium cromoglicate was used as a positive control.

2.4.6. Sugar identification for compounds 1, 2, and 3

Each compound [**1** (0.5 mg), **2** (1.0 mg), and **3** (1.0 mg)] was hydrolyzed with 2 M HCl (0.5 mL) at 80°C for 1 h. The reaction mixtures were concentrated to leave the sugar fractions. The sugar fractions were stirred with L-cysteine methyl ester (0.5~2 mg) in pyridine (0.5 mL), and *o*-tolyl isothiocyanate (5 μL for the sugar fractions from **1**, **2**, and **3**) was added to the mixtures (Tanaka et al., 2007). The reaction mixtures were analyzed by HPLC (column: J-pak shimphonia C18; JASCO, Tokyo, Japan; 4.6 × 250 mm; mobile phase: CH_3CN/0.2 % TFA in H_2O (1:3); flow rate: 1.0 mL/min; detection, UV at 250 nm). A D-glucose derivative (t_R = 18.1 min) and an L-rhamnose derivative (t_R = 31.0 min) were identified as the sugar moieties of **1**, **2**, and **3** and of **1**, and **3**, respectively, based on comparisons with derivatives of authentic D-glucose (t_R = 18.1 min), L-glucose (t_R = 16.6 min), and L-rhamnose (t_R = 31.0 min). Furthermore, the reaction mixtures were analyzed again by HPLC under different conditions (column: Develosil C30; 4.6 × 250 mm; mobile phase: CH_3CN/0.2 % TFA in H_2O (1:3); flow rate: 1.0 mL/min; detection, UV at 250 nm). A D-glucose derivative (t_R = 22.4 min) and a D-glucuronic acid derivative (t_R = 23.2 min) were identified as the sugar moieties of **1** and **3**, based on comparisons with derivatives

of authentic samples of D-glucose derivative (t_R = 22.4 min), L-glucose derivative (t_R = 20.7 min), and D-glucuronic acid derivative (t_R = 23.2 min).

2.4.7. Sodium salt of compound 3

One drop of saturated NaOH in methanol-d_4 was added to a methanol-d_4 solution of **3** (13.1 mg/0.5 mL) in the NMR tube to yield a sodium salt solution of **3** (**3a**).

*Sodium Salt of **3** (**3a**):* ^{1}H NMR (methanol-d_4, 400 MHz): δ_H 3.37 (overlapping, H-3), 5.35 (1H, brs, H-12), 1.28 (3H, s, H-23), 3.21 (1H, d, *J* = 11.0 Hz, H-24), 4.11 (1H, d, *J* = 11.0 Hz, H-24), 0.88 (3H, s, H-25), 0.97 (3H, s, H-26), 1.28 (3H, s, H-27), 1.04 (3H, s, H-28), 0.97 (3H, s, H-30), 4.46 (1H, d, *J* = 7.0 Hz, GlcA-1), 4.90 (1H, d, *J* = 7.5 Hz, Glc-1), 5.21 (1H, brs, Rha-1), 3.92 (1H, brd, *J* = 3.5 Hz, Rha-2), 1.25 (1H, d, *J* = 6.0 Hz, Rha-6); ^{13}C NMR (methanol-d_4, 100 MHz): see Table 1.

CHAPTER III. ABSOLUTE CONFIGURATION'S DETERMINATION OF SOME COMPOUNDS ISOLATED FROM *BRACHANTHEMUM GOBICUM*

3.1. Introduction

Figure 7. *Brachanthemum gobicum*

Scientific classification:
Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: *Brachanthemum*
Species: *B. gobicum*

The genus *Brachanthemum* in the family Asteraceae is distributed from Altai republic, Russian federation to Mongolia, down to Kazakhstan and Gansu province, the People's Republic of China, and which is represented by 12 species, including *Brachanthemum gobicum* (Smirnov 2013). *B. gobicum*, locally known as awful firewood, is a subshrub and 15-40 cm tall. This plant grows in thin desert sands and debris-pebble deserts (Tungalag 2016). Nomads who earn a living based on pasture farming and products of livestock origin utilize this plant as the fodder of livestock and for firewood (Batkhuu et al., 2005). Besides, the fume that is exhausted from burned *B. gobicum*, specifically its leaves and stems, is used to eliminate external parasites, including the parasitic louse *Linognathus*, from tamed sheep (Ligaa et al., 2005). According to previous studies (Shatar 2010; Khanina 1999), the essential oils were considered as the major constituents of the genus *Brachanthemum*. Our studies on the chemical constituents of *B. gobicum* could result in the identifications of the various compounds, including flavonoids and their glycosides, lignans and their acylated derivatives, phenolic

compounds, phenolic glucosides, quinic acid derivatives, coumarin glycosides, sesquiterpenoids, and an amino acid (Odonbayar et al., 2019). This research work on *B. gobicum* was mainly carried out by Drs B. Odonbayar and T. Murata. They isolated a total of 51 compounds from aerial parts of this plant and then elucidated their chemical structures using various spectroscopic techniques, such as HRFABM, UV and ECD spectra, ^{1}H and ^{13}C NMR. But there were several compounds which usually determined as racemic mixture due to their optical rotation values were close to zero. In order to isolate pure enantiomers from these racemic mixtures and identify absolute configurations for these compounds, I took part in this research work, and all of the investigations were carried out under the guidelines of Dr. T. Murata. The optically inactive sample combined in a 1:1 mixture of two enantiomers is called a racemic mixture. Although racemates rarely occur in natural products, there are several scientific reports that carried out on enantioseparations of lignans (Lu et al., 2015). In the present work, chiral column HPLC was used to purify enantiomers of these compounds, and their structures, including absolute configurations, were assigned by optical rotations and electronic circular dichroism (ECD) spectra.

3.2. Results and discussion

As noted earlier, Odonbayar and Murata mainly investigated the chemical constituents of *B. gobicum*. As a result, a total of 51 compounds was elucidated based on their spectroscopic data, including 2D NMR. All of the compounds were reported in Odonbayar's diploma thesis. Among these compounds, previously unreported eight isovaleryllignans (**37**-**40** and **44**-**47**), and three isovalerylphenylpropanoids (**41**-**43**) had a great interest more than the other 40 known compounds. But, these new compounds were considered as a racemic mixture because of optically inactive and no cotton effects in

their ECD spectra. Herein, we describe more comprehensively how these compounds were purified from their mixture and how absolute configurations were determined.

3.2.1. Absolute configurations for enantiomers

All of the new compounds were firstly analysed by chiral-phase analysis HPLC. The couple peaks detected in each sample indicated that these compounds were a mixture of enantiomers. For compounds **40**, **42**, **43**, **46,** and **47,** enantiomers of them were not obtained enough amount to study absolute configurations. Other compounds were discussed below.

The compound **37** was subjected to chiral-phase HPLC (Daicel Chiralpak AS-H), and enantiomers obtained from **37** were signed as **37a** and **37b**. The ECD curve of **37a** at ~200–350 nm was inverse of that of **37b** (Figure 9). The specific rotation of **37a** ($[\alpha]^{25}_{D}$ –17º) was the opposite of that of **37b** ($[\alpha]^{25}_{D}$ +15º). The positive Cotton effect around 202 nm and the negative Cotton effect at 289 nm suggested a (7′*R*, 8′*S*)-absolute configuration for **37a**. Similarly, the negative and positive Cotton effects at 202 and 288 nm, respectively, indicated (7′*S*, 8′*R*)-absolute configuration for **37b**. On the basis of these data, structures of **37a** and **37b** were assigned as (7′*R*, 8′*S*)-brachangobinan A and (7′*S*, 8′*R*)- brachangobinan A, respectively (Figure 8).

With the help of chiral-phase HPLC, enantiomers **38a** and **38b** were isolated from their scalemic mixture. The ECD curve of **38a** around 200-400 nm was the opposite of those of **38b** (Figure 9). The specific rotation of **38a** ($[\alpha]^{25}_{D}$ –54º) was the inverse of that of **38b** ($[\alpha]^{25}_{D}$ +32º). In accordance with the structural similarity of (–)-lucidenal (Sriyatep et al., 2014), **38a** was identified to have an 8*R*-configuration. Therefore, the structures of **38a** and **38b** were determined as shown in Figure 8 and named as (8*R*)-brachangobinan B and (8*S*)-brachangobinan B, respectively.

The specific rotation value of **39** ($[\alpha]^{23}_{D}$ 0º) indicated that **39** was (7′*RS*,8′*RS*) racemic mixtures. Similar to the racemate of **37**, enantioseparation of **39** was carried out using chiral-phase HPLC to obtain the enantiomers **39a** and **39b**. Although (7′*R*,8′*R*)-absolute configuration of the reported compound was determined by a negative Cotton effect at 226 nm (Hue et al., 2011), no clear Cotton effect was shown in the ECD spectra of **39a** and **39b** because of few yields. Compounds **39a** and **39b** have specific rotations of $[\alpha]^{24}_{D}$ +7º and $[\alpha]^{24}_{D}$ –7º, respectively. (7′*R*,8′*R*)-*threo*-(*E*)-4′,7′-dihydroxy-9,9′-diisovaleroyloxy-3,3′-dimethoxy-7-en-4-*O*-8′-neolignan has been reported previously and was found to have a positive optical rotation, similar to that of **39a** (Hue et al., 2011). Therefore **39a** was identified as (7′*R*,8′*R*)-*threo*-(*E*)-4′,7′-dihydroxy-9,9′-diisovaleroyloxy-3,3′-dimethoxy-7-en-4-*O*-8′-neolignan, and **39b** was determined as (7′*S*,8′*S*)-brachangobinan C (Figure 8).

By using chiral column HPLC, enantiomers **41a** and **41b** were purified from compound **41**. The specific rotation of **41a** ($[\alpha]^{25}_{D}$ –14º) was the inverse of that of **41b** ($[\alpha]^{25}_{D}$ +14º). By comparison with a specific rotation of alatusol A (Kim et al., 2013), **41a** also has 7*R*,8*R*-configuration. The ECD curve of **41b** at 200-260 nm was the mirror image of that of **41a** (Figure 9). From these data, these structures were determined as (7*R*,8*R*)-brachangobinan E (**41a**) and (7*S*,8*S*)-brachangobinan E (**41b**) (Figure 8).

Compounds **44a** and **44b** were obtained by chiral column HPLC separation of **44**, and the specific rotation of **44a** ($[\alpha]^{24}_{D}$ –15º) and **44b** ($[\alpha]^{25}_{D}$ +11º) were recorded. A negative ECD Cotton effect at 327 nm and a positive Cotton effect at 339 nm suggested the structures of (7′*R*,8′*S*)- **44a** and (7′*S*,8′*R*)- **44b**, respectively (Figure 9) (Lou et al., 2018). Based on these data, the structures of **44a** and **44b** were identified as (7′*R*,8′*S*)-brachangobinan H and (7′*S*,8′*R*)-brachangobinan H, respectively (Figure 8).

