Bioactive polysaccharides and phenolic compounds from two *Aconitum* plants: *Aconitum carmichaelii* and *Aconitum septentrionale*

PhD Thesis

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Being yourself and living well. 做好自己，好好生活

Oslo, October, 2022

Yu-Ping Fu

List of publications

I. **Yu-Ping Fu**, Yuan-Feng Zou, Fei-Yi Lei, Helle Wangensteen, Kari Tvete Inngjerdingen. *Aconitum carmichaelii* Debeaux: a systematic review on traditional use, and the chemical structures and pharmacological properties of polysaccharides and phenolic compounds in the roots. *Journal of Ethnopharmacology 2022, 291, 115148*.

II. **Yu-Ping Fu**, Cen-Yu Li, Yuan-Feng Zou, Xi Peng, Berit Smestad Paulsen, Helle Wangensteen, Kari Tvete Inngjerdingen. Polysaccharides with immunomodulatory effects from different plant parts of *Aconitum carmichaelii*. *Manuscript submitted to International Journal of Biological Macromolecules*

III. **Yu-Ping Fu**, Cen-Yu Li, Xi Peng, Yuan-Feng Zou, Frode Rise, Berit Smestad Paulsen, Helle Wangensteen, Kari Tvete Inngjerdingen. Polysaccharides from *Aconitum carmichaelii* leaves: structure, immunomodulatory and anti-inflammatory activities. *Carborhydrate Polymers 2022, 291, 119655.*

IV. **Yu-Ping Fu**, Cen-Yu Li, Xi Peng, Helle Wangensteen, Kari Tvete Inngjerdingen, Yuan-Feng Zou. Pectic polysaccharides from *Aconitum carmichaelii* leaves protects against DSS-induced ulcerative colitis in mice through modulations of metabolism and microbiota composition. *Biomedicine & Pharmacotherapy 2022, 1551, 113767.*

V. **Yu-Ping Fu**, Karl Egil Malterud, Anne Grethe Hamre, Kari Tvete Inngjerdingen, Helle Wangensteen. Bioactive phenolics and polysaccharides from the water extract of *Aconitum septentrionale* roots. *Manuscript*

Abbreviations

15-LO	15-Lipoxygenase
Ara	Arabinose
AG	Arabinogalactan
DHA	3-Deoxy-lyxo-2-heptulosaric acid
DMSO	Dimethyl sulfoxide
DSS	Dextran sulphate sodium
ESI/MS	Electrospray ionization-mass spectroscopy
Gal	Galactose
GalA	Galacturonic acid
GBIF	Global Biodiversity Information Facility
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
Glc	Glucose
GlcA	Glucuronic acid
HG	Homogalacturonan
HPLC	High performance liquid chromatography
IBD	Intestinal bowel diseases
IEC	Ion exchange chromatography
IFN-γ	Interferon γ
iNOS	Inducible nitric oxide synthase
KDO	3-Deoxy-manno-2-octulosonic acid
LPS	Lipopolysaccharides
Man	Mannose
MeOH	Methanol
Mw	Molecular weight
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NSPs	Non-starch polysaccharides
PRRs	Pattern-recognition receptors
Rha	Rhamnose
RG	Rhamnogalacturonan

SCFAs	Short chain fatty acids
SEC	Size exclusion chromatography
SR	Scavenger receptors
TCM	Traditional Chinese Medicine
TLR	Toll-like receptor
UC	Ulcerative colitis
XG	Xyloglucan
XGA	Xylogalacturonan
XO	Xanthine oxidase
Xyl	Xylose

Abstract

Aconitum plants are well known medicinal plants containing bioactive alkaloids and being poisonous to the neuro and cardiovascular system. However, non-alkaloid and low-toxic compounds have also been identified in these plants, especially phenolic compounds and polysaccharides, and these compounds have been reported with various biological activities. This thesis aimed to study non-alkaloid compounds present in two *Aconitum* plants, the commonly used *A. carmichaelii* and the less used *A. septentrionale*. Their biological activities of isolated compounds were determined by focusing on immunomodulatory and/or anti-inflammatory effects.

The lateral roots ("Fuzi") and mother roots ("Chuanwu") of *A. carmichaelii* are commonly used in Traditional Chinese Medicine, and different types of polysaccharides and 39 phenolic compounds like flavonoids, phenylpropanoids, lignans, neolignans, and benzoic acid derivatives have been isolated and identified from these roots. However, the rootlets and aerial parts are discarded after collection of the roots. In addition to the already known bioactive alkaloids, non-toxic polysaccharides could contribute to the immunomodulatory and anti-inflammatory effects observed by *A. carmichaelii*. However, there is limited knowledge on the detailed structural characterization and structure-activity relationships of polysaccharides from *A. carmichaelii*. In this study, six neutral and 16 acidic polysaccharide fractions were systematically isolated from different plant parts of *A. carmichaelii*, including the lateral roots, mother roots, rootlets, leaves, stems and the entire aerial parts. Monosaccharide composition and their linkage patterns indicated that the neutral fraction isolated from the rootlets was different from those isolated from the lateral and mother roots, consisting of less starch and more possible mannans, galactans, and/or xyloglucans, which was similar to those of the leaves and aerial parts. Pectic polysaccharides consisting of homogalacturonan (HG) and branched type I rhamnogalacturonan (RG)-I were present in all plant parts of *A. carmichaelii*. However, more of arabinogalactan (AG)-II side chains in the RG-I backbone were found in the aerial parts, especially in the leaves, while more of arabinans followed by AG-I/II were found in the roots and stems. One neutral and two acidic polysaccharides were obtained from the water extract of *A. septentrionale* roots. A high amount of starch and minor amounts of mannans or glucomannans were observed in the neutral fraction, while pectic polysaccharides composed of HG and RG-I, with branches of arabinans, AG-II and minor amounts of galactans were found in the acidic polysaccharide fractions. Starch and possibly other types of glucans might be tightly attached to the main structure of the acidic fractions.

The isolated polysaccharides from different plant parts of *A. carmichaelii* were studied regarding their *in vitro* complement fixation activity and inhibition of the transcription of pro-inflammatory cytokines. It was found that the presence of arabinan and AG-II were positively correlated with complement fixation activity, while correlations between anti-inflammatory effects and specific structural elements were not apparent. The major acidic polysaccharide isolated from the leaves (AL-I), being the fraction with the highest yield, exhibited both anti-inflammatory effect and complement fixation activity. This fraction was therefore chosen for a further comprehensive structural analysis by NMR and for investigation in an experimental ulcerative colitis (UC) mouse model, where UC was induced by dextran sulphate sodium (DSS). AL-I was found to alleviate symptoms and colonic pathological injury in colitis mice, and ameliorate the levels of inflammatory indices in serum and colon. The production of short- and branched-chain fatty acids was also restored by AL-I. The observed protective effect could be due to the inhibition of nucleotide-binding oligomerization domain 1 (NOD1) and Toll-like receptor 4 (TLR4) activation, the promotion of gene transcription of tight-junction proteins, and the modulation of gut microbiota composition like *Bacteroides*, *Dubosiella*, *Alistipes* and *Prevotella*. A regulation of serum metabolomic profiles being relevant to the bacterial change, such as D-mannose 6-phosphate, D-erythrose 4-phosphate and uric acid, was also observed. Polysaccharides from the water extract of *A. septentrionale* roots were shown to be inactive on the inhibition of NO release on LPS+IFN-γ activated dendritic cells as anti-inflammatory substances.

The presence of several phenolic compounds of *A. carmichaelii* roots have been reported, but those of *A. septentrionale* roots remains mostly unknown so far. In this study, phenolic compounds were isolated and identified from the water extract of *A. septentrionale* roots (WEAS). WEAS was initially fractionated by chromatography by Diaion HP-20, giving the main fractions D1-D5. Fractions D2 and D3 were further fractionated using reverse phase C18 flash chromatography, followed by Sephadex LH-20 and prep-HPLC. The obtained compounds were identified by NMR and ESI/MS. Fifteen phenolic compounds were obtained, and one of them is a new natural product. Three phenolic compounds exhibited inhibitory effects on nitric oxide (NO) release by LPS+IFN-γ activated dendritic cells. Two of the phenolic compounds showed higher inhibitory activity than the positive control, quercetin, towards 15-lipoxygenase, and one compound was a moderate inhibitor of xanthine oxidase.

Overall, the results in this thesis report on the composition and distribution of polysaccharides in different plant parts of *A. carmichaelii* and described the diversity of polysaccharides and

phenolics present in *A. septentrionale* roots for the first time. This study also highlighted the potential medicinal value of the unutilized parts of *A. carmichaelii*, especially the leaves, as a plant source of immunomodulatory and anti-inflammatory substances, and revealed *A. septentrionale* as a potential source of anti-inflammatory and anti-oxidative substances.

1 Introduction

1.1 A brief introduction to the *Aconitum* genus

Aconitum L. is a large genus of the Ranunculaceae family, consisting of over 300 species distributed all over the world. Most of them grow naturally at high altitudes in the northern hemisphere, and more than 200 of them are growing in China [1]. So far, only two *Aconitum* species are recorded in the Chinese Pharmacopoeia [2] and are used in Traditional Chinese Medicine (TCM), namely *Aconitum kusnezoffii* Reichb ("Caowu") and *Aconitum carmichaeli* Debeaux ("Chuanwu" and "Fuzi"). In Europe, *Aconitum* species have mainly been used as poisons [1].

Aconitum species contain a range of compounds that have shown various therapeutic effects. The majority of studies are related to the diterpene alkaloids, which are bioactive and toxic components [3]. So far, the *Aconitum*-derived alkaloids have been shown to possess arrhythmogenic (toxic), anti-arrhythmic, analgesic, anti-myocardial infarction, anti-epileptic, anti-microbial and anti-inflammatory effects, as well as alleviating neuropathic pain and treating rheumatoid arthritis [1, 3]. Non-alkaloid compounds have also been identified in these plants, including flavonoids, free fatty acids, phenylpropanoids, phenolics and acids, terpenoids, steroids, and polysaccharides. Some of these compounds have shown to exhibit anti-oxidative, anti-parasitic, anti-inflammatory, anti-neoplastic, hypoglycemic and immunomodulatory effects [4]. However, since *Aconitum* plants are toxic due to the content of alkaloids and their derivatives, affecting mainly the central nervous system, the heart, and the gastrointestinal system [1], relevant applications and scientific studies on these plants are relatively limited compared to other plant genera.

In the current study, two *Aconitum* species, *Aconitum carmichaelii* Debeaux collected in China and *Aconitum septentrionale* Koelle collected in Norway, were investigated. As shown in **Fig. 1-1**, *A. septentrionale* is more widely distributed around the world compared to *A. carmichaelii* [5]. However, the applications and scientific studies of *A. carmichaelii* is far more extensive compared to that of *A. septentrionale*, which could be due to the inclusion of the roots of *A. carmichaelii* in the Chinese Pharmacopoeia [2].

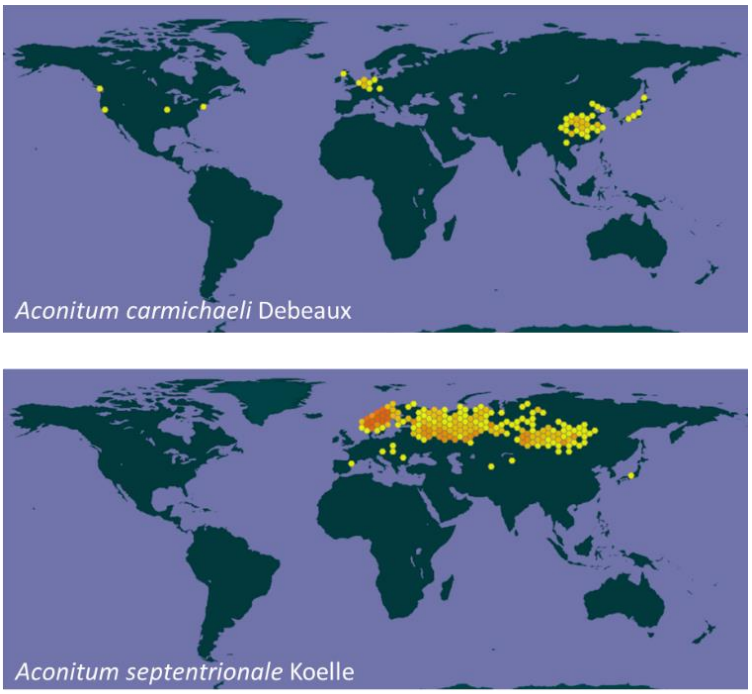


Fig. 1-1. Distribution of *A. carmichaelii* Debeaux and *A. septentrionale* Koelle in the world. Figures from Global Biodiversity Information Facility (GBIF) database [5].