The enantiomers of **45** (**45a** and **45b**) were obtained by chiral column HPLC separation of **45**. The specific rotation of **45a** ($[\alpha]^{28}_{D}$ –51º) was the inverse of that of **45b** ($[\alpha]^{28}_{D}$ +64º). A negative ECD Cotton effect at 297 nm indicated a structure of (7′*R*,8′*S*)-**45a** (Figure 9) (Wu et al., 2016). In contrast, a positive Cotton effect at 296 nm indicated the structure of (7′*S*,8′*R*)-**45b**. From these data, **45a** and **45b** were determined as (7′*R*,8′*S*)-brachangobinan I and (7′*S*,8′*R*)-brachangobinan I, respectively (Figure 8).

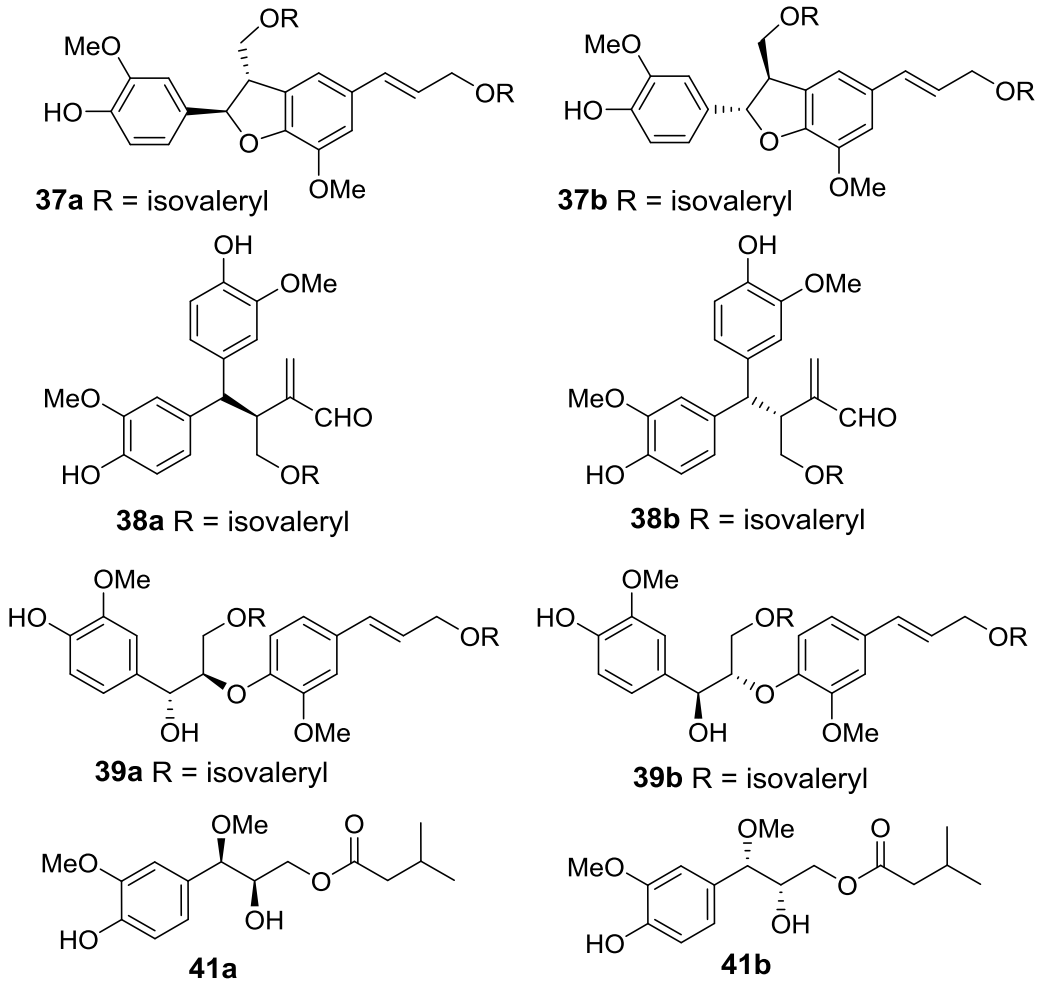


Figure 8. Chemical structures of **37a**/**37b**-**39a**/**39b**, **41a**/**41b**, **44a**/**44b**, and **45a**/**45b**

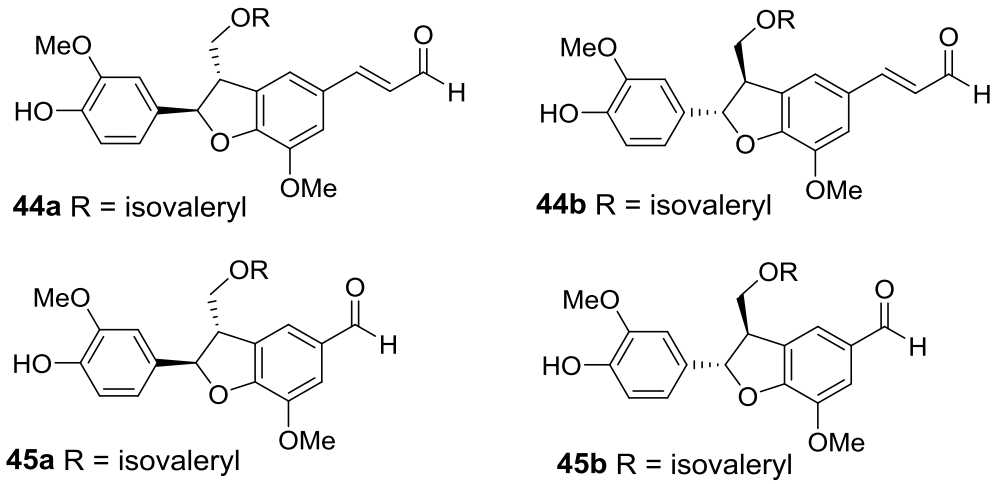


Figure 8. Continued

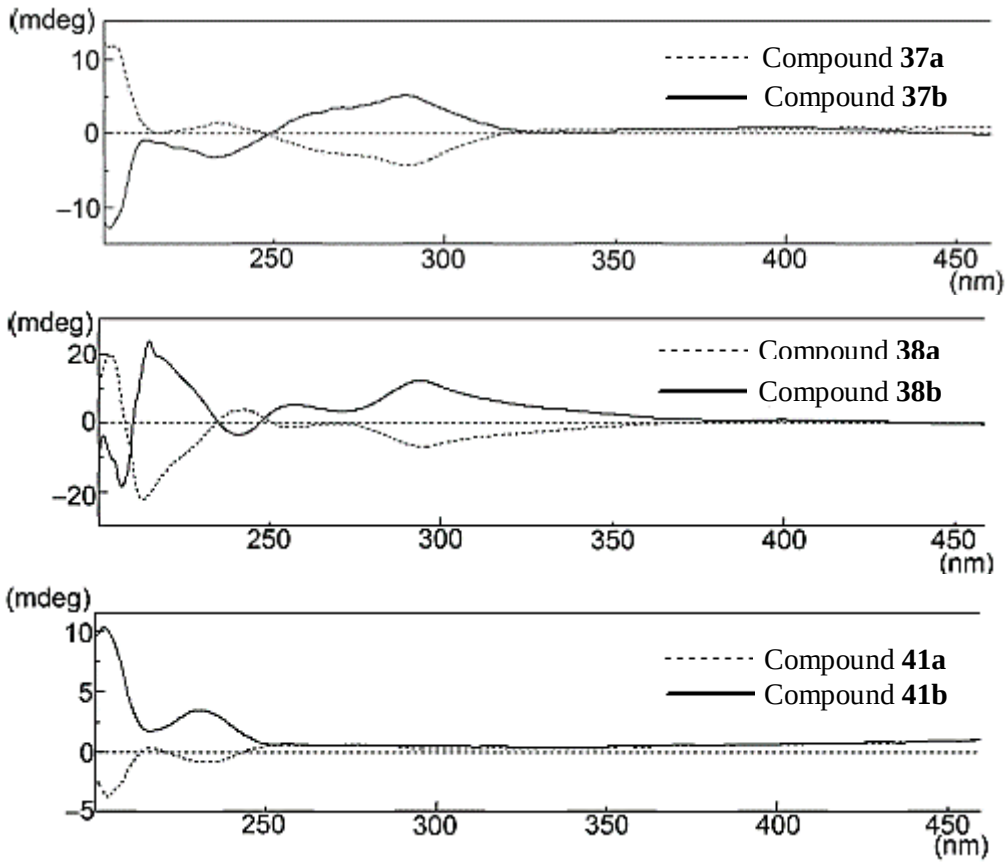


Figure 9. ECD spectra of compounds **37a**/**37b**, **38a**/**38b**, **41a**/**41b**, **44a**/**44b**, and **45a**/**45b**

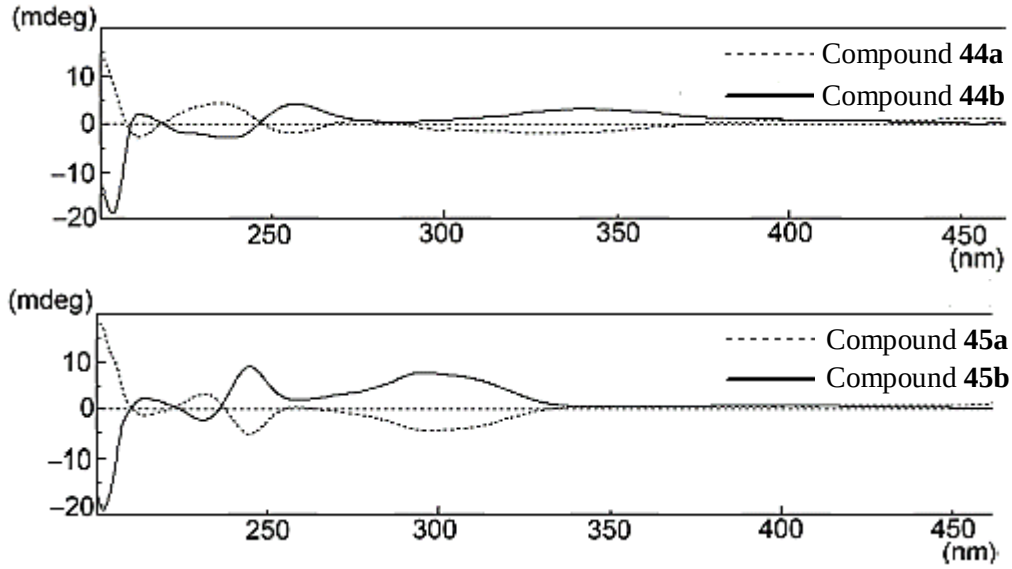


Figure 9. Continued

3.2.2. Anti-trypanosoma activity of isolated compounds

Compounds with racemic mixture were screened against *Trypanosoma congolense* because yields of enantiomers isolated from racemic mixtures were not enough to test. As a result of this investigation, compounds **37**, **38**, **39**, **40**, **44**, and **45** showed an inhibition activity.

Table 5. Evaluation of inhibitory activity against *T. congolense* for compounds isolated from aerial parts of *B. gobicum*

Compound	IC_{50} (μM)
37	13.4
38	2.8
39	17.4
40	9.6
44	2.4
45	19.9
Pentamidine	0.1
Diminazene	0.2

Compounds **41**-**43**, **46**, and **47** were inactive at 50 μg/mL. The treatment was replicated two or three times for each concentration

Among them, compounds **38** (IC_{50} = 2.8 μM) and **44** (IC_{50} = 2.4 μM) inhibited more efficiently than others. But it was moderate activity to compare positive control diminazene (IC_{50} = 0.1 μM) and pentamidine (IC_{50} = 0.2 μM). The main feature of compounds with comparatively high activity was a formyl group and diphenyl moieties in their structures because they seemed to play an important role in the trypanocidal activity.

3.3. Conclusion

To conclude, a total of 51 compounds were isolated aerial parts of *B. gobicum*. Among them, 11 compounds were reported as new in nature based on their various spectral data, such as 2D NMR and ECD. All of these compounds were isolated for the first time from this plant. For the compounds with the racemic mixture, they were separated from their enantiomers by using chiral phase HPLC. After that, absolute configurations of them were elucidated with the help of data obtained from specific rotation and ECD spectra. But the absolute configurations of compounds **40**, **42**, **43**, **46**, and **47** were not determined owing to the small yield of enantiomers. In terms of trypanocidal investigations, compounds **38** (IC_{50} = 2.8 μM) and **44** (IC_{50} = 2.4 μM) showed a potent inhibition activity to compare with other compounds.

3.4. Experimental section

3.4.1. General experimental procedures

Optical rotations were measured with a JASCO P-2300 polarimeter (JASCO, Tokyo, Japan). ECD spectra were recorded on a JASCO J-700 spectropolarimeter (JASCO). Preparative HPLC was performed using a JASCO 2089 with UV detection at 210 nm (JASCO), using Daicel chiralpak AS-H column (Daicel, Osaka, Japan, 4.6 × 250 mm).

3.4.2. Isolation of enantiomers

Compound **37** (3.9 mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (9:1), flow rate: 1.0 mL/min] to obtain **37a** (0.7 mg, t_R 18.3 min) and **37b** (0.8 mg. t_R 16.1 min). Compound **38** (2.6 mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (4:1), flow rate: 1.0 mL/min] to obtain **38a** (1.4 mg, t_R 9.0 min) and **38b** (0.9 mg. t_R 14.0 min). Compound **39** (1.2 mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (9:1), flow rate: 1.0 mL/min] to obtain **39a** (0.3 mg, t_R 16.8 min) and **39b** (0.3 mg. t_R 19.8 min). Compound **41** (2.6 mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (4:1), flow rate: 1.0 mL/min] to obtain **41a** (1.0 mg, t_R 9.4 min) and **41b** (1.0 mg. t_R 5.8 min). Compound **44** (3.2 mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (3:2), flow rate: 1.0 mL/min] to obtain **44a** (0.9 mg, t_R 13.8 min) and **44b** (0.9 mg. t_R 10.3 min). Compound **45** (2.7mg) was subjected to the chiral column [Daicel chiralpak AS-H, hexane-EtOH (3:2), flow rate: 1.0 mL/min] to obtain **45a** (0.7 mg, t_R 10.6 min) and **45b** (1.0 mg. t_R 6.9 min).

3.4.3. Evaluation of trypanocidal activity

To evaluate the trypanocidal activity of isolated compounds, the bloodstream form (BSF) of *T. congolense* IL3000 strain was applied. The BSF was cultivated in Hirumi's modified Iscove's medium (HMI)-9, which was prepared according to the previously reported method (Hirumi et al., 1991). Each compound was evaluated in the assay with concentrations ranging from 25 μg/mL to 1.6 ng/mL after fivefold serial dilution. After 72 hours of cultivation, 25 μL of CellTiter-Glo reagent was added to each well, and the luminesces were measured using a GloMax-Multi+ Detection System plate reader. The IC_{50} values of each compound were calculated using GraphPad PRISM 5 software. Pentamidine and diminazene were used as a positive control.

CHAPTER IV. CHEMICAL CONSTITUENTS OF *CALLIGONUM MONGOLICUM* AND ANTI PHENYLOXIDASE ACTIVITY OF CATECHIN

4.1. Introduction

Scientific classification:
Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Polygonales
Family: Polygonaceae
Genus: *Calligonum*
Species: *C. mongolicum*

Figure 10. *Calligoum mongolicum*

According to the literature data, as of 2020, approximately 158 species, including *Calligonum mongolicum*, belong to the genus *Calligonum*, which is one of the largest genera in the family Polygonaceae (Purohit et al., 2020). *C. mongolicum* have been commonly used for the treatment of nasal hemorrhagic, hemostatic, and the relief of menstruation. Asides from that, this plant is utilized for the fodder of livestock, specifically camels. Moreover, these shrub plants distributed in Middle Asia, including Mongolia, play a really essential role to prevent the drifting of sand, and nomads who live in the countryside use it for firewood (Jigjidsuren et al., 2003).

Studies on chemical constituents of this genus have resulted in the isolation of terpenoids (Samejo et al., 2013b), flavonoids (Ahmed et al., 2016), steroids (Samejo et al., 2013a), butanolides (Yawer et al., 2007), and stilbenes (Okasaka et al., 2004). Moreover, some of the above compounds showed important pharmacological activities, such as antioxidative, antibacterial, anti-lipoxygenase, and cytotoxicity (Ahmed, 2016; Yawer, 2007; Okasaka, 2004). For single species of *C. mongolicum*, took an interest in

us, there were no previous reports, especially on its chemical constituents.

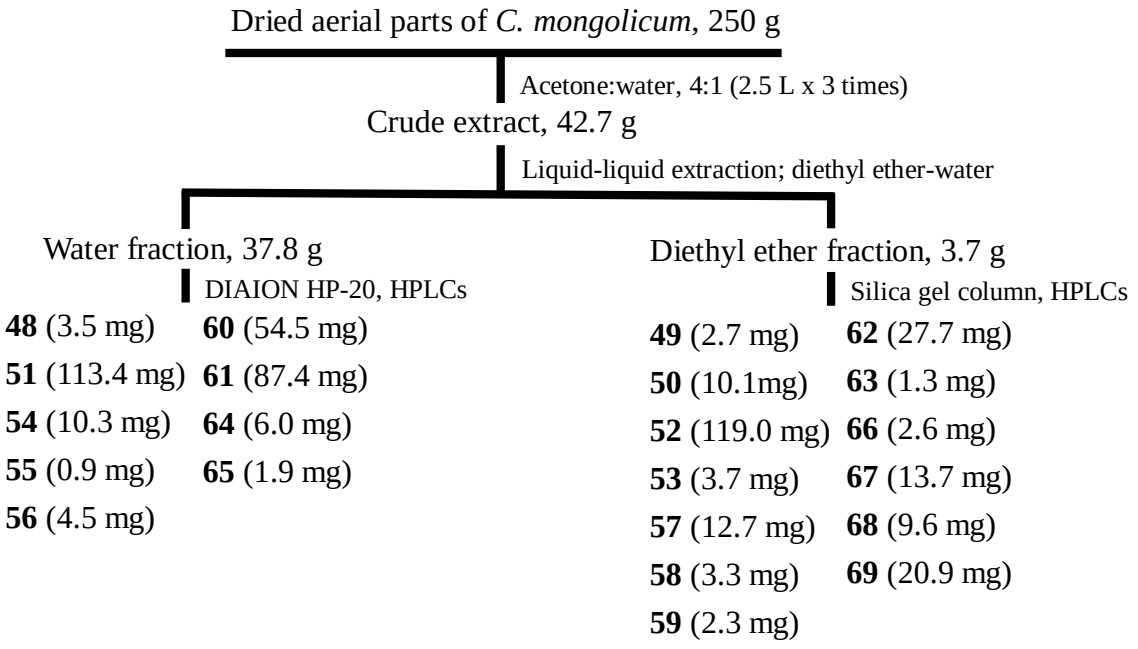
In this study, we aimed to describe the isolation and characterization of twenty-two (**48**-**69**) compounds from the aerial parts of this plant. As a result, two previously unreported compounds (**48** and **49**) and one compound (**50**) that was firstly obtained from natural sources were reported. As far as we know well, all compounds (**48**-**69**) were isolated from this species for the first time.

4.2. Results and discussion

4.2.1. Extraction and isolation

The aerial parts of *C. mongolicum* were extracted with 7.5 L of acetone-water (4:1) to provide 42 g of crude extract. This concentrated extract was suspended in water and then partitioned with diethyl ether. Furthermore, the water and diethyl ether extracts were subjected to HP-20 resin and silica gel column chromatography, respectively. These separations and purifications of extracts led to the isolation of two new and 20 known compounds.

Scheme 2. Extraction and isolation of aerial parts of *C. mongolicum*



4.2.2. Identification of known compounds

The chemical structures of the known compounds **50**–**69** were determined as (2*R*, 4a*S*, 8a*S*)-4a-hydroxy-2-methyl-3,4,4a,8a-tetrahydrobenzo-1 (2H)-pyran-7 (8)-one (**50**) (Barradas et al., 2009), rhododendrin (**51**) (Kim et al., 2011), (*R*)-(−)-rhododendrol (**52**) (Kim et al., 2011), 4-(2-oxobutyl)phenol (**53**) (Bunce and Reeves,1989), catechin (**54**) (Galotta et al., 2008), epicatechin (**55**) (Davis et al., 1996), dihydrokaempferol (**56**) (Lee et al., 2003), dihydroquercetin (**57**) (Keihlmann and Slade, 2003), kaempferol (**58**) (Chang et al., 2000), quercetin (**59**) (Chang et al., 2000), kaempferol 3-*O*-β-D-glucuronopyranoside (**60**) (Dini et al., 2004), quercetin 3-β-*O*-glucuronopyranoside (**61**) (Castillo-Muñoz et al., 2009), *N*-*trans*-feruloyltyramine (**62**) (Kim et al., 2005), *N*-*cis*-feruloyltyramine (**63**) (Fukuda et al., 1983), 2-(4-hydroxyphenyl)ethyl-β-D-glucopyranoside (**64**) (Shi et al., 2011), isopentyl β-D-glucopyranoside (**65**) (Kurashima et al., 2004), *p*-hydroxy-*trans*-cinnamic acid (**66**) (Satake et al., 1980), *p*-hydroxybenzoic acid (**67**) (Chang et al., 2000), protocatechuic acid (**68**) (Zhang et al., 1998), and gallic acid (**69**) (Gottlieb et al., 1991) by comparing their MS, ^{1}H and ^{13}C NMR data with those in the literature (Figure 11).