1.2 *Aconitum carmichaelii*

1.2.1 The roots of *A. carmichaelii*, phytochemistry and pharmacological activities

A. carmichaelii is a commonly used *Aconitum* species in China. It is 0.6–1.5 m high with apically stems and sparsely retrorse pubescent, and pentagonal blade leaves (6–11 cm long and 9–15 cm wide, **Fig. 1-2**). It normally flowers in June, and roots are collected from late June to early August [6]. The lateral roots (known as "Fuzi", Aconiti Lateralis Radix Praeparata) and its processed products are traditionally used to rescue *Yang*, reverse collapse, augment *Fire* and *Yang*, dispel *Cold* and relieve pain in TCM [2, 7], and is frequently used to treat shock resulting from acute myocardial infarction, low blood pressure, coronary heart disease, and chronic heart failure in combination with other herbs in formulation [7]. The mother roots of *A. carmichaelii* "Chuanwu" (Aconiti Radix), is utilized independently from "Fuzi". They have been demonstrated having abilities similar to "Fuzi", such as dispelling *Wind* and *Dampness*, dispersing *Cold*, and relieving pain, but it is more frequently used for the treatment of rheumatism, joint pain, cold, abdominal colic and anesthesia in TCM theory [2]. The presence of alkaloids has been reported to be responsible for the pharmacological properties of both "Fuzi" and "Chuanwu" [7, 8].

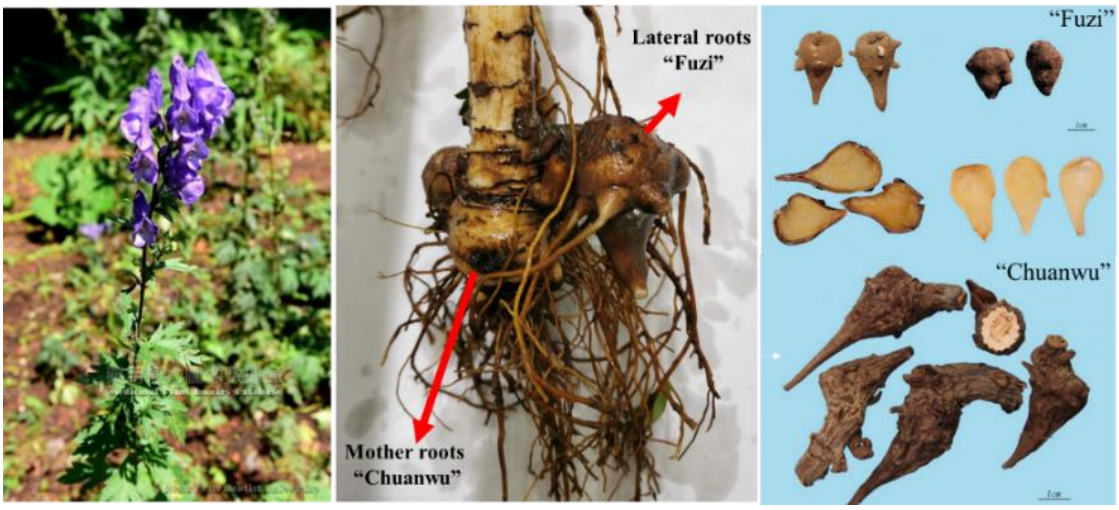
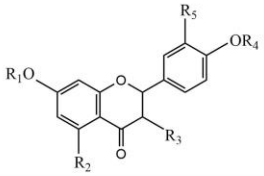


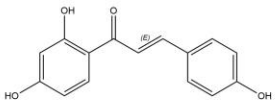
Fig. 1-2. The morphology of the aerial parts (left), roots (middle) and commercial roots (right) of *A. carmichaelii*. Pictures refer to the Chinese Medicinal Material Images Database [9].

In addition to alkaloids, a range of natural compounds have been reported isolated from roots of *A. carmichaelii*. From the aspect of polysaccharides, glucans were the first ones reported isolated from the roots of *A. carmichaelii*, including starch, 1→6 linked α-$_D$-glucan branched at *O*-3, and unclearly identified α- and β-glucans. Neutral heteropolysaccharides in different root parts of *A. carmichaelii* have been reported consisting of rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), mannose (Man), or xylose (Xyl) [10]. Only one neutral heteropolysaccharide isolated from the lateral roots ("Fuzi") has been reported and identified as a 1,4 linked-glucan with Ara side chains [11]. Acidic polysaccharides containing galacturonic acid (GalA) along with Gal, Ara, Rha, and minor amounts of other monomers, or glucuronic acid (GlcA) have also been reported in different root parts of *A. carmichaelii*. So far, most acidic polysaccharides have been isolated from the lateral roots ("Fuzi") with primary characterization, including polysaccharide content and monosaccharide composition, whereas less is known about polysaccharides from the mother roots ("Chuanwu") [10]. The isolated acidic polysaccharides lack information on detailed glycosidic linkage types. Various pharmacological properties of the isolated polysaccharides have been observed, such as hypoglycemic, hypolipidemic, immunomodulatory, anti-tumor, neuro-protective and anti-oxidative activities [10]. However, the structural characterization of the polysaccharides isolated from these roots needs to be further clarified, and also their biological properties. Especially polysaccharides isolated from the mother roots are not sufficiently studied. Various other secondary metabolites, especially phenolic compounds, have also been reported to be

present in these plants and may be involved in the medicinal functions of the *Aconitum* plants [4]. So far, 39 phenolic compounds have been isolated and identified from the roots of *A. carmichaelii*, including four flavonoids (compounds **1**-**4**), eighteen phenylpropanoids (compounds **5**-**22**), four lignans (compounds **23**-**26**), one neolignan (compound **27**), seven benzoic acid derivatives (compounds **28**-**34**), and five other phenolic compounds (compounds **35**-**39**), as listed in **Fig. 1**-**3**.



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	H	H
2	H	H	H	β-D-Glc*p*	H
3	H	H	H	6-*O*-Acetyl-β-D-Glc*p*	H



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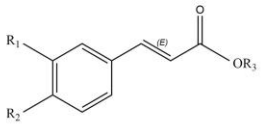
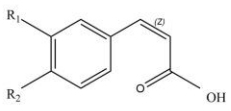
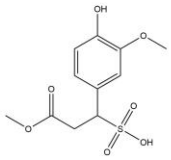


	R_1	R_2	R_3
5	H	*O*H	H
6	*O*-Me	OH	H
7	*O*-Me	OH	Me
8	*O*-Me	OH	*n*-Bu
9	*O*-β-D-Glc*p*	H	Me
10	*O*-Me	*O*-β-D-Glc*p*	H
11	*O*-Me	*O*-β-D-All*p*	Me
12	H	*O*-β-D-Glc*p*	Me
13	*O*-Me	*O*-β-D-Glc*p*	Me



	R_1	R_2
14	*O*-Me	*O*-β-D-Glc*p*
15	H	*O*-β-D-Glc*p*



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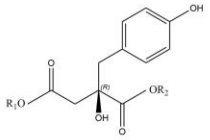
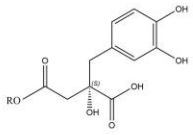
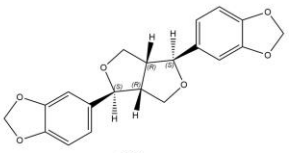


	R_1	R_2
16	H	H
17	Me	H
18	Me	Me



	R
19	H
20	Me
21	*n*-Bu



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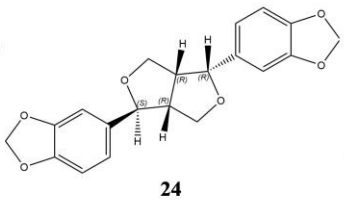

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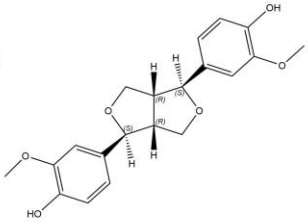

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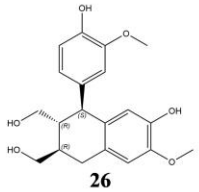

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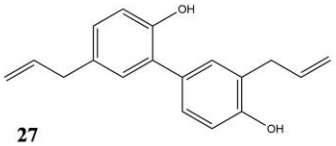

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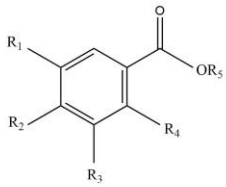
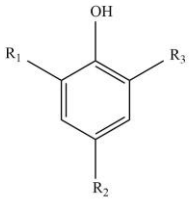
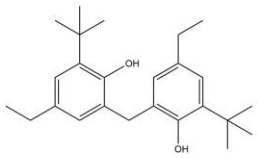
Fig. 1-3. Flavonoids (compound **1-4**), phenylpropanoids and derivatives (compound **5-22**), lignans (compound **23-26**), and neolignan (compound **27**), benzoic acids and derivatives (compound **28-34**) and other phenolic compounds (compound **35-39**) obtained from the roots of *A. carmichaelii* (figures refer to Paper I).



	R_1	R_2	R_3	R_4	R_5
28	H	H	H	OH	H
29	OH	*O*-Me	H	H	H
30	*O*-Me	OH	H	H	4-Butyl-1-methyl (+)-(R)-2-*O*-malate
31	H	OH	*O*-Me	H	1-Butyl-4-methyl (+)-(R)-2-*O*-malate
32	H	OH	H	H	Dimethyl-(+)-(R)-2-*O*-malate
33	*O*-Me	OH	H	H	Dimethyl-(+)-(R)-2-*O*-malate
34	H	H	H	OH	2-carbamoyl-4-hydroxy benzyl





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	R_1	R_2	R_3
35	*t*-Bu	*t*-Bu	H
36	*t*-Bu	H	*t*-Bu
37	*t*-Bu	Me	*t*-Bu
38	*t*-Bu	1,1,3,3-tetramethylbutyl	H

Fig.1-3. *(Continued)*

1.2.2 Nontraditionally used plant parts of *A. carmichaelii*, phytochemistry and pharmacological activities

In general, most phytochemical studies of *A. carmichaelii* have been performed on the root parts as they have been utilized in clinical traditional medicine, whereas limited information is available concerning extraction of phytochemicals from other plant parts. Therefore, it would be of importance to investigate the unutilized medicinal plant parts for possible bioactive compounds with potential pharmacological applications [12]. *A. carmichaelii* is a widely commercially traded plant, industrially grown in Sichuan and Shaanxi provinces in China, generating a huge amount of waste when processing “Fuzi” and “Chuanwu”. The rootlets and the aerial parts that include stems (60-150 cm high) and pentagonal leaves (6-11 cm long and 9-15 cm wide) are normally discarded when the roots are collected. These unutilized plant parts are potential medicinal materials that should be recycled and converted into valuable products. From the aspect of phytochemistry, the rootlets of *A. carmichaelii* has been reported occupying around 11% of the biomass of the traditional used “Fuzi”, and containing similar types of

natural product as those present in “Fuzi”, such as alkaloid and polysaccharides [13-17]. Further, the monosaccharide composition of the polysaccharides from rootlets was proposed being different from those isolated from the lateral and mother roots [14]. As for the aerial parts, polysaccharides [15, 16], alkaloids [16-21] and flavonoids [22, 23] have also been isolated, and this plant part was suggested to exhibit similar analgesic and anti-inflammatory activities as those of the traditionally used roots [19]. However, a systematic comparison of the chemical and biological properties of these polysaccharides is limited, and no detailed structural information and their differences has been reported, which could broaden the application of the whole plant as an alternative medicinal plant source.

1.3 *Aconitum septentrionale*

A. septentrionale is one of the *Aconitum* species (Ranunculaceae family) distributed widely in Norway, Sweden and Russia [5] with two subspecies *A. septentrionale* subsp. *rubicundum* (Fisch.) Vorosch. and *A. septentrionale* subsp. *septentrionale* [24]. The plant distributed in Norway (**Fig. 1-4**) is named *tyrihjelm* or *blå tyrihjelm* in Norwegian, or *ahkáras* in Northern Sami, as recorded in GBIF [5]. *A. septentrionale* is a perennial herb up to 2 m high, with a tall and straight flower stem covered with sparsely downward pubescent and blooms in July-August. The leaves are hand-lobed and 10-20 cm wide. This plant is traditionally used as a poison against flies and lice, and a decoction of the plant has been used to clean livestock and dogs externally [25].