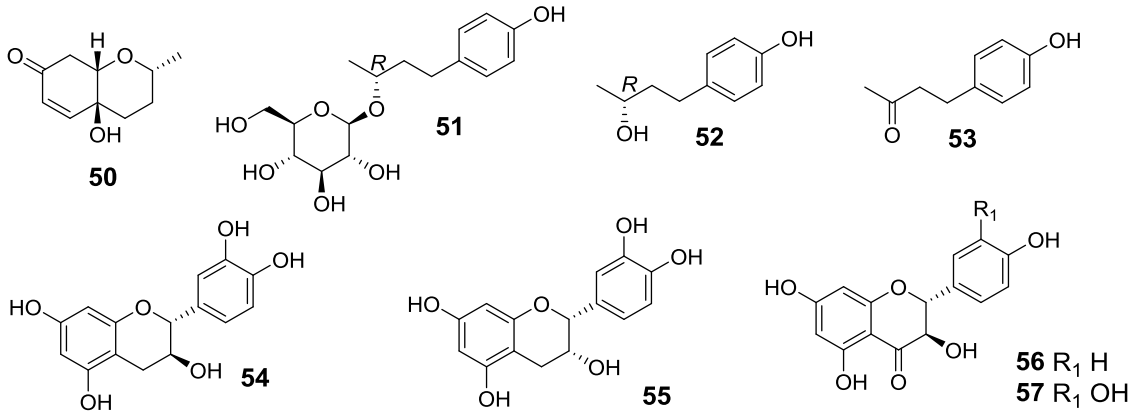


Figure 11. Chemical structures of known compounds isolated from *C. mongolicum*

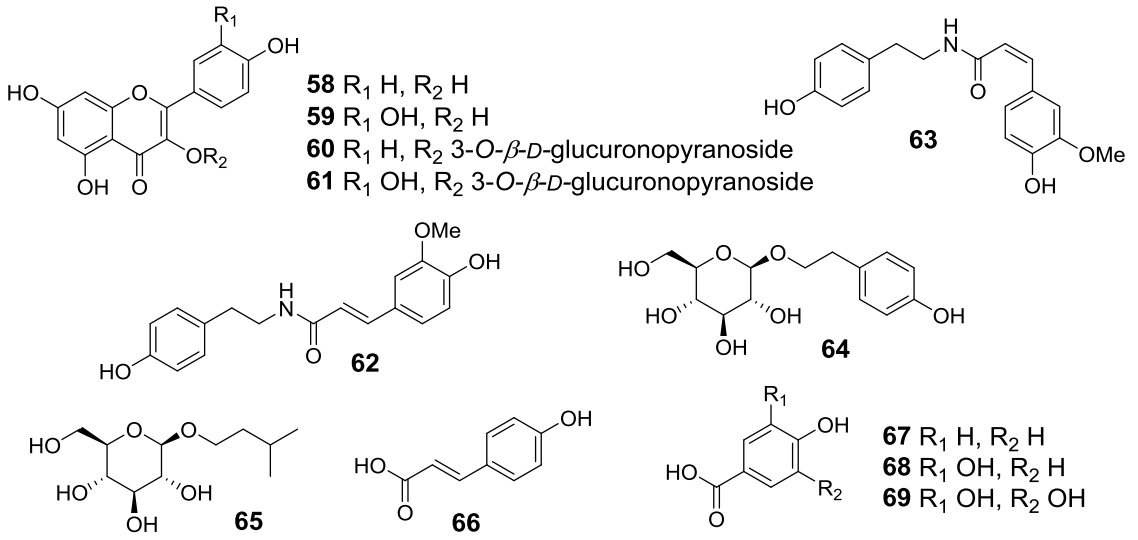


Figure 11. Continued

4.2.3. Structural elucidation of new compounds 48 and 49

Compound **48** and **49** were considered to be new compounds. The structure determination procedures of them were described below.

Compound **48** demonstrated a molecular formula of $C_{23}H_{29}O_{11}$ based on the molecular ion peak $[M+H]^+$ at *m/z* 481.1709, calcd for $C_{23}H_{29}O_{11}$ at *m/z* 481.1710 in HRFABMS. ^{1}H NMR spectrum of **48** showed a singlet proton at δ 7.11 and a set of *o*-coupling doublet methine protons at δ 6.91 and 6.59 in its aromatic field. In the HBMC spectrum (Figure 12), singlet proton at δ 7.11 was long-range correlated with carbons at δ 121.6 (C-1‴), 146.6 (C-3‴), 139.9 (C-4‴), 110.3 (C-6‴), and 168.5 (C-7‴), which indicated the presence of a galloyl moiety. Furthermore, the *o*-coupling doublet methine protons and aromatic carbon resonances at δ 134.7 (C-1′), 130.4 (C-2′ and 6′), 116.0 (C-3′ and 5′), and 156.1 (C-4′) suggested the presence of a *p*-substituted benzene ring. The lower field shifted C-4′ carbon suggested it was oxygenated. In the ^{13}C NMR spectrum of **48,** 10 carbon resonances, including six methines, three methylene, and one methyl, were displayed in its aliphatic field. Among these resonances, the oxygenated five

methine carbons at δ 102.6 (C-1′′), 75.2 (C-2′′), 78.2 (C-3′′), 71.9 (C-4′′), and 75.4 (C-5′′), and one methylene carbon at δ 64.9 (C-6′′) suggested the presence of a 6-acylated glucopyranosyl moiety (Shikishima et al., 2001). As a result of sugar analysis that was carried out using HPLC after acid hydrolysis of **48**, sugar moiety was assigned as D-glucose. The HMBC spectrum exhibited long-range correlations between H-6′′ and C-7′′′ displayed that the acyl group was the galloyl group. In the ^{1}H-^{1}H COSY spectrum of **48** (Figure 12), protons at δ 1.17, 3.80, 1.78, 1.65, and 2.52 indicated the presence of 2-oxygenated butyl moiety. The HMBC long-range correlation from H-1" to C-2 suggested that the D-glucopyranosyl moiety connected to C-2. The β-orientation of this sugar moiety was verified by the coupling constant (J = 7.5 Hz) of the anomeric proton in the glucosyl moiety. The long-range correlations from H-4 to C-1', C-2', and C-6' in the HMBC spectrum indicated that the *p*-substituted benzene ring connected to C-4. Based on the above data, the chemical structure of **48** was identified as 4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside. However, another compound with that same molecular structure with **48** was previously reported and named as (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (Shikishima et al., 2001). But, the NMR data of both compounds were not similar. By applying the rule of glycosidation shifts (Seo et al., 1978), the absolute configuration of C-2 in **48** was indicated to be (2*R*)-configuration. It was verified by comparison with (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (Shikishima et al., 2001), 2*R*-rhododendron (**51**) (Kim et al., 2011) and (*R*)-(–)-rhododendrol (**52**) (Kim et al., 2011). Hence, compound **48** was established as (*R*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside.

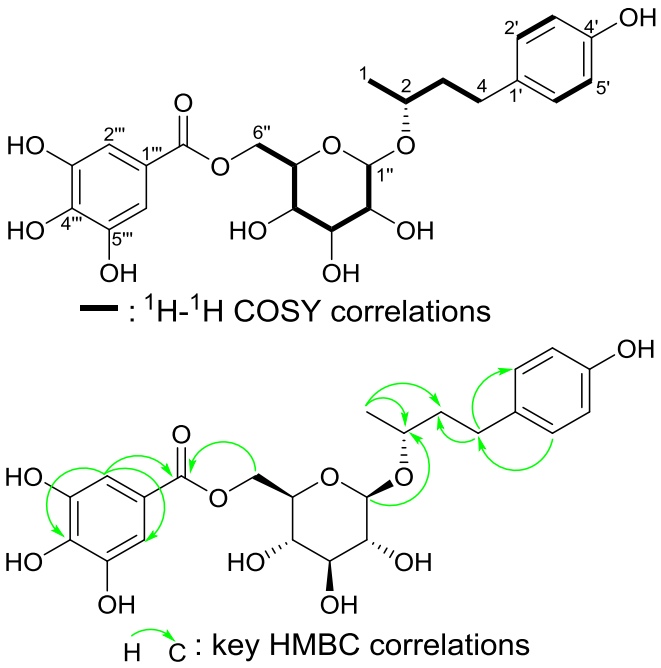


Figure 12. ^{1}H-^{1}H COSY and key HMBC correlations of compound **48**

Compound **49** showed a molecular formula of $C_{11}H_{12}O_3$ based on the molecular ion peak at *m/z* 192.0790 $[M]^+$; calcd for $C_{11}H_{12}O_3$, 192.0786 in HREIMS. In the ^{1}H NMR spectrum of **49**, two doublet methine protons at δ 7.00 (2H, d, *J* = 8.5 Hz, H-2′, 6′) and 6.69 (2H, d, *J* = 8.5, H-3′, 5′), and two olefinic protons at δ 5.78 (1H, d, *J* = 15.5 Hz, H-2) and 6.91 (1H, m, H-3), and two methylene protons at δ 2.46 (2H, m, H-4) and 2.67 (2H, t, *J* = 7.0 Hz, H-5) were observed. The ^{1}H–^{1}H COSY spectrum of **49** (Figure 13) showed the long-range correlations from H-3 with H-2 and H-4, H-5 with H-4, which indicating the presence of an aliphatic butyl chain. The coupling constant of the two olefinic proton resonances (*J* = 15.5 Hz) between H-2 and H-3 suggested their *E* configuration. Moreover, the ^{13}C NMR spectrum exhibited a quaternary carbon resonance at δ 171.0 (C-1), indicating the presence of the carboxylic acid moiety, and it was long-range correlated with H-2 and H-3 in its HMBC spectra (Figure 13). In addition, the ^{13}C NMR spectrum showed four aromatic carbon resonances at δ 133.2 (C-1′), 130.4 (C-2′

and 6′), 116.2 (C-3′ and 5′), and 156.6 (C-4′), which suggested the presence of 4-oxygenated benzene ring. The HMBC correlations from H-5 to C-1', C-2', and C-6' established that the 4-hydroxy phenyl moiety connected to C-5. From these data, **49** was determined as 5-(4-hydroxyphenyl) 2-pentenoic acid.

Table 6. ^{1}H and ^{13}C NMR spectroscopic data of compounds 48-49

48			**49**		
position	δ_H (*J* in Hz)	δ_C	position	δ_H (*J* in Hz)	δ_C
1	1.17, d (6.0)	20.2	1		171.0
2	3.80, m	75.6	2	5.78, d (15.5)	123.8
3	1.65, m 1.78, m	40.6	3	6.91, m	149.4
4	2.52, m	31.8	4	2.46, m	35.4
1'		134.7	5	2.67, t (7)	34.7
2'	6.91, d (8.5)	130.4	1'		133.2
3'	6.59, d (8.5)	116.0	2'	7.0, d (8.5)	130.4
4'		156.1	3'	6.69, d (8.5)	116.2
5'	6.59, d (8.5)	116.0	4'		156.6
6'	6.91, d (8.5)	130.4	5'	6.69, d (8.5)	116.2
1"	4.34, d (7.5)	102.6	6'	7.0, d (8.5)	130.4
2"	3.22, dd (7.5, 9.0)	75.2			
3"	3.39, t (9.0)	78.2			
4"	3.45, t (9.0)	71.9			
5"	3.53, m	75.4			
6"	4.45, dd (12.5, 5.5) 4.50, dd (12.5, 2.5)	64.9			
1‴		121.6			
2‴	7.11, s	110.3			
3‴		146.6			
4‴		139.9			
5‴		146.6			
6‴	7.11, s	110.3			
7‴		168.5			