Fig. 1-4. Distribution and morphology of *Aconitum septentrionale* in Norway. The map of plant distribution in Norway was obtained from Norwegian Biodiversity Information Center (https://www.biodiversity.no/). Photos were taken in August 2021 and 2022 by Yu-Ping Fu and Professor Emeritus Karl Egil Malterud.

Several alkaloids have been isolated and characterized from *A. septentrionale*, like lappaconitine, lappaconine, septentrionaline and cynoctonine that were isolated from the fresh plant in Norway [26] and from the roots in Russia [27-29]. The root part has been used as a source of lappaconitine hydrobromide, which is the basis of the antiarrhythmic drug allapinine [30]. Lipids [30, 31], amino acids [32] and organic acids [26, 33] have also been reported to be present in *A. septentrionale*. Ethanol extracts [34] and water-alcohol extracts [35] of *A. septentrionale* have shown inhibitory effects of development and symptoms of inflammation. However, no further research was conducted to investigate the possible active compounds with anti-inflammatory activities, and the presence of other types of natural products in this plant mostly remains unknown. Compared to other *Aconitum* plants [1, 4], especially *A. carmichaelii* [10, 22], the knowledge about non-alkaloid compounds in *A. septentrionale* is limited.

1.4 Polysaccharides and pectins from higher plants

1.4.1 Structures

Polysaccharides, classified as long-chain carbohydrates, are made up of simple sugar molecules connected by glycosidic linkages. A wide range of polysaccharide molecular structures with different functional properties are existing in nature, and they differ by monosaccharide composition, type of glycosidic linkages, degree of polymerization, three-dimensional structure, and the presence of functional groups. These plant polysaccharides are commonly categorized as starches and non-starch polysaccharides (NSPs) [36]. Cellulose, an insoluble polysaccharide forming microfibrils, as well as hemicelluloses (xyloglucans, xylans, mannans, β-glucan and xyloglucans) and pectins (**Fig. 1-5**) are all matrix components in plant cell wall [37] and members of NSPs. Covalent cross-link between cellulose and hemicellulose is reported to provide the cell wall tensile strength, while the pectic polysaccharides, as the most dynamic polymers in the plant cell wall, are thought to lubricate microfibrils motions [38], and are crucial for remodeling and tissue softening [39]. In addition, fructans are another energy-reserve carbohydrate in about 15% of flowering plants [40]. So far, these polysaccharides have been used for different purposes. For instance, starch and cellulose are valuable building blocks in preparation of composites, bioplastics, or within drug delivery system, while hemicellulose polymers are utilized as biomaterials for food packaging. NSPs have been applied for pharmaceutical use as human nutrition and prebiotics/dietary fibers, or in the potential treatment of cancer or intestinal diseases, or as anti-inflammatory, immunomodulatory, anti-virus, hypoglycemic and anti-oxidative substances [40, 41].

Pectin, as one of the major plant cell wall components, is probably the most complex macromolecule in nature, as it can be composed of as many as 17 different monosaccharides containing more than 20 different linkages in addition to methyl and acetyl groups. It influences various cell wall properties such as porosity, surface charge, pH and ion balance, and therefore is of importance to the ion transport in the cell wall. In general, pectin is a hetero-polysaccharide predominantly consisting of GalA residues, and contains moieties such as homogalacturonan (HG) and rhamnogalacturonan I/II (RG-I/II) with substitutions [42].

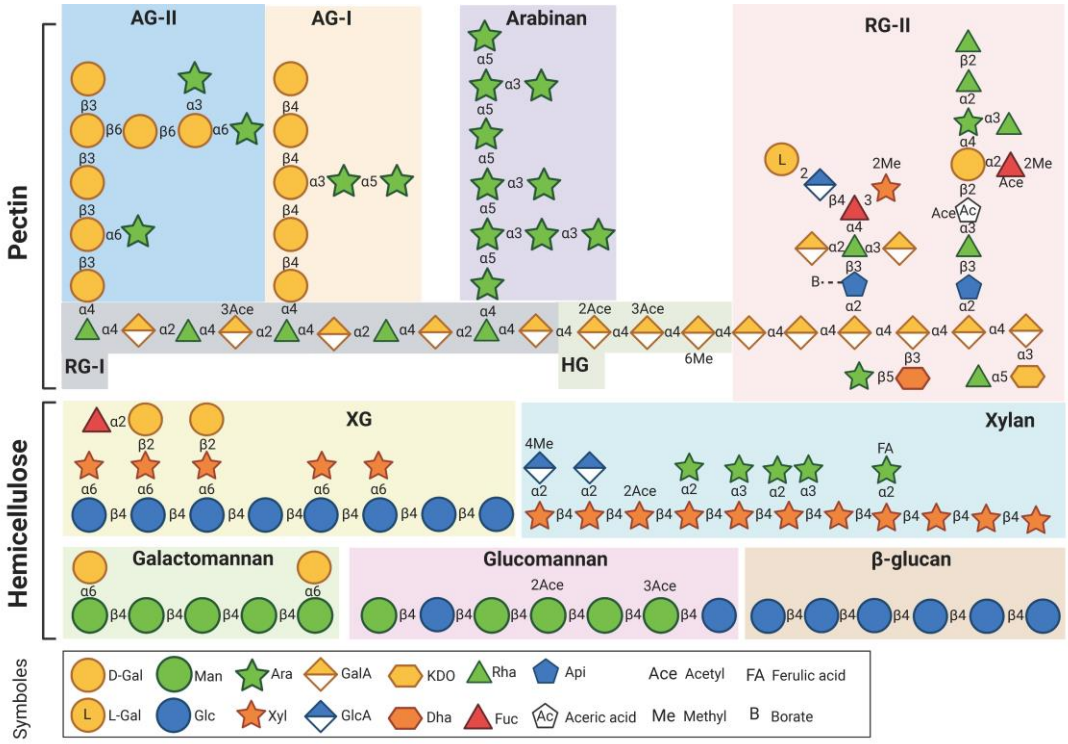

Fig. 1-5. Schematic structures of plant cell wall polysaccharides, including cellulose (β-glucan), xyloglucan (XG), xylan, mannans, rhamnogalacturonan I (RG-I), homogalacturonan (HG), arabinans (Ara), arabinogalactan (AG) and rhamnogalacturonan II (RG-II). Figure was made in Biorender according to a review of Martens, Lowe, Chiang, Pudlo, Wu, McNulty, Abbott, Henrissat, Gilbert, Bolam and Gordon [43].

HG is the major backbone of pectin in cell walls, accounting for approximately 60% of the total pectin and consist of α-1,4-linked Gal*p*A, which may be methyl esterified at C-6 or acetylated at *O*-2 and/or *O*-3. Substitution of HG could also occur at *O*-3 with single Xyl*p* unit as side chains, forming xylogalacturonan (XGA) [42]. The RG-I backbone is composed of repeating →2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→ disaccharides with various branches or linear side-

chains mainly being neutral glycosyl residues at *O*-4 of Rha*p* [39, 42]. These side chains are mainly composed of galactosyl and/or arabinosyl residues, such as β-1,4-linked galactan, arabinogalactan I (AG I, 1,4-linked β-D-Gal*p* backbone with α-L-Ara*f* residues attached to *O*-3 of Gal*p*), AG-II (1,3-linked β-D-Gal*p* backbone with short side chains of α-L-Ara*f* at *O*-6 of Gal*p*) and arabinan (1,5-linked α-L-Ara*f*), as shown in **Fig. 1-5** [42, 43]. RG-II is more complex than RG-I as a distinct region within HG, containing clusters of four different side chains with very rare sugar residues, such as apiose, aceric acid, 3-deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO) [42].

1.4.2 Pharmacological properties

Polysaccharides have attracted increasing attention due to their wide range of applications as natural resources, low toxicity and diverse biological activities, such as antitumor, immunomodulatory, anti-oxidative and anti-inflammatory effects [41, 44].

A wide range of *in vivo* and *in vitro* studies have been performed evaluating the anti-inflammatory effects of natural polysaccharides, and most of them are carried out on colitis models that displays certain key characteristics similar to human intestinal bowel disease (IBD) [44]. Plant NSPs are not degraded by the human digestive system, but can enter the large intestine with the peristalsis of the intestine and then be utilized by microorganisms. For example, *Bacteroidetes* that are rich in carbohydrate metabolic pathways can ferment NSPs into short chain fatty acids (SCFAs), and correspondingly improve intestinal microecology by repairing intestinal barrier function, regulating the composition of intestinal flora and cytokine levels [45]. To date, pectins have shown benefits in IBD models, through inhibiting oxidative stress, down-regulating proinflammatory cytokines and signaling pathways, improving the composition and function of intestinal microorganism and reducing the damage of colonic mucosal barrier [46, 47], as shown in **Fig. 1-6**. Furthermore, pectins have also shown a potential role in the treatment of other inflammatory diseases besides IBD, such as gastritis, cerebral inflammation and other inflammatory diseases induced by high-fat diet or infection [48]. In addition, i*n vitro* studies have also indicated their direct inhibitory effects on inflammation of the immune system without involvement of the intestinal microbiota. All these effects by pectin have shown to be related to HG, RG-I, RG-II or XGA domains through direct or indirect ways [48]. However, systematic studies on the exact structure-activity relationships of anti-inflammatory pectin are limited.

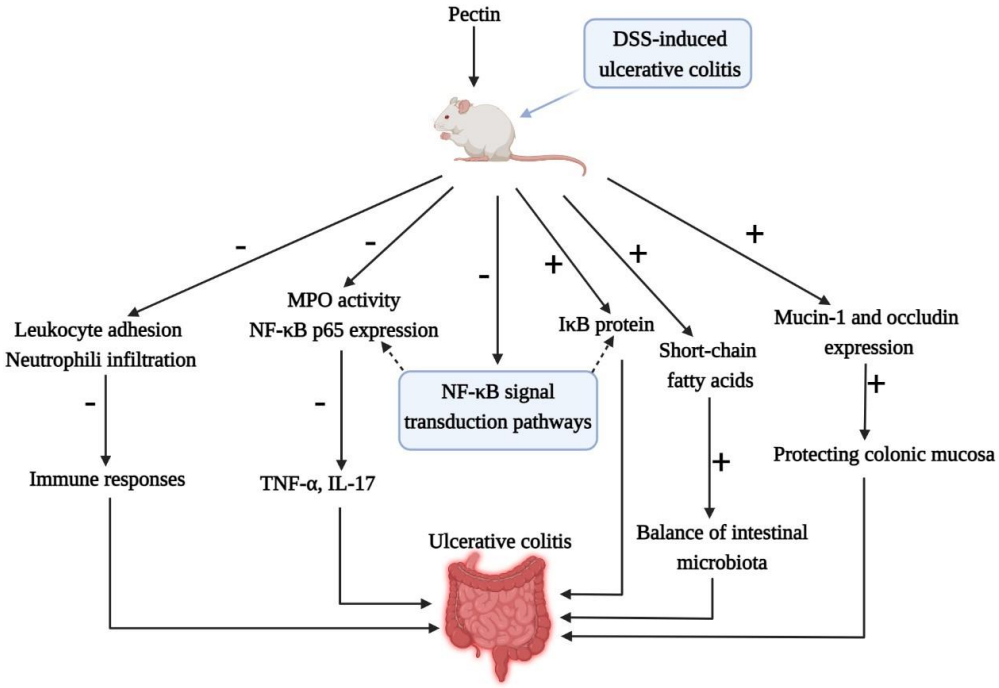

Fig. 1-6. Effects of pectin (extracted from *Rauvolfia verticillata* (Lour.) Baill var. hainanensis Tsiang) in the treatment of dextran sulfate sodium (DSS)-induced ulcerative colitis mice. Figure was made in Biorender according to a review of Niu, Chen, Xu, Dong, Yang, Wang, Zhang and Ju [47].

NSPs are not only known for anti-inflammatory activities, but also function as immunomodulators, including complement fixating effects and pro-inflammatory effects on immune cells (macrophages, dendritic cells, neutrophils and lymphocytes). They are reported to increase the secretion of nitric oxide (NO) and proinflammatory cytokines, through binding receptors like scavenger receptors (SRs), Dectin-1, mannose receptor, CR3, and TLR4 [49-51], as displayed in **Fig. 1-7**. Structural features like conformation, molecular weight (*Mw*), presence of functional groups like acetyl and sulfate groups, and degree of branching are connected with the immune effects [51]. Particularly, pectin rich in AG-II domains (arabino-3,6-galactan structure) have been studied for their complement-fixating activities, and those with RG-I regions expressed most potent effects on activation of macrophages [52, 53]. Moreover, multiple structural characteristics of pectin could exhibit diverse effects that enhance the intestinal immune barrier through a direct (*in vitro*), microbiota-independent interaction with immune cells. They may enhance the intestinal immune barrier by strengthening the mucus layer, enhancing epithelial integrity or activating and inhibiting macrophage and dendritic cell

responses, which may be governed through interactions with recognition receptors [54] (**Fig.1-8**). Nevertheless, specific patterns of pectin for promoting such effects are still unclear.

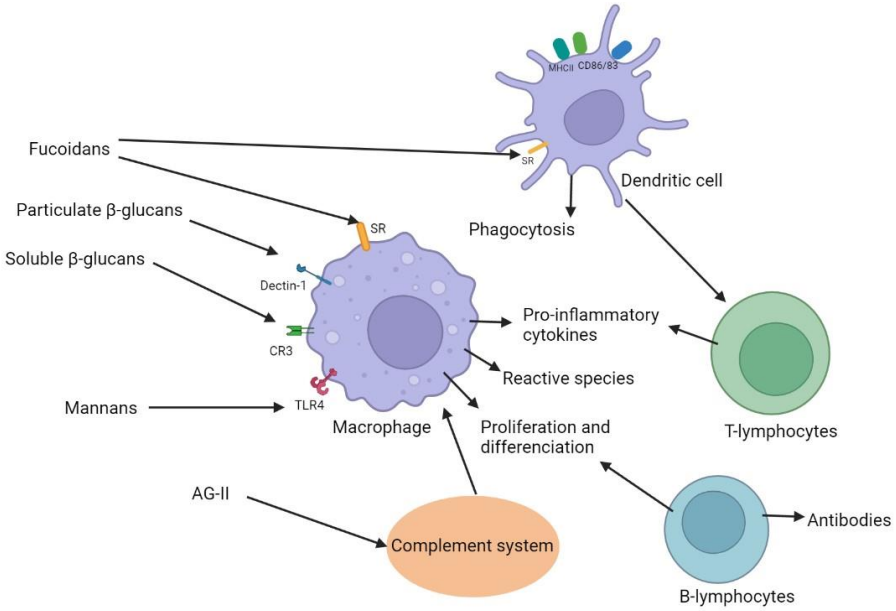

Fig. 1-7. Illustration of immune system activation by immunostimulatory polysaccharides after interaction and triggering of several molecular/cellular events. Figure was made in Biorender according to a review of Ferreira, Passos, Madureira, Vilanova and Coimbra [51].

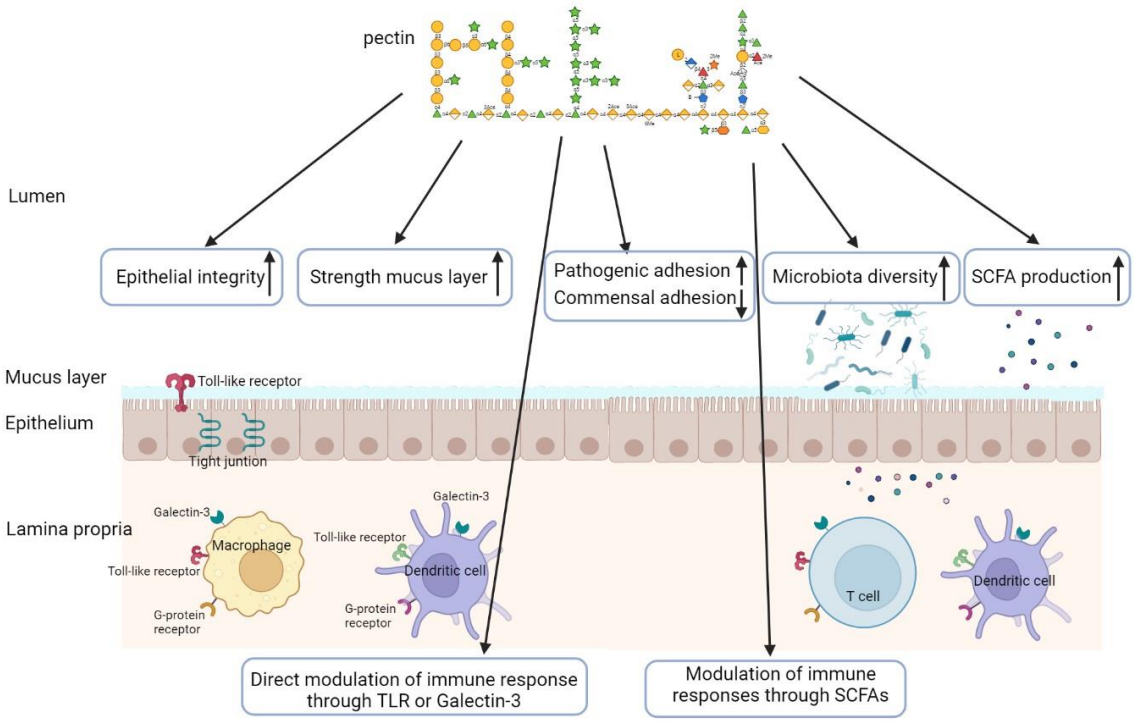

Fig. 1-8. Influence of pectins on the gastrointestinal immune barrier. Figure was made in Biorender according to a review of Beukema, Faas and de Vos [54].

Regarding the toxic *Aconitum* plants, the understanding of the patterns and the biological activities of such non-toxic polysaccharides in *A. carmcichaelii* or *A. septentrionale* would be beneficial in order to lay foundation for their future applications. A determination of the structure-activity relationship is undoubtfully crucial for a comprehensive investigation of their biological activities, which remains unclear so far for any *Aconitum* plant. Even if the anti-tumor and immunomodulatory effects have been suggested to be related to GalA and Ara units of polysaccharides from "Fuzi" [55], more detailed and comprehensive studies should be performed to understand the exact structural information and the correlations to bioactivities. Further, the effect of these polysaccharides derived from *Aconitum* plants on intestinal inflammation would be an interesting direction to explain more about their potential use as medicinal plants.

1.5 Phenolic compounds in plants

Polyphenols and phenolic compounds are common secondary metabolites from plants. They are often classified as flavonoids and nonflavonoids with different main structural formats. Phenolic compounds share a common structural feature, an aromatic ring with at least one hydroxyl substituent that might be conjugated (as shown in **Fig. 1-9**), while polyphenols contain two or more phenolic rings [56, 57]. Briefly, flavonoids are characterized by a diphenyl propane skeleton formed by two benzene rings joined by a linear bridge constituted by three-carbon (C_6-C_3-C_6) (**Fig. 1-9 A**). They are divided according to the degree of oxidation of the C ring, or present as various substituents with sugars to form glycosides. Since no flavonoid compounds were identified in the current project, details of this type of compound and its bioactivities are not introduced here.

Non-flavonoid phenolic compounds have similar main skeleton. Herein, simple phenols are characterized as a C_6 ring with one or more hydroxy group in the aromatic ring (**Fig. 1-9 B**). While, phenolic acids are the most common non-flavonoid phenolics in the human diet, formed by C_6-C_1 carbon structure (**Fig. 1-9 C**), or a C_6-C_2 structure such as phenylacetic acids (**Fig. 1-9 D**), C_6-C_3 structure like hydroxycinnamic acids (**Fig. 1-9 E**), phenylpropenes (**Fig. 1-9 F**) and their derivatives, and polyphenolic C_6-C_2-C_6 stilbenes (**Fig. 1-9 G**) [57].

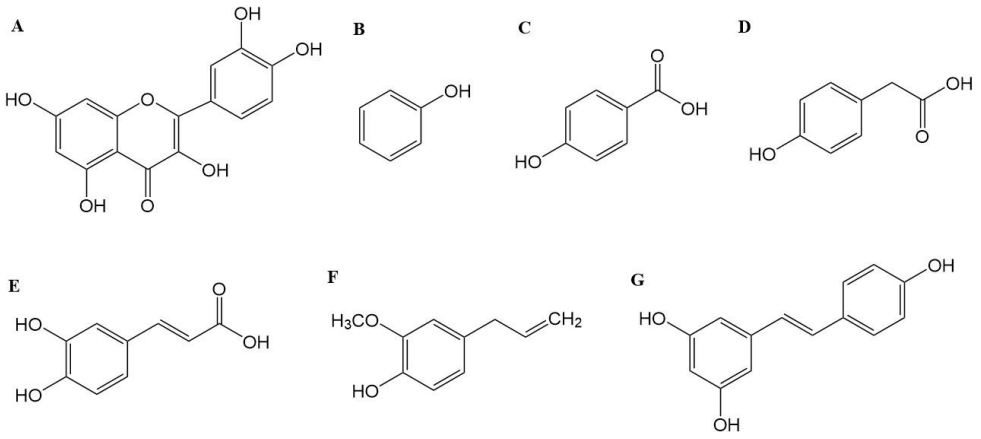

Fig. 1-9. Basic chemical structures of flavonoids (A, eg. quercetin), simple phenols (B), phenolic acids (C, eg. 4-hydroxybenzoic acid), hydroxyphenylacetic acids (D), hydroxycinnamic acids (E, eg. caffeic acid), phenylpropenes (F, eg. eugenol) and stilbenes (G, eg. resveratrol).

Phenolic compounds are demonstrated with benefits against human diseases, including cardiovascular diseases, cancer, obesity, diabetes, and infectious diseases, through reduction of oxidative stress, prevention of inflammatory signaling pathways, inhibition of inflammatory enzymes, and glucose and fructose transport, etc [58]. The anti-oxidative effects of phenolics have been known for decades, which is due to the presence of an aromatic ring, the electron donation and hydrogen atom transfer to free radicals, or the activation of endogenous antioxidant pathways [59]. The anti-inflammatory activities of phenolic acids have been reported through regulation of cytokines secretion both *in vivo* and *in vitro*, and their anti-microbial effects have also been suggested against gram-negative bacteria, which seems to be related to the pyrogallol group [59]. Both phenolics and polyphenols are suggested to have a therapeutic potential in the treatment of various cancer [60, 61]. For instance, the improvement of colon cancer by phenolic acids has been reported possibly by scavenging free radicals, inducing enzymes involved in the metabolism of xenobiotics, modulating cellular signaling pathways (including those involved in DNA damage repair, cell proliferation, apoptosis and invasion), and inhibiting transcription factors linked to inflammation (pro-inflammatory cytokines, lipoxygenases, inducible nitric oxide synthase (iNOS) [62]. Therefore, the investigation of phenolic compounds, especially their anti-inflammatory and anti-oxidative activities would lay a solid foundation for more clinical applications on human diseases in the future.

2 Aims of the thesis

The aims of this thesis were as follows:

- To give a systematic review on the phytochemical and pharmacological studies of non-alkaloid compounds in *A. carmichaelii* (**Paper I**)
- To investigate the polysaccharide patterns present in the traditionally used *A. carmichaelii*, both in roots and the unutilized plant parts (**Paper II and III**)
- To determine the immunomodulatory and intestinal anti-inflammatory effects of polysaccharides in different plant parts of *A. carmichaelii* (**Paper II and III**)
- To identify polysaccharides isolated from *A. carmichaelii* with protective effects against ulcerative colitis (**Paper IV**)
- To determine the presence of non-alkaloid compounds in *A. septentrionale*, with focus on phenolic and polysaccharide compounds (**Paper V**)

3 Summary of papers

Paper I *Aconitum carmichaelii* Debeaux: a systematic review on traditional use, and the chemical structures and pharmacological properties of polysaccharides and phenolic compounds in the roots

This review paper aimed to give a systematic overview on the phytochemical and pharmacological studies of non-alkaloid compounds that have been reported in the traditionally used *A. carmichaelii*, with focus on polysaccharides and phenolic compounds. This would lay a solid foundation and further research directions for this Ph.D. project.