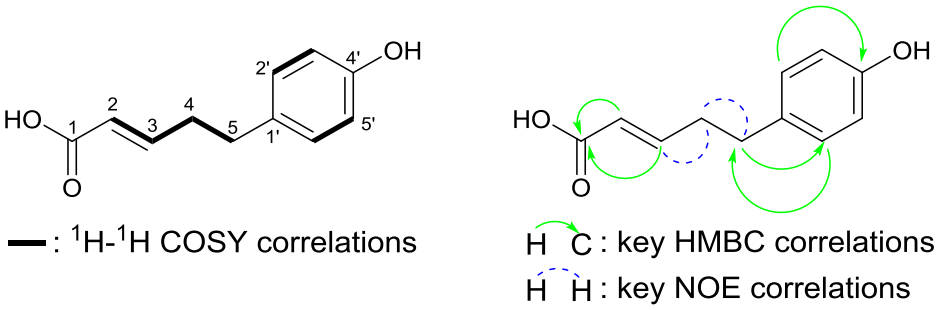


Figure 13. ^{1}H-^{1}H COSY, key HMBC, and NOE correlations of compound **49**

4.2.4. Insect phenoloxidase inhibitory activity of isolated compounds

According to the report of Sae-leaw et al. (2017), some phenolic compounds showed inhibitory activity against phenoloxidase by interacting with the active sites of the enzymes. Some compounds isolated from *C. mongolicum* contained benzene rings in their chemical structures. Based on this evidence, anti-phenoloxidase assay was carried out for compounds with catechol groups. By the time conducted biological experiments on the isolated compounds, not only were the anti-phenoloxidase activity investigated but also were acetylcholinesterase and tyrosinase inhibitory activities estimated. Consequently, no compounds exhibited specific activities on electric eel acetylcholinesterase and mushroom tyrosinase. But, compounds **54** and **55** displayed inhibitory activities on insect phenoloxidase (Table 5). From these compounds, catechin (**54**) (9.1 μM) exhibited ten times stronger IC_{50} value compared with epicatechin (**55**) (148.3 μM). In order to verify this result, guaranteed pure substances, (+)-catechin hydrate (18.6 μM) and (–)-epicatechin (195.8 μM), were used. Generally, there was a similar molecular structure in compounds **54** and **55**. So, it can be clearly seen that stereochemistry of C-2 and C-3 in their chemical structure may influence the inhibition activity against the phenoloxidase. Previously, Odonbayar et al. (2016) reported that gallocatechin exhibited a phenoloxidase inhibitory activity and more effective than epigallocatechin. They indicated that

pyrogallol B-ring showed a high effect than that of catechol B-ring. Even so, catechin (**54**) with catechol B-ring exhibited a potent activity than gallocatechin in this study. Moreover, it is the best way to continue further studies on the structure-activity relationship of catechin derivatives. By doing experiments about the insect phenoloxidase inhibitors, it can result to control of pests and understanding of the interactions between plant chemicals and insect immune systems.

Table 7. Insect phenoloxidase inhibitory activities for identified compounds from the aerial parts of *C. mongolicum*

Compound	IC_{50} (μM)
54[a]	9.1 ± 0.3
catechin[b]	18.6 ± 0.6
55[a]	148.3 ± 2.6
epicatechin[c]	195.8 ± 4.8
N-phenylthiourea	0.053 ± 0.001

[a]Compounds were isolated from *C. mongolicum*. [b]The compound was guaranteed by the Tokyo chemical industry. [c]The compound was guaranteed by the Fujifilm. The treatment was replicated three times for each concentration

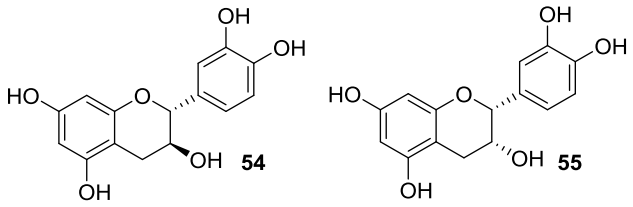


Figure 14. Chemical structures of compounds **54** and **55** with anti-phenoloxidase activity

4.3. Conclusion

To summarise, chemical investigations of an extract from the aerial parts of *C. mongolicum* led to the isolation and identification of two new and 20 known compounds, including flavonoids, alkaloids, monoterpenoid, phenol, and glycosides. As far as we know, compounds **48** and **49** were not reported previously to the literature. Compound **50**

was newly obtained from natural sources. Asides from that, all of the compounds (**48**-**69**) were isolated from this plant for the first time. Some of the isolated compounds were estimated for enzyme inhibitory activity tests. Among them, catechin (**54**) showed ten times higher phenoloxidase inhibitory activity (IC_{50} 9.1 μM) than epicatechin (**55**) (IC_{50} 148.3 μM). Compounds **54** and **55** have a common molecular structure except for their stereochemistry, and this result was supported by a reproducibility test using pure guaranteed authentic samples.

4.4. Experimental section

4.4.1. General experimental procedures

The specific rotation was taken on a JASCO P-2300 polarimeter (JASCO, Tokyo, Japan). NMR experiments were carried out using a JEOL JNM-AL400 FT-NMR spectrometer (JEOL, Tokyo, Japan) operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, and chemical shifts were given as δ values with TMS as an internal standard at 25 °C (measured in methanol-d_4, chloroform-*d*, and pyridine-d_5). HMQC (optimized for $^{1}J_{C–H}$ = 145 Hz) and HMBC (optimized for $^{n}J_{C–H}$ = 8 Hz) pulse sequences with a pulsed field gradient. HRFABMS, and HREIMS data were processed using a JEOL JMS700 mass spectrometer (JEOL) with a glycerol matrix. Preparative and analytical HPLC was performed using a JASCO 2089 (JASCO) with UV detection at 210 nm, using the following columns: Ultra Pack ODS-SM-50C-M (Yamazen, Osaka, Japan, 37 × 100 mm), TSKgel ODS-120T (Tosoh, Tokyo, Japan, 21.5 × 300 mm), Mightysil RP-18 GP (Kanto Chemical, Tokyo, Japan, 10 × 250 mm), Cosmosil 5C_{18} AR-II (Nacalai Tesque, Kyoto, Japan, 20 × 250 mm) and Develosil C_{30}-UG-5 (Nomura Chemical, Aichi, Japan, 20 × 250 mm).

4.4.2. Plant materials

The aerial parts of *Calligonum mongolicum* were collected in July 2012 from Segs Tsagaan Bogd Mountain, Shine Jinst soum, Bayankhongor province, Mongolia. A voucher specimen was deposited at the herbarium of Laboratory of Bioorganic Chemistry and Pharmacognosy, National University of Mongolia, and identified by Prof. Ch. Sanchir, Institute of Botany, Mongolian Academy of Sciences.

4.4.3. Extraction and isolation

The powdered and air-dried aerial parts (250 g) were extracted with 7.5 L of acetone-water (4:1) at room temperature for 4 days. After filtration, the extracts were combined and evaporated *in vacuo* to yield 42 g of extract. The evaporated extract was suspended in water (0.5 L) and then partitioned with diethyl ether (3 × 0.5 L). The aqueous extract (37.8 g) was subjected to DIAION HP-20 column chromatography with a gradient eluent of water-methanol (1:0 to 0:1, v/v) to give five fractions (1A-1E). Fraction 1C (4.0 g) was chromatographed over a reverse-phase ODS-SM-50C-M column eluted with water-methanol (gradient system from 4:1 to 3:2, v/v) to afford subfractions 2A-2L. Subfraction 2C (82.5 mg) was separated by preparative HPLC to yield compounds **54** (10.3 mg) and **64** (6.0 mg) [TSKgel ODS-120T, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA]. Subfractions 2F-H (622.0 mg) were purified by preparative HPLC to obtain compounds **51** (113.4 mg), **55** (0.9 mg), **56** (4.5 mg), and **65** (1.9 mg) [TSKgel ODS-120T, CH_3CN–H_2O (4:16, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (6:14, v/v) containing 0.2% TFA]. Fraction 1D (12.5 g) was loaded on a reverse-phase ODS-SM-50C-M column eluted with water-methanol (gradient system from 4:1 to 1:1, v/v) to produce subfractions 3A-T. Subfractions 3J-K (172.4 mg) were separated by preparative HPLC to obtain compounds **48** (3.5 mg) and **60** (54.5 mg) [TSKgel ODS-120T, CH_3CN–H_2O (1:4, v/v)

containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (1:4, v/v) containing 0.2% TFA] and subfractions 3H-I (169.9 mg) were subjected to preparative HPLC to obtain compound **61** (87.4 mg) [TSKgel ODS-120T, CH_3CN–H_2O (1:4, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (1:4, v/v) containing 0.2% TFA].

The diethyl ether extract (3.7 g) that was subjected to silica gel column chromatography was eluted with *n*-hexane-acetone (gradient system from 1:0 to 0:1, v/v) and aqueous methanol (1:1 and 0:1, v/v) to generate 37 fractions (4A-4K1). Fractions 4M-P (150.8 mg) were applied to the column using HP-20 resin with a gradient eluent of water-methanol (1:4 and 0:5, v/v) to isolate two subfractions (5A-5B). 5A (39.9 mg) was purified by HPLC on the 5C_{18}-AR-II column with CH_3CN–H_2O (1:4, v/v) to achieve compounds **50** (10.1 mg) and **53** (3.7 mg). Fractions 4S-T (118.2 mg), 4U (213.4 mg), and 4-X (315.8 mg) were isolated using the same procedure as 4M-P to yield compounds **49** (2.7 mg), **52** (119.0 mg), **57** (12.7 mg), **58** (3.3 mg), **59** (2.3 mg), **62** (27.7 mg), **63** (1.3 mg), **66** (2.6 mg), **67** (13.7 mg), **68** (9.6 mg), and **69** (20.9 mg).

4.4.3.1. *R*-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (**48**)

Yellowish, amorphous solid; $[\alpha]^{22}_D$ –15.7° (*c* 0.05, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz): δ 7.11 (2H, s, H-2‴, 6‴), 6.91 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.59 (2H, d, *J* = 8.5 Hz, H-3', 5'), 4.50 (1H, dd, *J* = 12.5, 2.5 Hz, H-6"), 4.45 (1H, dd, *J* =12.5, 5.5 Hz, H-6"), 4.34 (1H, d, *J* = 7.5 Hz, H-1"), 3.80 (1H, m, H-2), 3.53 (1H, m, H-5"), 3.45 (1H, t, *J* = 9.0 Hz, H-4"), 3.39 (1H, t, *J* = 9.0 Hz, H-3"), 3.22 (1H, dd, *J* = 7.5, 9.0 Hz, H-2"), 2.52 (1H, m, H-4), 1.78 (1H, m, H-3), 1.65 (1H, m, H-3), 1.17 (1H, d, *J* = 6.0 Hz, H-1); ^{13}C NMR (methanol-d_4, 100 MHz): δ 168.5 (C-7‴), 156.1 (C-4'), 146.6 (C-3‴, 5‴), 139.9 (C-4‴), 134.7 (C-1'), 130.4 (C-2', 6'), 121.6 (C-1‴), 116.0 (C-3', 5'), 110.3 (C-2‴, 6‴), 102.6

(C-1"), 78.2 (C-3"), 75.6 (C-2), 75.4 (C-5"), 75.2 (C-2"), 71.9 (C-4"), 64.9 (C-6"), 40.6 (C-3), 31.8 (C-4), 20.2 (C-1); HMBC (methanol-d_4, 400 MHz): from 1.17 (H-1) to 75.6 (C-2), and 40.6 (C-3); from 2.52 (H-4) to 40.6 (C-3), 134.7 (C-1'), and 130.4 (C-2', 6'); from 6.91 (H-2') to 31.8 (C-4), 130.4 (C-6') and 156.1 (C-4'); from 6.59 (H-3') to 134.7 (C-1'); from 4.34 (H-1") to 75.6 (C-2); from 3.22 (H-2") to 102.6 (C-1"); from 4.45 (H-6") to 168.5 (C-7'"); from 7.11 (H-2'") to 121.6 (C-1'"), 146.6 (C-3'"), 139.9 (C-4'"), 110.3 (C-6'"), and 168.5 (C-7'"); HRFABMS (positive) *m/z* 481.1709 $[M+H]^+$ (calcd for $C_{23}H_{29}O_{11}$, 481.171).