After a comprehensive search in the literature, it was found that different types of polysaccharides, both neutral and acidic, and 39 phenolic compounds like flavonoids, phenylpropanoids, lignans, neolignans, and benzoic acid derivatives have been isolated and identified from the roots (see details in 1.2.1). Pharmacological studies of the isolated polysaccharides have demonstrated various biological effects such as hypoglycemic, hypolipidemic, cardiovascular, immunomodulatory, anti-tumor, and neuropharmacological activities. Studies on pharmacological effects of the phenolic compounds isolated from the roots are, however, limited. This review showed that polysaccharides could be one of the active components in the roots of *A. carmichaelii*, and they are promising for future applications due to their pharmacological properties. In addition, polysaccharides are generally non-toxic, biocompatible, and biodegradable. A more detailed structural characterization of polysaccharides from different root parts of *A. carmichaelii*, and their structure-activity relationships are required. Additionally, their pharmacological properties as immunomodulators in the intestinal system should be investigated. Further, more knowledge about the pharmacological effects and molecular mechanisms of the phenolic compounds that have been identified are needed.

According to this overview, the most aforementioned research gaps were further studied in the following papers. Since several phenolic compounds have been identified from the roots earlier, the isolation of such compounds in *A. carmichaelii* was not performed in the current project.

Paper II Polysaccharides with immunomodulatory effects in different plant parts of *Aconitum carmichaelii*

According to the research background in Paper I, the lateral and mother roots of *A. carmichaelii* are the commonly used plant parts, but fewer studies were performed on the mother roots compared to the lateral roots. Studies have also suggested that polysaccharides were present in the other plant parts of *A. carmichaelii*, and these plant parts, especially the aerial parts, have exhibited similar biological activities to those of the traditionally used roots (see details in 1.2.2). Thus, the aim of this paper was to isolate and compare the similarities and differences of the polysaccharides from different plant parts of *A. carmichaelii* (lateral roots, mother roots, rootlets, entire aerial parts, stems and leaves).

In this paper, both neutral and acidic polysaccharide fractions were obtained from the water extract of different plant parts after ion exchange chromatography (IEC) and size exclusion chromatography (SEC). It was found that the neutral fraction isolated from the rootlets (ARL-N) was different from those from the lateral and mother roots (ALR-N and AMR-N), consisting of less starch and more possible mannans, galactan, and/or xyloglucan, and being similar to those of aerial parts (AAP-N or AS-N). Pectic polysaccharides containing homogalacturonan and branched type I rhamnogalacturonan (RG)-I were present in all plant parts of *A. carmichaelii*. However, more of arabinogalactan (AG)-II side chains were found in the RG-I backbone in the aerial parts, while more of arabinans followed by AG-I/II were found in the roots. *In vitro* complement fixation activity and inhibition of pro-inflammatory cytokines' transcription of these isolated polysaccharide fractions were also determined. The content of arabinan and AG-II were positively correlated with complement fixation activity, but correlations of structural characteristics with anti-inflammatory effects were not apparent. This study showed the diversity of polysaccharides present in *A. carmichaelii*, and highlighted the potential value of the unutilized parts of *A. carmichaelii* which are normally discarded and generate huge waste of medicinal plant sources.

The polysaccharide fractions isolated from the leaves of this plant were shown to be different due to the highest yield and/or potent immunomodulatory and anti-inflammatory effects compared to those isolated from the other plant parts, and were further studied in detail, as introduced in Paper III and IV.

Paper III Polysaccharides from *Aconitum carmichaelii* leaves: structure, immunomodulatory and anti-inflammatory activities

This study aimed to investigate the polysaccharide fractions in the unutilized *Aconitum carmichaelii* leaves, which are discarded in the processing of the roots used in TCM.

After removal of pigments and small *Mw* molecules, the leaves of *A. carmichaelii* were extracted with boiling water. One neutral (AL-N) and two purified acidic polysaccharide fractions (AL-I-I and AL-I-II) were obtained after IEC and SEC, using the same methods as applied in Paper II. Their monosaccharide composition, glycosidic linkage patterns and *Mws* were determined, and NMR of AL-I-I and AL-I-II was performed for a comprehensive elucidation of their structures. AL-N appeared to be a mixture of heteromannans, and AL-I-I and AL-I-II were shown to be pectins containing a HG backbone substituted with terminal β-Xyl*p*-units. In addition, AL-I-I consisted of an RG-I core, with arabinan and AG-II domains attached, while AL-I-II was less branched. AL-N and AL-I-I were able to modulate the complement system, while AL-I-II was inactive. Interestingly, AL-N, AL-I-I and AL-I-II were shown to exert anti-inflammatory effects on porcine enterocyte IPEC-J2 cells through inhibiting expression of TLR4 and NOD1. This study presents a comprehensive chemical characterization of polysaccharides from *A. carmichaelii* leaves, and unravels their promising medicinal use as natural immunomodulatory and anti-inflammatory substances.

Paper IV Pectic polysaccharides from *Aconitum carmichaelii* leaves protects against DSS-induced ulcerative colitis in mice through modulations of metabolism and microbiota composition

As mentioned in the introduction, NSPs can enter the colon and be utilized by microorganisms as nondigestible polymers. This study aimed to investigate the protective effect of previously obtained pectin AL-I from the leaves of *A. carmichaelii* (Paper III) on UC mice for the possible application in the treatment of intestinal inflammatory diseases.

AL-I was given to DSS-induced experimental ulcerative colitis mice by gastric gavage. AL-I was found to alleviate symptoms and colonic pathological injury in colitis mice, and ameliorate the levels of inflammatory indices in serum and colon. The production of short-chained and branched fatty acids was also restored by AL-I. The observed protective effect could be due to the inhibition of NOD1 and TLR4 activation, the promotion of gene transcription of tight-junction proteins, and the modulation of gut microbiota composition like *Bacteroides*, *Dubosiella*, *Alistipes* and *Prevotella*. A regulation of serum metabolomic profiles being relevant to the bacterial change, such as D-mannose 6-phosphate, D-erythrose 4-phosphate and uric acid, was also observed.

Combining the biological activities observed both *in vitro* and *in vivo*, it was suggested that the leaves of *A. carmichaelii* are valuable plant materials that could be utilized as a medicinal plant resource for the treatment of intestinal inflammatory diseases.

Paper V Bioactive phenolics and polysaccharides from the water extract of *Aconitum septentrionale* roots

A. septentrionale, a medicinal plant widely distributed in Norway, Sweden and Russia, is well known for the presence of alkaloids and their toxicity. However, knowledge about other natural products in this species was limited, especially on phenolics and polysaccharides and their biological properties. This study aimed to isolate non-alkaloid natural products, from the aspect of phenolic compounds and polysaccharides from the water extract of *A. septentrionale* roots (WEAS).

WEAS was initially fractionated by Diaion HP-20, giving the main fractions D1-D5. Fifteen phenolic compounds were obtained and identified from D2 and D3. All of them are reported for the first time in this plant species, and one of them is a new natural product. Three main polysaccharide fractions ASP-N, ASP-I-I and ASP-I-II were obtained from D1 using the same methods as applied in Paper II. The structural characterization including monosaccharide composition, glycosidic linkage types and *Mws* were determined. ASP-N contained starch and minor amounts of mannans, and the starch was also present together with pectic polysaccharides in ASP-I-I and ASP-I-II. A more branched pectin was observed in ASP-I-I compared to ASP-I-II.

Three phenolic compounds exhibited inhibitory effects on nitric oxide (NO) release by LPS+IFN-γ-activated dendritic cells. Two of the phenolic compounds showed higher inhibitory activity than the positive control, quercetin, on 15-lipoxygenase (15-LO), and one compound was a moderate inhibitor of xanthine oxidase (XO). The polysaccharide fractions were not able to inhibit NO release by dendritic cells.

All these findings contribute with new knowledge about the diversity of natural compounds in the roots of *A. septentrionale*, and their anti-inflammatory and anti-oxidative potentials.

4 General discussion

A. carmichaelii and *A. septentrionale* are distributed in different regions in the world (see introduction). Besides the identification of alkaloids, studies on *A. septentrionale* are very limited, and barely mentioned in a recent review of *Aconitum* plants [63]. Therefore, it was of interest to expand the knowledge of this plant, and also to make a primary comparison to the widely used *A. carmichaelii*. Combining all results in aforementioned sections would inspire more research interest into *A. septentrionale*.

4.1 Water extracts of the roots of *A. carmichaelii* and *A. septentrionale*

The roots of these two species were normally used as decoctions in traditional medicine. The water extracts (boiling water) of the roots of *A. septentrionale* (WEAS), and the traditionally used lateral (WELR) and mother roots (WEMR) of *A. carmichaelii* are here compared and discussed. These results are not included in the publications. As shown in **Table 4-1**, the yield of WEAS is higher than that of WELR and WEMR, might be due to the roots of *A. septentrionale* were not separated into different parts as *A. carmichaelii*, and WEAS contains higher amounts of phenolic compounds than WELR and WEMR, probably contributing to the better DPPH scavenging effect of WEAS (Fig. S4 in Paper V). The total amount of alkaloids of WEAS was not determined in the current study, and there are so far no studies reporting the isolation of alkaloids from the water extract. WEAS displayed no toxicity on brine shrimp larvae (see supplementary methods of Paper V) or dendritic cells. However, both WELR and WEMR presented significant toxicities on dendritic cells at 20 and 100 μg/mL (**Fig. 4-1**). Interestingly, a toxic reaction on brine shrimp larvae was caused by a 100% MeOH-fraction of WEAS obtained from Diaion HP-20 (fraction D5, Fig. S5 in Paper V), but not on dendritic cells after co-cultivating for the same period. D5 fraction might contain higher concentration of toxic alkaloids compared to WEAS. Lappaconitine, one of the alkaloids reported in *A. septentrionale* roots, is probably present in D5 (results from ^{13}C and ^{1}H NMR). Different types of alkaloids existing in *A. septentrionale* and *A. carmichaelii* could be the reason of the toxicity differences between WEAS and WELR or WEMR. This will be discussed in section 4.4. Collectively, these findings revealed a potential medicinal value of *A. septentrionale* roots, due to the higher contents of phenolic compounds and anti-oxidative activities, as well as the relatively lower degree of toxicity compared to the widely used *A. carmichaelii* roots. These findings gave a foundation for the further fractionation of water-soluble compounds, phenolics and polysaccharides.

Table 4-1 Yields, chemical composition, DPPH scavenging activities and toxicity of WEAS, WELR and WEMR

	WEAS	WELR	WEMR
Yields (%) [a]	39.6	22.6	28.3
Carbohydrate (%)	62.4	75.8	60.7
Total phenolic compounds (%)	6.8	0.1	1.9
IC_{50}/μg/mL (5 min incubation)	88.4±0.3	>250	>250
IC_{50}/μg/mL (15 min incubation)	78.1±6.1	>250	>250
IC_{50}/μg/mL (30 min incubation)	72.7±5.4	>250	>250
Toxicity to brine shrimp larvae [b]	-	-	-
Toxicity to dendritic cells [b]	-	+	+

WEAS: water extract of the roots of *A. septentrionale*; WELR: water extract of the lateral roots of *A. carmichaelii*; WEMR: water extract of the mother roots of *A. carmichaelii*. [a], yields related to dried plant material (w/w); [b], "-", no toxicity was observed at final concentrations of 100 μg/mL; "+", toxicity was observed; detailed methods are described in the supplementary methods of Paper V.

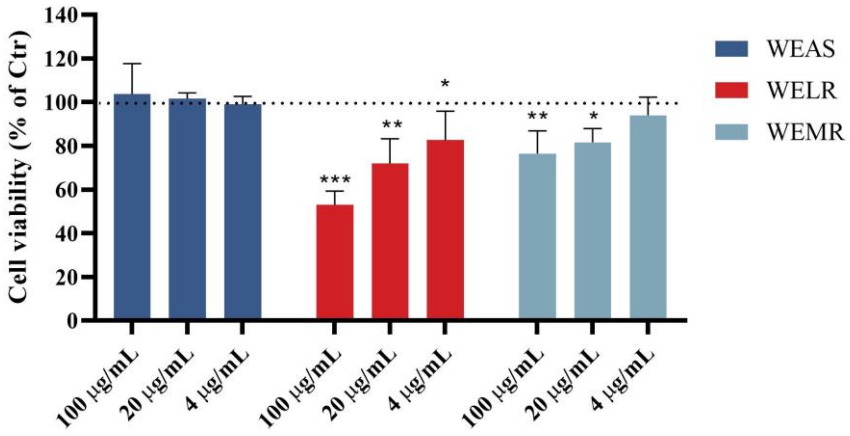

Fig. 4-1 Cell viability of dendritic cells treated with WEAS, WELR and WEMR; statistical analysis was performed compared to the cell viability of the negative control (100%), *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.2 Polysaccharides isolated from the roots of *A. carmichaelii* and *A. septentrionale*

In this study, several polysaccharide fractions were isolated from different plant parts of *A. carmichaelii* (Paper II and III) and the roots of *A. septentrionale* (Paper V). Herein, the fractions isolated from the main root parts of *A. carmichaelii*, the lateral and mother roots, were different from those isolated from roots of *A. septentrionale*, as various elution profiles were observed after both IEC and SEC (Fig. 1 in Paper II and Fig. S22 in Paper V). The yield of the ethanol-precipitated fraction ASP from *A. septentrionale* is lower than that of the ethanol-precipitated fractions ALR or AMR from *A. carmichaelii*. This is probably due to different processing before ethanol precipitation (**Fig. 4-2**), or the removal of a large amount of an insoluble fraction composed of Glc when re-dissolving D1 with dH_2O (Paper V). Even though the crude polysaccharides from these two *Aconitum* plants were not obtained in the exact same way, the polysaccharide fractions were isolated and purified using the same columns and matrixes on both IEC and SEC (**Fig. 4-2**). However, the higher yield of ASP-I from *A. septentrionale*, compared to that of the main fractions ALR-II and AMR-II from *A. carmichaelii*, might be due to the contamination of a large amount of starch that was probably tightly attached to the main chain of ASP-I-I and ASP-I-II, or was co-eluted with these two fractions. This was further shown after the determination of monosaccharide composition and corresponding glycosidic linkage types, as introduced below.

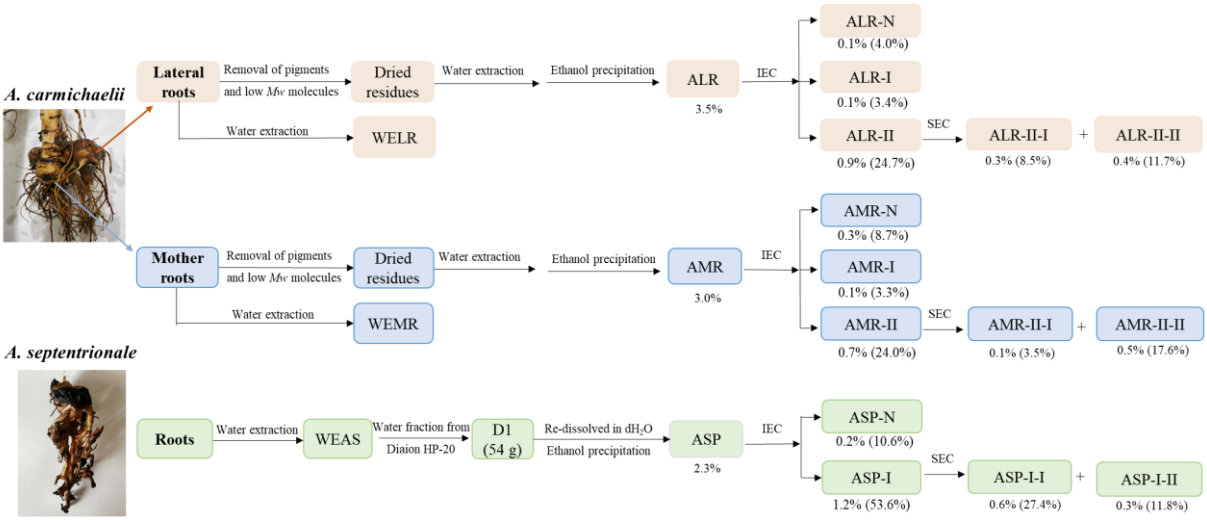

Fig. 4-2 Overview of the workflow and yields of polysaccharides from the root parts of *A. carmichaelii* and *A. septentrionale*. IEC, ion exchange chromatography; SEC, size exclusion chromatography; Yields (%): mass percent related to dried plant materials (crude polysaccharides).

Clustering analysis showed that for the neutral fractions isolated from the roots of these two *Aconitum* plants, ASP-N from *A. septentrionale* roots was more similar to the one isolated from the mother roots of *A. carmichaelii* (AMR-N) (**Fig. 4-3 A**) compared to the one isolated from the lateral roots (ALR-N). All three neutral fractions contained mainly Glc (**Table 4-2**), and the presence of starch was determined in all these three fractions (**Table 4-2**). However, ALR-N contained less Man and more Ara compared to ASP-N and AMR-N, even though all of them were obtained in the same way. The arabinan residues 1,5- and 1,3,5-linked Ara*f* were responsible for the higher amounts of Ara in ALR-N compared to ASP-N (**Table 4-3**).

The diversity of acidic polysaccharide fractions present in ASP was less than for ALR or AMR. The content of Glc in ASP-I-I and ASP-I-II were higher compared to all acidic fractions isolated from *A. carmichaelii* roots, resulting in relatively lower amounts of other monosaccharides, with the exception of Gal. The amount of Gal in ASP-I-I and ASP-I-II was higher than that of ALR-II-I and ALR-II-II (**Table 4-2**). As observed in the clustering analysis in **Fig. 4-3 A**, ASP-I-I was grouped together with ALR-II-I and AMR-II-I due to the similar pattern of monosaccharide composition and *Mw*, but differed from them by the high amount of Glc and smaller amounts of Ara and GalA. The differences turned out to be significant in the patterns of glycosidic linkage types, as ASP-I-I contained more than 30 mol% of 1,4-Glc*p* and relatively smaller amounts of other residues, which was consistent with the presence of starch in both ASP-I-I and ASP-I-II (**Table 4-3**) and their independent cluster different from theses acidic fractions of ALR or AMR in **Fig. 4-3 B.** The presence of pectin in all the acidic polysaccharides from these two *Aconitum* plants could be identified due to the high amounts of GalA, as well as the presence of 1,4-linked Gal*p*A (**Table 4-3**), as the major component of the HG region in the pectin. RG-I domains composed of 1,2-linked Rha*p* and 1,4-linked Gal*p*A disaccharides were present in a relative less amount in ASP-I-I compared to AMR-II-I or ALR-II-I (**Table 4-3**). But the ratio of Rha to GalA (Rha/GalA) of ASP-I-I (0.23) was similar to that of ALR-II-I (0.25) and AMR-II-I (0.30), as shown in **Fig. 4-3 C,** suggesting that they should have similar relative amounts of RG-I cores if the starch in ASP-I-I has been removed. Residues belonging to arabinan in ASP-I-I, a neural side chain of RG-I core, were present in smaller amounts compared to AMR-II-I or ALR-II-I. Whereas, ASP-I-I was composed of more residues belonging to AG-II domains (**Table 4-3** and **Fig. 4-3 B, C**). The pectic structural pattern of ASP-I-II was different from fractions ALR-II-II or AMR-II-II (**Fig. 4-3 C**) due to the higher relative amounts of AG-II residues (**Table 4-3**). A high amount of 1,4-Glc*p* co-existing with pectic polysaccharide*s* was seldom observed. It could be part of starch or other glucans attached

tightly to the pectin structure (Paper V), or co-eluted with these pectic polysaccharide fractions on SEC. This was the most obvious difference between ASP and ALR/AMR, even though all polysaccharide fractions were isolated according to the same procedure.

Collectively, both starch and pectic type polysaccharides seems to be present in the roots of both *A. septentrionale* and *A. carmichaelii*, as well as possibly other types of polysaccharides in minor amounts, such as mannans or xyloglucans (Paper II and V). The presence of pectic polysaccharides composed of RG-I core and HG domains have not been reported isolated from many *Aconitum* plants, so far.

Table 4-2 Monosaccharide composition and average *Mw* of polysaccharide fractions isolated from the roots of *A. carmichaelii* and *A. septentrionale.*

	Ara	Rha	Fuc	Xyl	Man	Gal	Glc	GlcA	GalA	Starch	*Mw*/kD
ALR-N[a]	12.4	Tr.	Tr.	Tr.	0.8	0.7	85.1	n.d.	Tr.	+	12.9
ALR-I[a]	30.4	Tr.	Tr.	0.8	0.8	4.6	60.6	Tr.	2.0	+	41.6
ALR-II-I[a]	50.0	7.0	0.8	2.6	Tr.	8.9	1.6	0.8	28.1	-	>475
ALR-II-II[a]	6.4	3.4	1.1	4.6	Tr.	2.9	4.9	0.6	75.8	-	105.9
AMR-N[a]	8.6	Tr.	Tr.	Tr.	2.5	1.1	87.0	n.d.	Tr.	+	10.2
AMR-I[a]	18.3	0.9	Tr.	1.5	1.7	8.1	61.5	Tr.	7.5	+	26.0
AMR-II-I[a]	33.9	9.6	0.9	4.2	Tr.	14.0	3.9	0.7	32.3	-	>475
AMR-II-II[a]	4.2	3.9	0.9	6.3	Tr.	2.3	3.4	Tr.	78.4	-	52.5
ASP-N[a]	3.1	Tr.	Tr.	0.8	3.1	1.6	89.7	n.d.	0.9	+	9.6
ASP-I[a]	18.3	4.3	0.6	1.9	1.0	10.6	40.3	1.3	21.8	+	n.t.
ASP-I-I[a]	20.1	4.3	0.5	1.7	n.d.	12.0	41.1	1.3	19.0	+	293.1
ASP-I-II[a]	5.8	4.3	0.9	2.3	1.8	6.4	42.1	1.7	34.8	+	16.3

Notes: [a], ALR, the lateral roots of *A. carmichaelii*; AMR, the mother roots of *A. carmichaelii*; ASP, the roots of *A. septentrionale*; -N, neutral polysaccharide fractions; -I, -I-I/II acidic polysaccharide fractions. The content of monosaccharide composition (mol%) was related to the total content of Ara, Rha, Fuc, Xyl, Man, Gal, Glc, GlcA and GalA; Tr., traces: less than 0.5mol%; n.d., not detected, n.t., not tested.

Table 4-3 Major glycosidic linkage types of polysaccharide fractions isolated from the roots of *A. carmichaelii* and *A. septentrionale*, and a suggestion of structural domains present in each fraction.

Structural domain	Linkage types	ALR series				AMR series				ASP series		
		ALR-N	ALR-I	ALR-II-I	ALR-II-II	AMR-N	AMR-I	AMR-II-I	AMR-II-II	ASP-N	ASP-I-I	ASP-I-II
	T-Ara*f* [a]	2.9	11.7	19.3	4.5	1.8	8.6	13.1	2.9	1.1	8.7	4.2
Arabinan	1,5-Ara*f*	3.5	7.4	8.1	0.7	3.5	5.1	8.6	0.7	0.9	5.8	1.1
	1,3,5-Ara*f*	6.0	10.9	22.3	1.1	3.2	4.2	11.7	Tr.	1.0	5.1	Tr.
RG-I and HG	1,2-Rha*p*	n.d.	n.d.	4.9	2.0	n.d.	n.d.	5.9	0.7	n.d.	2.7	4.2
	1,2,4-Rha*p*	n.d.	n.d.	1.8	Tr.	n.d.	Tr.	2.4	Tr.	n.d.	1.0	n.d.
	1,4-Gal*p*A	n.d.	2	25.1	65.2	Tr.	7.5	26.6	63.3	n.d.	17.1	32.0
XGA	T-Xyl*p*	Tr.	Tr.	2.6	4.3	Tr.	Tr.	3.4	6.3	0.6	0.8	2.3
	1,3,4-Gal*p*A	n.d.	n.d.	2.5	5.0	Tr.	n.d.	5.2	9.7	n.d.	1.9	2.8
Mannan	1,4-Man*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.6	n.d.	n.d.
	T-Gal*p* [b]	n.d.	Tr.	1.6	n.d.	Tr.	0.5	2.1	0.7	Tr.	1.1	0.6
AG-II	1,3-Gal*p*	n.d.	0.5	1.5	Tr.	n.d.	1.1	1.7	n.d.	n.d.	1.4	n.d.
	1,6-Gal*p*	n.d.	Tr.	Tr.	Tr.	n.d.	0.6	Tr.	Tr.	n.d.	0.7	2.3
	1,3,6-Gal*p*	n.d.	2.2	3.8	1.2	n.d.	2.9	3.4	0.5	Tr.	6.5	2.6
AG-I/galactan	1,4-Gal*p*	0.6	0.7	1.4	0.5	1.0	1.8	4.9	0.2	0.8	1.8	Tr.
Starch or glucan	T-Glc*p*	4.4	3.1	Tr.	n.d.	5.7	3.3	Tr.	n.d.	7.8	2.5	3.8
	1,4-Glc*p*	79.8	55.3	1.1	4.7	79.5	56.0	3.3	3.4	77.0	36.9	36.3
	1,4,6-Glc*p*	1.6	1.9	n.d.	Tr.	2.4	2.2	Tr.	n.d.	4.7	1.7	2.0

Note: [a], can be classified into both arabinan and AG-I/II domains; [b], can be classified into both AG-I and AG-II domains; Tr, traces: less than 0.5 mol%; n.d., not detected.