4.4.3.2. 5-(4-hydroxyphenyl) 2-pentenoic acid (49)

Yellowish, amorphous solid; ^{1}H NMR (methanol-d_4, 400 MHz): δ 7.00 (2H, d, *J* = 8.5, H-2', 6'), 6.91 (1H, m, H-3), 6.69 (2H, d, *J* = 8.5, H-3', 5'), 5.78 (1H, d, *J* = 15.5, H-2), 2.67 (1H, t, *J* = 7.0, H-5), 2.46 (1H, m, H-4); ^{13}C NMR (methanol-d_4, 100 MHz): δ 171.0 (C-1), 156.6 (C-4'), 149.4 (C-3), 133.2 (C-1'), 130.4 (C-2', C-6'), 123.8 (C-2), 116.2 (C-3', C-5'), 35.4 (C-4), 34.7 (C-5); HMBC (methanol-d_4, 400 MHz): from 5.78 (H-2) to 171.0 (C-1), and 35.4 (C-4); from 6.91 (H-3) to 171.0 (C-1), and 35.4 (C-4); from 2.46 (H-4) to 123.8 (C-2), 149.4 (C-3), 34.7 (C-5) and 133.2 (C-1'); from 2.67 (H-5) to 149.4 (C-3), 35.4 (C-4), 133.2 (C-1'), and 130.4 (C-2'); from 7.0 (H-2') to 34.7 (C-5), 130.4 (C-6'), 116.2 (C-3'), and 156.6 (C-4'); from 6.69 (H-3') to 130.4 (C-2'), 116.2 (C-5'), and 156.6 (C-4'); HREIMS (positive) *m/z* 192.0790 $[M]^+$ (calcd for $C_{11}H_{12}O_3$, 192.0786).

4.4.4. Sugar identification

In accordance with a method described previously, hexose moiety identification was accomplished (Tanaka et al., 2007). Compound **48** (1.0 mg) was separately hydrolyzed with 7% HCl (1 mL) at 60°C for two hours, and then the mixtures were subjected to aqueous sugar fractionation. The concentrated sugar fraction was stirred with L-cysteine

methyl ester (3 mg) in pyridine (0.5 mL) at 60ºC, one hour. Then *o*-tolyl isothiocyanate (3 μL) was added to the solution (60ºC, one hour). The reaction mixtures were analyzed by HPLC (Siseido, Capcel Pak C_{18}, 4.6 × 250 mm; CH_3CN–H_2O (1:3, v/v) containing 0.2% TFA, 1.0 mL/min; detection at 250 nm). The peaks of authentic L-glucose (*t*R = 15.8 min) and D-glucose (*t*R = 17.0 min) derivatives were used to identify the *O*-glucosidic moieties in **48** as D-glucose based on the corresponding retention times of 17.0 min.

4.4.5. *A. pisum* phenoloxidase assay

Insect phenoloxidase-containing crude enzyme solution from *A. pisum* was prepared, and tests were performed using the previously reported method (Odonbayar et al., 2016). Adults and the last instars of *A. pisum* (295 mg) were collected; H_2O (7.3 mL) was added, and they were frozen and stored at –20 °C for 24 h. After pestle homogenization and centrifugation (7,000 rpm, 5 min), the solution was filtered (0.22 μm, 25 mm, hydrophilic nylon, Starlab Scientific, China). The phenoloxidase activity was assayed spectrophotometrically using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as the substrate as described in the previous report (Odonbayar et al., 2016). The plate was incubated at 25 ˚C, and the absorbance was measured at 510 nm after 120 and 240 min. The percentage inhibition of insect phenoloxidase was calculated as follows: Inhibition percent (%) = [1 – (Sample $Abs_{510nm,240min}$ – Sample $Abs_{510nm,120min}$)/(Control $Abs_{510nm,240min}$ – Control $Abs_{510nm,120min}$)] x 100. Each sample (final concentration: 1,000, 500, 100, 50, 10 μM) was used for the assay, and *N*-Pahenylthiourea (final concentration: 1.0, 0.1, 0.01, 0.001 μM) was used as a positive control.

CHAPTER V. CHEMICAL CONSTITUENTS OF *APOCYNUM PICTUM*

5.1. Introduction

Figure 15. *Apocynum pictum*

Scientific classification:
Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Gentianales
Family: Apocynaceae
Genus: *Apocynum*
Species: *A. pictum*

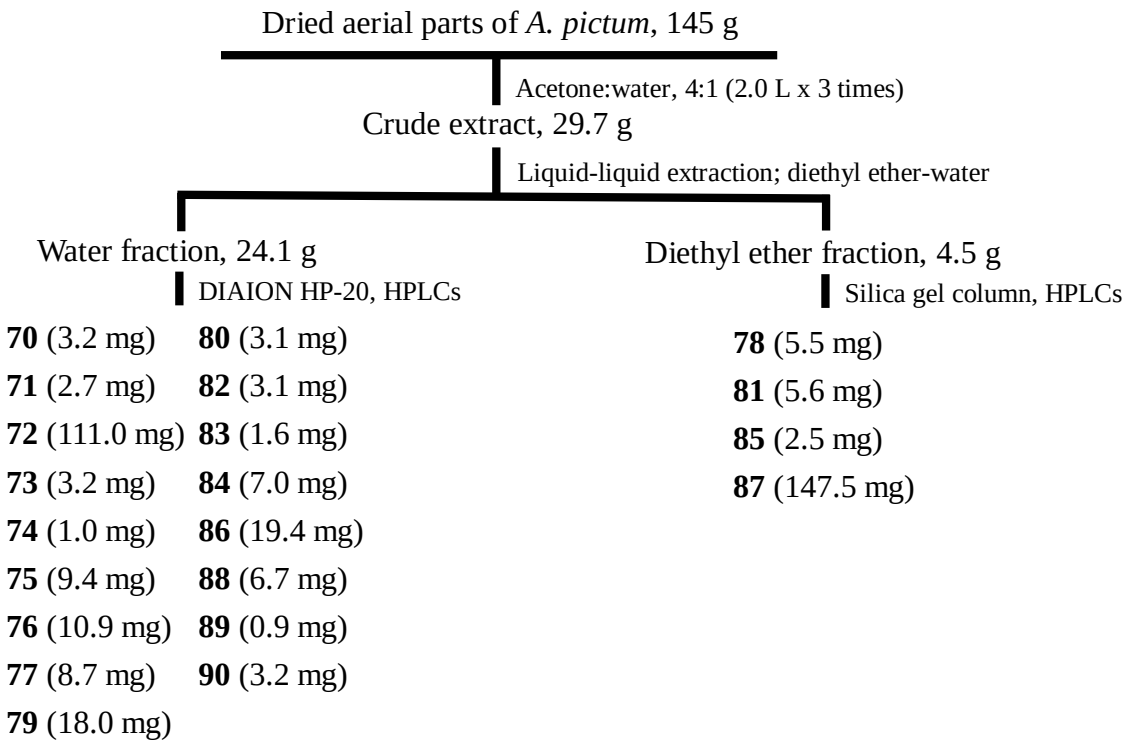
The genus *Apocynum* belongs to the family Apocynaceae and comprises five species, including *Apocynum pictum*, which are distributed in Central Asia, including Mongolia, Kyrgyzstan, Tajikistan, Northwest China, and Kazakhstan (Thevs 2012; Oyuntsetseg 2018). *A. pictum* that is locally known as White Hemp is a perennial herbaceous mainly grows in desert margins, riversides, and salt-barren zone (Oyuntsetseg 2018; Jiang 2018). Typically, this species is mixed up easily with *A. venetum* due to the fact that both are similar in terms of plant morphological characteristics and geographical distributions (Chan et al., 2015). These plants are popular in traditional Uygur and Chinese medicine, which uses to clear heat, calm the liver, promote diuresis, and diminish inflammation (Xie et al., 2012). Moreover, in the industries, *A. pictum* is used as fiber plants for spinning and papermaking. *Apocynum* fibers show an anti-microbial effect because it made by tanning agents (Chan et al., 2015). According to the phytochemical studies of this genus, it is known to contain numerous flavonoids, phytosterols, organic acids, and glycosides (Xie et al., 2012). However, quite a few studies have been carried out on the chemical constituents of *A. pictum*. Interestingly, regarding the sesquiterpene derivative, it reported

for the first time in not only *A. pictum* but also this genus.

5.2. Results and discussion

5.2.1. Extraction and isolation

Scheme 3. Extraction and isolation of aerial parts of *A. pictum*



By doing various separations and purifications on the extract of *A. pictum*, its phytochemical constituents were demonstrated to have one new and 20 known compounds.

5.2.2. Identification of known compounds

The structures of known compounds were identified as desacylcynaropicrin (**71**) (Rustaiyan et al., 1981), cynaropicrin (**72**) (Choi et al., 2005), desacylcynaropicrin 8-*O*-(*S*)-3-hydroxy-2-methylpropionate (**73**) (Marco et al., 1992), cebellin F (**74**) (Choi et al., 2005), 8α-hydroxy-11α,13-dihydrozaluzanin C (**75**) (Choi et al., 2005), 3α-hydroxy-11β,13-dihydrodehydrocostuslactone 8-*O*-β-D-glucose (**76**) (Li et al., 1989), (6*S*,9*R*)-6-

hydroxy-3-oxo-α-ionol-9-*O*-β-D-glucopyranoside (**77**) (Kuang et al., 2008), hispidulin (**78**) (Nagao et al., 2002), apigenin 7-*O*-β-D-glucuronide (**79**) (Ahmed et al., 1989), apigenin 7-*O*-β-D-glucuronide methyl ester (**80**) (Lee et al., 2002), apigenin 7-rutinoside (**81**) (Wang et al., 2003), secoisolariciresinol β-D-glucoside (**82**) (Baderschneider et al., 2001), 2-phenylethyl β-glucopyranoside (**83**) (Yoneda et al., 2008), (*E*)-8-*O*-D-glucopyranosylcinnamic acid (**84**) (Cho et al., 2014), 4-hydroxy-3-methoxycinnamic acid (**85**) (Xing et al., 2003), 3-caffeoylquinic acid (**86**) (Janda et al., 2009), 1-*O*-,5-*O*-dicaffeoylquinic acid (**87**) (Murata et al., 1995), 3-*O*-,5-*O*-dicaffeoylquinic acid (**88**) (Pauli et al., 1998), 4-hydroxyacetophenone (**89**) (Ding et al., 2000), and tryptophan (**90**) (Yan et al., 1999) by comparing their MS, ^{1}H and ^{13}C NMR data with those in the literature (Figure 16).