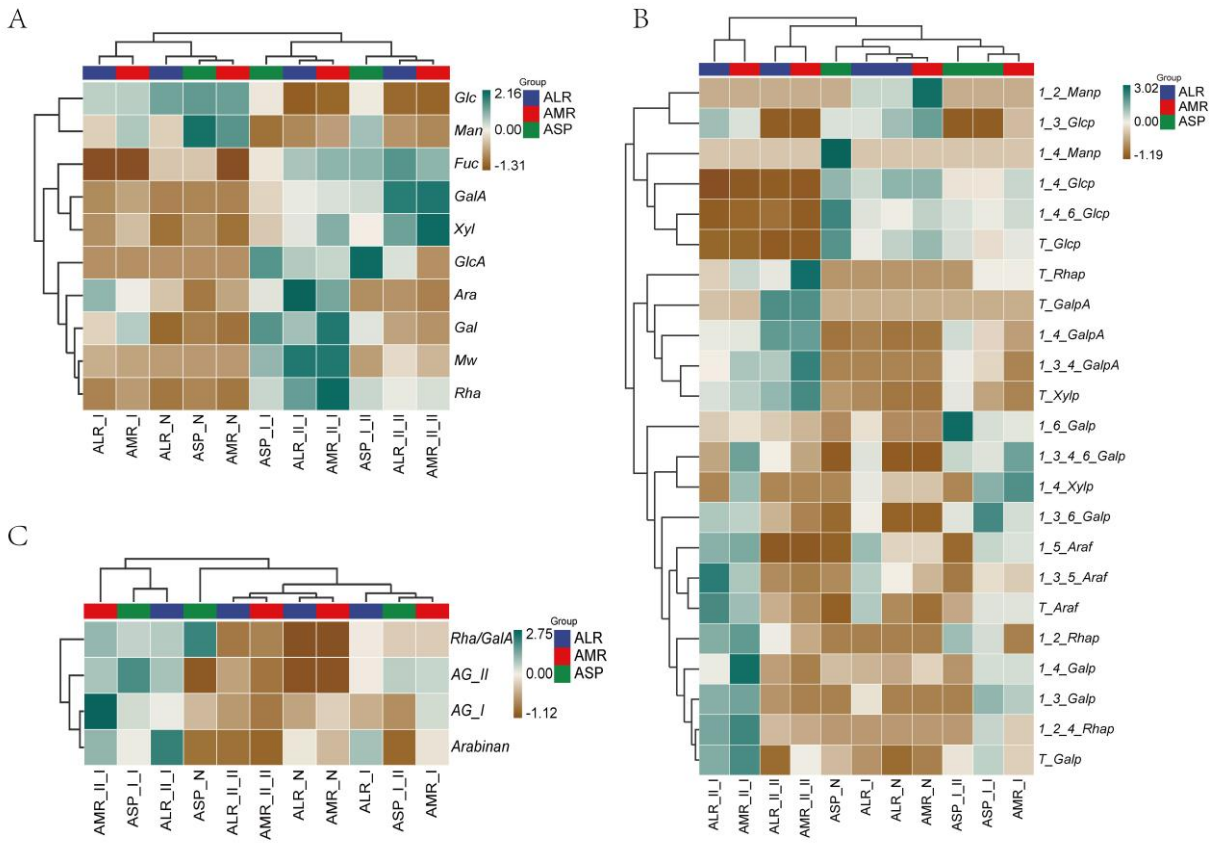

Fig. 4-3. Heatmap with dendrogram of monosaccharide composition (A), glycosidic linkages (B) and distribution of arabinan and AG regions (C) of polysaccharides from roots of *A. carmichaelii* and *A. septentrionale*. Hierarchical clustering was analyzed based on Euclidean distance, and the value represents the row Z scores. The amount of AG-I was represented by the sum molar amount of 1,4- and 1,3,4-linked Gal*p* residues; AG-II was represented by the sum molar amount of 1,3-, 1,6, and 1,3,6-linked Gal*p* residues; arabinan was represented by the sum molar amount of 1,5- and 1,3,5-linked Ara*f* residues.

4.3 Phenolic compounds isolated from the roots of *A. carmichaelii* and *A. septentrionale*

The non-alkaloid small molecules in *A. septentrionale* have not been well studied so far, while much more is known about these compounds in *A. carmichaelii*. Due to this, it is hard to find strong correlations between these two *Aconitum* species from the aspect of chemical constituents, as fewer compounds have been reported for *A. septentrionale*. However, there are, so far, two phenolic compounds that have been identified both in the roots of *A. carmichaelii* and *A. septentrionale*, a phenylpropanoid glucoside: lavandoside (compound **9**), and a

dianthramide glucoside: benzoic acid, 2-[[2-(β-D-glucopyranosyloxy)-5-hydroxybenzoyl] amino]-4-hydroxy-3-methoxy-, methyl ester (compound **12**) (Fig. 3 in Paper V). Tryptophan (compound **3**), deoxyadenosine (compound **4**), bracteanolide A (compound **7**), β-D-glucopyranoside, [(*2S,3R*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-5-[(*1E*)-3-hydroxy-1-propen-1-yl]-7-methoxy-3-benzofuranyl] methyl (compound **13**), and the new natural product – benzoic acid, 2-[[2-(β-D-glucopyranosyloxy)-5-hydroxybenzoyl] amino]-4, 5-dihydroxy-, methyl ester (compound **14**) are present in *A. septentrionale*, but have not been reported in other *Aconitum* plants so far (Fig. 3 in Paper V). Hydroxytyrosol (compound **1**) and hydroxytyrosol-1-*O*-β-glucoside (compound **2**) isolated from *A. septentrionale* in this study could be the main contributors of the observed NO inhibitory effects of D2, the 25% MeOH fraction from Diaion HP-20, while bracteanolide A (compound **7**) was the only effective one isolated from D3 fraction, the 50% MeOH fraction from Diaion HP-20 (Fig. 4 in Paper V). Compound **7,** compound **8** (magnoflorine) and compound **12** (benzoic acid, 2-[[2-(β-D-glucopyranosyloxy)-5-hydroxybenzoyl] amino]-5-hydroxy-, methyl ester) could be responsible for the moderate inhibitory activities on 15-LO or XO observed by D3 (Table 3 in Paper V).

4.4 Toxicity of the isolated compounds from *Aconitum* plants

As is well-known and mentioned in the introduction, *Aconitum* plants are normally toxic due to the content of alkaloids. Toxicity does not, however, apply to the polysaccharide fractions isolated from both *A. carmichaelii* (Paper II) and *A. septentrionale* (Paper V). Polysaccharides are well known as non-toxic polymers [47]. However, toxicity on brine shrimp larvae and dendritic cells were observed by fraction D5 (100% MeOH fraction from Diaion HP-20, Fig. S5 in Paper V) and two isolated compounds from *A. septentrionale* (compound **1** and **9**, Fig. 4 in Paper V), respectively. Further, as mentioned in section 4.1, WEAS was not toxic in either of the bioassays at 100 μg/mL. Considering that the alkaloids that have been previously reported from this plant were mostly isolated from organic solvent-extracts or weak-alkaline solvents [27, 29], there is a possibility that only minor amounts of toxic alkaloids are extracted by water (in WEAS), and are present in a relative higher concentration in D5 fraction after exclusion of other more hydrophilic compounds (D1-D4). Several alkaloids are previously reported to be present in the roots of *A. septentrionale* (as introduced in section 1.3). For instance, lappaconitine, which constitutes about 80% of the total alkaloid in the roots, has been reported to give toxic effects on mice after intravenous, intraperitoneal or oral administration [64]. In the current thesis, the signals in the ^{13}C NMR spectrum of fraction D5 were close to those of lappaconitine (**Fig. 4-4 B, C**), while for the ^{1}H NMR spectrum, proton signals in the aromatic

region (7.0-8.5 ppm) were comparable (**Fig. 4-4 A**). This can suggest that the toxic reaction of D5 could be due to the presence of lappaconitine. Aconitine, a C19-norditerpenoid alkaloid present in the water extract of *A. carmichaelii* [65, 66], is more toxic than lappaconitine [64], which likely explained the less toxicity of WEAS than WELR or WEMR on brine shrimp larvae (in section 4.1).

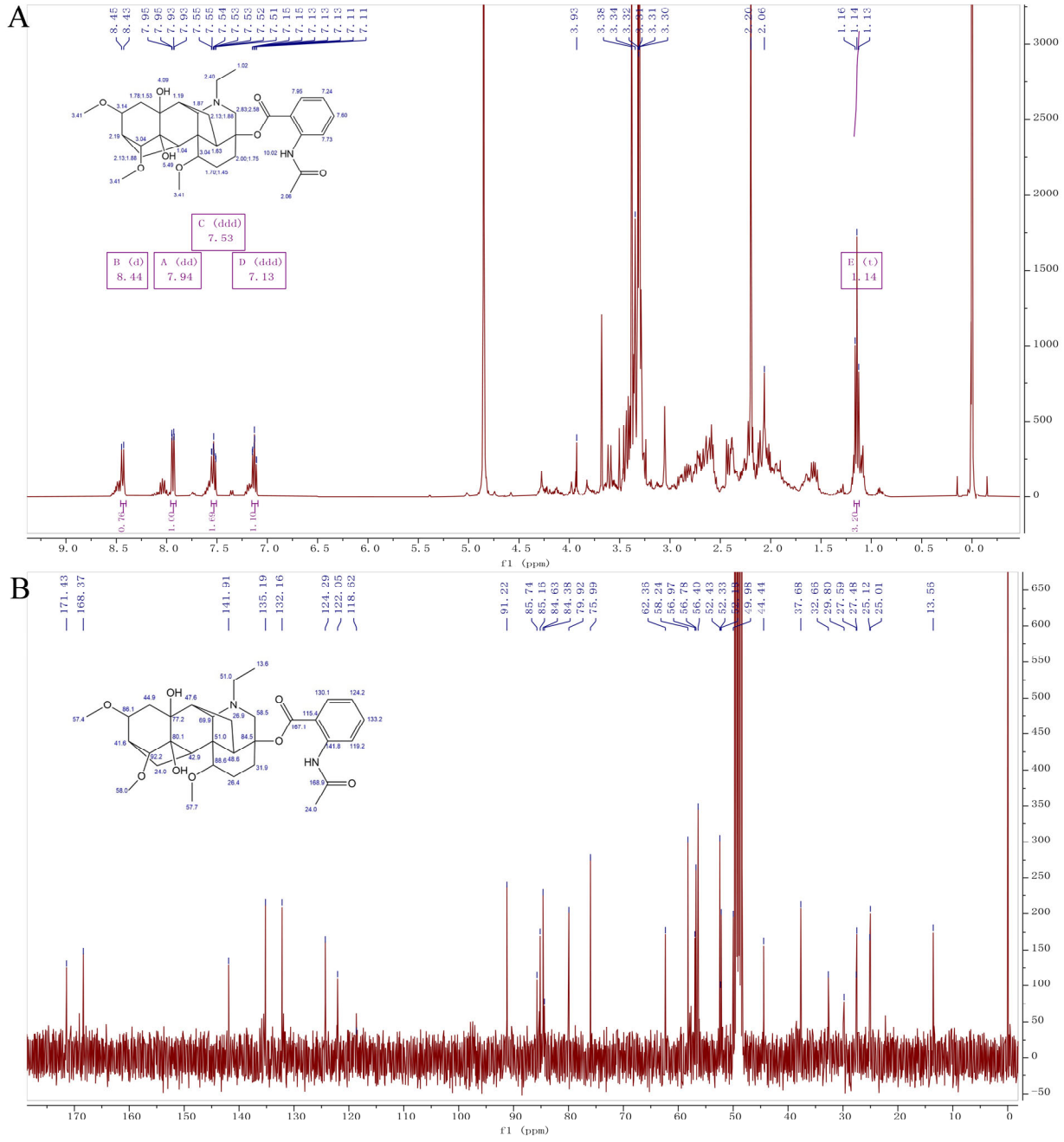

Fig. 4-4. ^{1}H (A) and ^{13}C (B) NMR spectra of D5 (400 MHz, solvent CD_3OD, TMS as reference) and the predicted chemical shift values of lappaconitine on ChemDraw (Pro. 16.0).