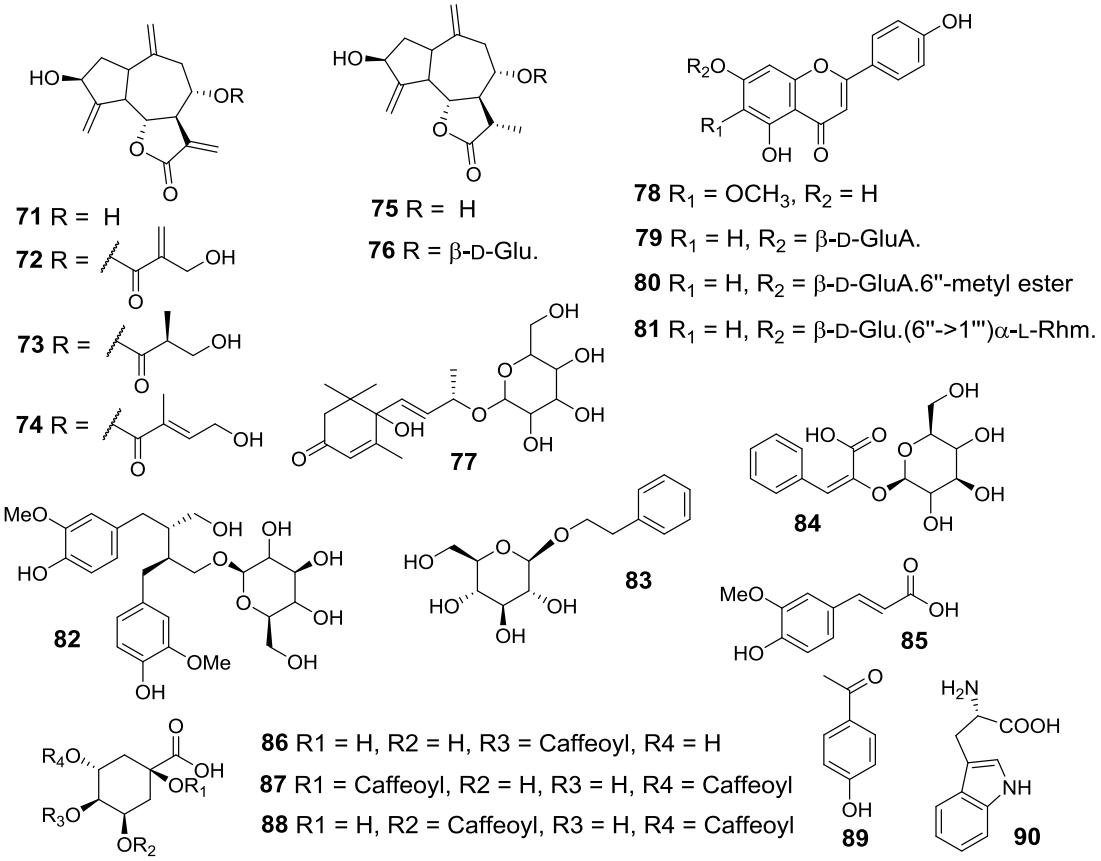


Figure 16. Chemical structures of known compounds isolated from *A. pictum*

5.2.3. Structural elucidation of new compound 70

Compound **70** demonstrated a molecular formula of $C_{20}H_{26}O_6$ based on the molecular ion peak $[M+H]^+$ at *m/z* 362.1700 $[M]^+$; calcd for $C_{20}H_{26}O_6$, 362.1700 in HREIMS. The ^{1}H-NMR spectrum of **70** showed the presence of one methyl protons at δ 1.0 (3H, d, *J* = 6.7 Hz, H-4′), three oxygenated methine protons at δ 4.30 (1H, dd, *J* = 2.0 and 7.5 Hz, H-6), 4.49 (1H, t, *J* = 8.8 Hz, H-3), and 5.05 (1H, m, H-8), three pairs of exomethylene protons at δ 4.93 (1H, d, *J* = 2.0 Hz, H-14a), 5.14 (1H, brs, H-14b), 5.33 (1H, brs, H-15a), 5.43 (1H, brs, H-15b), 5.70 (1H, d, *J* = 3.0 Hz, H-13a), 6.14 (1H, d, *J* = 3.5 Hz, H-13b). In the ^{13}C NMR spectrum, there were observed two quaternary carbon resonances at δ 171.3 (C-12) and 174.0 (C-1′), which indicated the presence of the carbonyl group moiety, and three quaternary carbon resonances at δ 139.8 (C-11), 144.1 (C-10), and 154.1 (C-4), which correlated with H-13ab, H-14ab, and H-15ab in its HMQC spectrum, respectively. In addition, the ^{13}C NMR spectrum exhibited another 15 carbon resonances, including one methyl, seven methylenes (three olefinic and one oxygenated), and seven methines (three oxygenated) carbons. Moreover, analysis of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectra confirmed assignments of all ^{1}H and ^{13}C NMR data of **70**. Furthermore, these spectral data were compared with previously reported data of cebelin F (**74**) (Choi et al., 2005), which was almost identical to those of **70**. As a result, these pieces of evidence suggested that **70** was a derivative of quaiane-type sesquiterpene with a C-8 ester side chain. The main difference in the ^{1}H NMR spectra was the presence of methyl proton at δ 1.0 and methylene protons at δ 2.24 (1H, m, H-2′) and 2.56 (1H, dd, *J* = 6.0 and 15.0 Hz, H-2′) in **70**, while cebelin F (**74**) showed resonances at δ 6.89 (1H, t, *J* = 5.5 Hz, H-3′) and 1.88 (3H, s, H-5′). In terms of NOESY experiments, strong NOE cross-peaks were observed between H-1 and H-3; and H-5 and H-7, which indicated that H-1, H-3, H-5,

and H-7 were in α-orientation while H-6 and H-8 were β-oriented. The absolute configuration of the acyl group in this compound was not determined owing to a small quantity. Based on these pieces of evidence, compound **70** was determined to be (1α,3α,5α,6β,7α,8β)-8-hydroxy-3,6,9-trimethylene-2-oxododecahydroazuleno[4,5] furan-4-yl 4-hydroxy-3-methylbutanoate and named choninhorin (Figure 17).

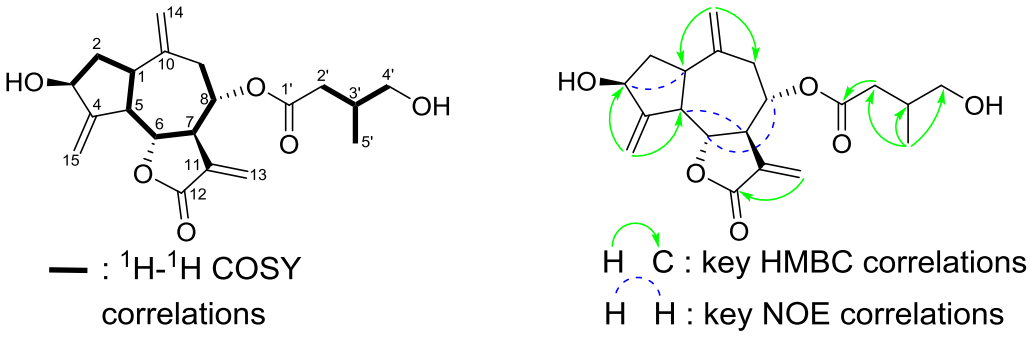


Figure 17. ^{1}H-^{1}H COSY, key HMBC and NOE correlations of compound **70**

5.3. Conclusion

Phytochemical investigations of an extract from the aerial parts of *A. pictum* led to the isolation and identification of one new and 20 known compounds, including flavonoids, sesquiterpenoids, glycosides, and amino acids. All of the compounds were isolated from this plant for the first time. Therefore, quaiane-type sesquiterpenes were reported newly in not only this species but also in the genus of *Apocynum*.

5.4. Experimental section

5.4.1. General experimental procedures

The specific rotation was taken on a JASCO P-2300 polarimeter (JASCO, Tokyo, Japan). NMR experiments were carried out using a JEOL JNM-AL400 FT-NMR spectrometer (JEOL, Tokyo, Japan) operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, and chemical shifts were given as δ values with TMS as an internal standard at 25 °C (measured in methanol-d_4, chloroform-d, and pyridine-d_5). HMQC (optimized for $^1J_{C–H}$

= 145 Hz) and HMBC (optimized for $^{n}J_{C–H}$ = 8 Hz) pulse sequences with a pulsed field gradient. HRFABMS and HREIMS data were processed using a JEOL JMS700 mass spectrometer (JEOL) with a glycerol matrix. Preparative and analytical HPLC was performed using a JASCO 2089 (JASCO) with UV detection at 210 nm, using the following columns: Ultra Pack ODS-SM-50C-M (Yamazen, Osaka, Japan, 37 × 100 mm), TSKgel ODS-120T (Tosoh, Tokyo, Japan, 21.5 × 300 mm), Mightysil RP-18 GP (Kanto Chemical, Tokyo, Japan, 10 × 250 mm), Cosmosil 5C_{18} AR-II (Nacalai Tesque, Kyoto, Japan, 20 × 250 mm) and Develosil C_{30}-UG-5 (Nomura Chemical, Aichi, Japan, 20 × 250 mm).

5.4.2. Plant materials

The whole plant of *A. pictum* was collected from Tsagaan Tohoi in the territory of Bayanundur soum, Bayankhongor province, Mongolia, at 1444 m above the sea level, in May 2017. The plant species was authenticated by Dr. Shagdar Dariimaa, Mongolian State University of Education. A voucher specimen (No.83.01.02.17A) was deposited at the Laboratory of Bioorganic Chemistry and Pharmacognosy, National University of Mongolia.

5.4.3. Extraction and isolation

The powdered plant of *A. pictum* (145 g) was extracted with 6 L of acetone-water (4:1) at room temperature for one week. The solvent extract was concentrated under reduced pressure to give 29 g of dry extract. The concentrated crude extract was suspended in water (1.5 L) and partitioned successively with diethyl ether (0.5 L). The aqueous extract (24.1 g) was subjected to DIAION HP-20 column chromatography with a gradient eluent of water-methanol (1:0 to 0:1, v/v) to provide five fractions (1A-1E). Fraction 1E (922.1 mg) was chromatographed over a reverse-phase ODS-SM-50C-M

column eluted with water-methanol (gradient system from 2:3 to 0:1, v/v) to yield fractions 2A-2F. Subfraction 2D (93.6 mg) was separated by preparative HPLC and then purified using the Mightysil C_{18} column and $CH_3CN–H_2O$ (3:7, v/v) as the mobile phase to obtain compounds **70** (3.2 mg), **73** (3.2 mg), and **74** (1.0 mg). Compound **72** (111.0 mg) was isolated from the subfraction 2C (247.6 mg) after purified via preparative HPLC with a C_{30}-UG-5 column and $CH_3CN–H_2O$ (3:7, v/v) as isocratic elution. Separation of fraction 1D (787.8 mg) over a reverse-phase ODS-SM-50C-M column with water-methanol (gradient system from 1:1 to 0:1, v/v) yielded subfractions 3A-3I. Subfraction 3E (208.1 mg) was subjected to C_{30}-UG-5 column eluted with $CH_3CN–H_2O$ (1:4, v/v) to afford compounds **71** (2.7 mg), **75** (9.4 mg), **79** (18.0 mg), **80** (3.1 mg), and **89** (0.9 mg). According to the same isolation procedure as above, compounds **82** (3.1 mg) and **88** (6.7 mg) were obtained from subfractions 3D (46.3 mg) and 3B (143.3 mg), respectively. Isolations of fraction 1C (703.5 mg) and 1B (536.8 mg) was performed on the reverse-phase ODS-SM-50C-M column using water-methanol (gradient system from 1:4 to 0:1, v/v) as eluent to obtain subfractions 4A-J and 5A-J, respectively. By purifying subfractions 4D (57.0 mg), 4G (102.8 mg) and 4I (21.4 mg) on the C_{30}-UG-5 column eluted with $CH_3CN–H_2O$ (1:4, v/v), compounds **76** (10.9 mg), **77** (8.7 mg), **83** (1.6 mg), and **90** (3.2) were isolated. Further purifications of subfraction 5C on a C_{30}-UG-5 column using $CH_3CN–H_2O$ (1:19, v/v) prepared with 0.2% TFA gave compounds **84** (7.0 mg) and **86** (19.4 mg).

The diethyl ether extract was fractionated via silica gel column chromatography using n-hexane-ethylacetate (gradient system from 1:0 to 0:1, v/v) and ethylacetate-methanol (1:1 and 0:1, v/v) to produce 14 fractions (6A-6N). Fractions 6L (2.0 g) were applied to the column using HP-20 resin with a gradient eluent of water-methanol (1:4

and 0:1, v/v) to generate two subfractions (7A-7B). Subfraction 7A was loaded on a reverse-phase ODS-SM-50C-M column eluted with CH_3CN–H_2O (3:7, v/v) to produce subfractions 8A-H, and then these subfractions were purified via preparative HPLC using C_{30}-UG-5 column and CH_3CN–H_2O (3:7, v/v) as a mobile phase to afford compounds **78** (5.5 mg), **81** (5.6 mg), **85** (2.5 mg), and **87** (147.5 mg).

5.4.3.1. (1α,3α,5α,6β,7α,8β)-8-hydroxy-3,6,9-trimethylene-2-oxododecahydroazuleno[4,5]furan-4-yl 4-hydroxy-3-methylbutanoate (70)

Colorless oil; ^{1}H NMR (methanol-d_4, 400 MHz): δ 6.14 (1H, d, *J* = 3.5 Hz, H-13b), 5.70 (1H, d, *J* = 3.0 Hz, H-13a), 5.43 (1H, brs, H-15b), 5.33 (1H, brs, H-15a), 5.14 (1H, brs, H-14b), 5.05 (1H, m, H-8), 4.93 (1H, d, *J* = 2.0 Hz, H-14a), 4.49 (1H, t, *J* = 8.8 Hz, H-3), 4.30 (1H, dd, *J* = 2.0 and 7.5 Hz, H-6), 3.47 (1H, d, *J* = 5.6 Hz, H-4′), 3.41 (1H, d, *J* = 6.5 Hz, H-4′), 3.19 (1H, m, H-7), 2.99 (1H, m, H-1), 2.87 (1H, t, *J* = 10.0 Hz, H-5), 2.71 (1H, dd, *J* = 5.0 and 15.0 Hz, H-9), 2.56 (1H, dd, *J* = 6.0 and 15.0 Hz, H-2′), 2.37 (1H, dd, *J* = 3.2 and 14.0 Hz, H-9), 2.24 (1H, m, H-2′), 2.17 (1H, m, H-3′), 2.08 (1H, m, H-2), 1.73 (1H, m, H-2), 1.0 (3H, d, *J* = 6.7 Hz, H-4′); ^{13}C NMR (methanol-d_4, 100 MHz): δ 174.0 (C-1'), 171.3 (C-12), 154.1 (C-4), 144.1 (C-10), 139.8 (C-11), 122.5 (C-13), 118.2 (C-14), 112.8 (C-15), 80.4 (C-6), 75.4 (C-8), 74.2 (C-3), 67.5 (C-4'), 52.1 (C-5), 46.2 (C-1), 40.1 (C-2), 39.4 (C-2'), 37.9 (C-9), 34.3 (C-3'), 17.1 (C-5'); HREIMS (positive) *m/z* 362.1700 $[M]^+$ (calcd for $C_{20}H_{26}O_6$, 362.1700).

Conclusion

In this thesis, the chemical constituents and biological activities of four species of plants growing in Mongolia, named *Oxytropis lanata*, *Brachanthemum gobicum*, *Calligonum mongolicum*, and *Apocynum pictum*, were investigated. Our main goal was to study the chemical constituents of these plants utilized in the traditional medicine of Mongolia. Asides from that, for us, it was really essential to investigate the trypanocidal activities of the compounds isolated from these species. As a result, just 90 compounds were identified from these plants (*O. lanata* 36; *B. gobicum* 11, *C. mongolicum* 22, and *A. pictum* 21), and almost all of them were tested against *T. congolense*, the causative agent of African trypanosomosis in animals. During this research, three new oleanane-type saponins (**1**, **2** and **3**) from *O. lanata*, two new compounds (**48** and **49**) from *C. mongolicum*, and a new quaiane-type sesquiterpene (**70**) from *A. pictum* were reported for the first time. The compounds **25** (IC_{50} = 10.5 μM) from *O. lanata* and **38** (IC_{50} = 2.8 μM) and **44** (IC_{50} = 2.4 μM) from *B. gobicum* showed an inhibitory activity against *T. congolense*. Other compounds obtained from them did not exhibit a potent activity against this parasite. Also, anti-hyaluronidase and anti-phenoloxidase assays were done for the compounds isolated from *O. lanata* and *C. mongolicum*, respectively. When it comes to the anti-hyaluronidase assay, compounds **3** and **9** showed significant inhibitory activity. This result suggests that these saponins can be beneficial in explaining the anti-inflammatory properties of *O. lanata*. In the anti-phenoloxidase screening, compound **54** showed ten times higher phenoloxidase inhibitory activity (IC_{50} 9.1 μM) than compound **55** (IC_{50} 148.3 μM). This active compound can contribute to understand the interactions between plant chemicals and insect immune systems.

With regard to *O. lanata*, both its roots and aerial parts have been investigated by our team since 2014. Roots mainly contained oxazole-type alkaloids and isoflavonoids. Seven of the oxazole derivatives were previously unreported. One of them, 2-(2′,3′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)oxazole, showed potent inhibitory activity against *T. congolense* (IC_{50} 1.0 μM). Based on these results derived from the roots of *O.lanata*, we decided to study aerial parts of this plant. We firstly expected that other oxazole derivatives might be obtained from the aerial parts. But oleanane-type saponins, isoflavonoids, and glycosides were isolated from the aerial parts. Also, there were no oxazoles except only two derivatives obtained before. The trypanocidal activity of 5,7,4′-trihydroxyisoflavone (**25**) isolated from the aerial parts was ten times lower to compare with 2-(2′,3′-dihydroxyphenyl)-5-(2′′-hydroxyphenyl)oxazole isolated from the roots. Numerous saponins have been considered to show anti-hyaluronidase activity, which is one of the most commonly used screenings for anti-inflammatory activity. Some oleanane-type saponins that we reported in this study demonstrated significant inhibitory activity, and their effect was stronger than that of sodium cromoglicate, which is used as a reference drug. In the future, the processes of total synthesis or semi-synthesis to modify the functional groups of the oxazoles will be necessary to generate novel lead compounds with potent trypanocidal activity and with fewer adverse effects. As mentioned before, *O. lanata* is traditionally used to treat bone fractures. To investigate the interaction between active constituents and bone formation can lead to new medication for broken bones. So, we will proceed with further studies on this species and also plan to cultivate *O. lanata* for protecting plant resources.

Our investigation on the aerial parts of *B. gobicum* led to the isolation and identification of previously undescribed 11 compounds, including eight isovaleryllignans

(**37**-**40** and **44**-**47**) and three isovalerylphenylpropanoids (**41**-**43**). But these compounds were considered as a racemic mixture because of optically inactive and no cotton effects in their electronic circular dichroism spectra. Separations of compounds **37**, **38**, **39**, **41**, **44**, and **45** with racemic mixture were carried out by using chiral column HPLC, and their absolute configurations were assigned by optical rotations and electronic circular dichroism spectra. For these purified compounds, trypanocidal screening was not conducted owing to their small amount of yield. Typically, it has been recognized that enantiomers show different biological activities depending on their stereochemistry. Studying other bioactivities for these purified compounds, depending on their structural features can expand the scientific understanding of this plant. Therefore, we will continue additional studies on *B. gobicum*.

Our further study tends to focus mainly on pasture plants. Pastoral animal husbandry is one of the important sectors of the Mongolian economy. As of 2019, approximately 70.9 million livestock, which usually feeds on pasture plants, were counted in Mongolia. Studying pasture plants and their effect on the livestock is significant to the developments of animal farming and product's quality of animal origin. To cite an example, pastoral animals feed the snow-covered grasses in the winter to survive in the Mongolian extreme continental climate, with heavy snow and long cold winter. Asides from that, in the spring season, exhausted animals can rejuvenate by feeding some tonic plants, such as *Pulsatilla flavescens* and *Artemisia sieversiana*. In terms of these mentioned two plants, our research team already investigated their chemical constituents and biological activity. Currently, the investigations of histology, biochemistry, physiology, and hematology are being carried out on the goats fed with *Pulsatilla flavescens*. By doing these experiments, the chance that explains how to influence this plant to regain livestock can be found.

Therefore, we have been attracting our attention to study the bioactivity of isolated compounds against zoonotic diseases, which are one of the big problems in the livestock husbandry of Mongolia. Trypanosomosis caused by *trypanosoma* species is an example of these diseases. These research works can lead to demonstrate scientific profitableness of plants and open the possibilities to find more highly effective compounds from Mongolian native plants.

List of Abbreviations

$[\alpha]_D$	specific rotation
A. pictum	*Apocynum pictum*
A. pisum	*Acyrthosiphon pisum*
B. gobicum	*Brachanthemum gobicum*
C. mongolicum	*Calligonum mongolicum*
CD	circular dichroism
CH_3CN	acetonitrile
COSY	correlation spectroscopy
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one
DMSO	dimethyl sulfoxide
Glc.	glucopyranosyl
GlcA.	glucuronopyranosyl
H_2O	water
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HPLC	high-performance liquid chromatography
HREIMS	high-resolution electron ionisation mass spectrometry
HRFABMS	high-resolution fast atom bombardment mass spectrometry
IC_{50}	50% inhibitory concentration
MeOH	methanol
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
O. lanata	*Oxytropis lanata*
Over.	overlapping
ROESY	rotating frame Overhauser effect spectroscopy
Rhm.	rhamnopyranosyl
T. congolense	*Trypanosoma congolense*
TFA	trifluoroacetic acid
TMS	tetramethylsilane
UV	ultraviolet
WHO	World Health Organization
δ_C	carbon delta scale that expresses chemical shifts
δ_H	proton delta scale that expresses chemical shifts

List of definitions

°C	celsius
kDa	kilodaleton
M	mole
m	meter
mg	milligram
MHz	megahertz
mL	millilitr
mM	millimole
ng	nanogram
rpm	Revolutions per minute
μg	microgram
μL	microliter
μM	micromole

List of figures

List of tables

List of schemes

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