4.5 Applications of the unutilized parts of *Aconitum* plants

The potential use of TCM in treating many diseases such as cancers, gastrointestinal and cardiovascular diseases has been attractive all over the world [67]. However, the industrial applications and processing of TCM generate a huge waste of TCM residues [67, 68]. Components of the residues of Chinese herbal medicines include lignin, fat, protein and polysaccharide (including cellulose and hemicellulose) [68]. These compounds could be valuable sources utilized in medicine and pharmacy and therefore should be recycled from waste materials. There is an increased interest concerning unutilized plant parts of TCM, especially polysaccharides in the leaves accounting for the largest portion of plant biomass [69], in addition to the stems [70]. Several natural compounds have been reported in the non-traditionally used parts of *Aconitum* plants, such as flavonol glycosides from *A. angustifolium* flowers and leaves [71], alkaloids from *A. coreanum* leaves [72], alkaloids, lipids, flavonoids, polysaccharides and lignans from the aerial parts of *A. carmichaelii* [15, 21-23], as well as the diterpene steviol in the leaves of *A. heterophyllum* [73]. The polysaccharides and flavonoids, for instance, are promising bioactive compounds as anti-inflammatory and anti-oxidative substances, as introduced in section 1.4 and 1.5, respectively. These unutilized plant parts are not only sources with potential medicinal values, but also increase the possibility for isolating new natural product, like a new flavonol glycoside [71] and a new alkaloid [72] that have been identified from the non-traditionally used plant parts of *Aconitum* plants.

As introduced in section 1.2.2, *A. carmichaelii* is a widely traded plant in Asian countries, while the rootlets and the entire aerial parts are normally discarded when the roots are collected. A large amount of medicinal plant parts containing potential bioactive compounds, especially polysaccharides, are going to waste. Reports on comprehensive isolation, purification and structural characterization of polysaccharides from *A. carmichaelii* are rare, as majority of studies focus on the isolation and biological activities of crude polysaccharides from the root parts, or only pay attention to the isolated glucans (Paper I). The current study concluded that, for the first time, pectic type polysaccharides were present in all plant parts of *A. carmichaelii*. The pectic structural domains AG-I/II and arabinans were distributed differently in roots and aerial parts of *A. carmichaelii*, and AG-II and arabinan domains were shown having strong correlations with the immunomodulatory effects *in vitro* (Paper II and III). Further, the pectic polysaccharide isolated from *A. carmichaelii* leaves, AL-I, exhibited promising inhibitory effects on LPS-induced inflammation *in vitro* (Paper III), and protective effects against DSS-induced UC *in vivo*, where the gut microbiota and serum metabolites levels were significantly

modulated (Paper IV). All these results demonstrated the robust anti-inflammatory and immunomodulatory potentials of *A. carmichaelii* leaves as a medicinal resource. In addition, some polysaccharide fractions from other unutilized plant parts of this plant, the stems and rootlets, showed immunomodulatory and anti-inflammatory activities *in vitro*, revealing their potential values as medicinal plant sources (Paper II). Therefore, the current project suggests that the non-traditionally used plant parts of *A. carmichaelii* could be promising sources of bioactive polysaccharides.

Similarly, several alkaloids and lipids have been identified from the seeds and the epigeal parts of *A. septentrionale* [30, 74-77], which are processing wastes after collecting the root parts for the antiarrhythmic preparation allapinine. Thus, the aerial parts of *A. septentrionale* could be a potential medicinal source of bioactive compounds, including polysaccharides and phenolic compounds, which would be important for the comprehensive understanding and applications of *A. septentrionale* in the future.

4.6 The inclusion of gut microbiota into the study of anti-inflammatory activities of pectic polysaccharides

As shown in Paper II and III, some pectic polysaccharide fractions were demonstrated to exhibit potent anti-inflammatory effects on intestinal epithelial cells. However, there was a weak correlation between different structural characteristics and anti-inflammatory activity observed by pectic polysaccharides. There is still a challenge to fully understand specific structural features of anti-inflammatory pectic polysaccharides, even though many pectic polysaccharides containing HG and/or RG-I/II regions have been reported with anti-inflammatory effects [48]. Possible mechanisms (for *in vitro* bioassays) have been previously reported, and suggests effects through electrostatic forces due to the negative charge on pectin [48, 78], or through binding of receptors and thereby regulating signal pathways by their side chains [48] (details in the discussion of Paper II). However, combined with the robust anti-inflammatory effects observed *in vivo* in our project (Paper IV), the inclusion of microorganisms seems to be necessary to better understand such activity of pectic polysaccharide as a nondigestive polymer, as has been summarized much frequently in the last few years [44-47]. Gut microbiota could be the link between pectic polysaccharides and a range of bioactivities [45]. Several bacteria have been identified with specific carbohydrate-degrading enzymes, including glycoside hydrolases/transferases, polysaccharide lyases, or carbohydrate esterase identified from human bacteria in *Bacteroidetes*, *Lachnospiraceae* or *Ruminococcaceae* families [79]. These enzymes,

as well as the enzymatic degradation products deriving from pectins, in my opinion, could be the final targets for understanding how pectic polysaccharides are utilized step by step in human intestine.

For a clearer understanding of structure-activity relationship of anti-inflammatory pectins, enzymatic degradation for removal of specific structural regions of pectin, and then applying these derivatives to specific *in vivo* assays, as for instance an animal model by gastric gavage, would be useful to investigate the correlations. A study performed by Ishisono, Mano, Yabe and Kitaguch [80], is a good example, where the protective effects against colitis of pectins with different side chains were investigated. The pectin containing the highest degree of side chains exhibited best activity on the amelioration of clinical symptoms and colonic tissue damage. The use of animal models with deficiency of certain receptors, like TLR4 or NOD1/2, or that are bacteria-free, are important to verify the function of these active targets. And if possible, a systematic structural characterization of the dynamic change on polymers after fermentation by gut microbiota at different time points, might be more accurate to explain what happened with the pectins. However, the gut microbiota is a complex ecosystem including millions of bacteria and their metabolites which makes it difficult. Therefore, most studies only focus on the microbiota and metabolites changes compared to controls, like in Paper IV. It is obvious that a simple *in vitro* evaluation on certain cell lines or bioassays is not sufficient in order to understand the exact mechanisms and bioactivities of such biopolymers.

4.7 Limitations of this thesis

As mentioned in the former section, a structure-activity relationship of anti-inflammatory polysaccharides was not apparent (Paper II). A removal of specific structural regions, such as HG, terminal Ara*f* and/or 1,4-galactan backbones (AG-I) by enzymatic degradation using *endo*-α-(1–4)-polygalacturonase (pectinase), *exo*-α-L-arabinofuranosidase and *endo*-1,4-β-galactanase, respectively [81], should be considered in future studies. Further, the removal of starch specifically for polysaccharides isolated from *A. septentrionale* should be included in this study, which was not performed here due to time limitation. This could also be helpful for further investigating bioactivities of these polysaccharide fractions, which had no effect on the inhibition of NO release of dendritic cells (Paper V). Additionally, a full understanding of the physical characteristics (chain conformation, particle shape and size), as well as the use of a molecular docking model to predict the binding between polysaccharides/certain structural domains with receptors such as TLR4 or NOD2, might be of interest to further investigate a clear correlation of anti-inflammatory effects and pectic structure, as performed in earlier

studies [82, 83]. The charge of pectic polysaccharides (carboxyl group of GalA) has been reported to prevent the formation of hydrogen bonds and intestinal mucin–pectic aggregates, which is beneficial for the penetration of pectin through the mucin layer and promotes interaction between pectin and intestinal epithelial cells [78]. This is also important for intestinal anti-inflammatory activities, and therefore, the degree of esterification on carboxyl groups (or the degree of free carboxyl group) should be determined as well.

On the other hand, most polysaccharide fractions included in this thesis exhibited promising anti-inflammatory activities on intestinal epithelial cells. However, the isolated phenolic compounds were shown to have moderate or weak anti-inflammatory activities on dendritic cells. The active range of concentration used for each compound should be tested independently before starting the experiment. This was unfortunately not performed due to the low yields of tested samples. Other types of phenolics, like flavonoids, could be promising bioactive substances generated from *A. septentrionale*. They were not found in WEAS in the current project, and were not further studied due to the time limitation. More compounds could have been found in the other types of crude extracts of both the roots and the aerial parts of *A. septentrionale*.

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5 Conclusion and future perspectives

Most studies on *Aconitum* plants are limited to alkaloids and their poisonous feature. However, studies on less-toxic phytochemicals should also be performed in order to understand and broaden the medicinal use of these plants.

In this study, a variety of polysaccharides present in different plant parts of *A. carmichaelii* was compared, including those in the traditionally used roots of *A. carmichaelii*, lateral and mother roots, as well as the unutilized parts including the entire aerial parts, stems, leaves and rootlets. The polysaccharide patterns in the neutral fraction from the rootlets was different from those from lateral and mother roots, but was similar to those from the aerial parts, being a mixture of starch, arabinans, galactans, mannans and/or xyloglucans. RG-I regions possibly branched with arabinans and AG side chains, in addition to HG regions, were found in all plant parts of *A. carmichaelii*. However, the acidic polysaccharide fractions in the aerial parts, predominantly from leaves, were composed of more AG-II moieties compared to the acidic fractions from the root parts.

Polysaccharides isolated from the roots of *A. septentrionale* was similar to those from the traditionally used roots of *A. carmichaelii*, being mainly composed of starch, as well as pectic polysaccharides consisting of both HG and RG-I regions. Further purification of the polysaccharide fractions of *A. septentrionale* like removal of starch, would be important for a comprehensive characterization of the pectin, and for investigating various pharmacological properties.

Biological activities of the isolated polysaccharides of *A. carmichaelii*, such as their immunomodulatory properties and anti-inflammatory abilities on intestinal epithelial cells, indicates a potential medicinal value of this plant. Specifically, the major acidic fraction isolated from the leaves of *A. carmichaelii* exhibited potent amelioration on inflammatory colitis, which laid a foundation for future medicinal use of these unutilized plant sources. However, a structure-activity relationship for the anti-inflammatory polysaccharides were not apparent in the current study. Enzymatic degradation of specific regions of polysaccharides, a full understanding of physical characteristics, as well as molecular docking models between specific pectic structural domains with TLRs or NODs, might be of interest to further investigate a clear correlation for anti-inflammatory effects and structure.

Phenolic compounds identified in *A. septentrionale* roots in this study enrich the knowledge about the phytochemical diversity of this plant besides the already known alkaloids. Isolated

compounds showed potential anti-inflammatory and anti-oxidative activities. As mentioned in section 4.5, exploration of phenolic compounds from the other plant parts of *A. septentrionale* would be of interest to expand the knowledge about phytochemicals being present in the whole plant.

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Scientific papers I-V

Paper I

Aconitum carmichaelii Debeaux: a systematic review on traditional use, and the chemical structures and pharmacological properties of polysaccharides and phenolic compounds in the roots.

Yu-Ping Fu, Yuan-Feng Zou, Fei-Yi Lei, Helle Wangensteen, Kari Tvete Inngjerdingen.

Journal of Ethnopharmacology 2022, 291, 115148.

Paper II

Polysaccharides with immunomodulatory effects in different plant parts of *Aconitum carmichaelii*.

Yu-Ping Fu, Cen-Yu Li, Yuan-Feng Zou, Xi Peng, Berit Smestad Paulsen, Helle Wangensteen, Kari Tvete Inngjerdingen.

Manuscript submitted to International Journal of Biological Macromolecules

Paper III

Polysaccharides from *Aconitum carmichaelii* leaves: structure, immunomodulatory and anti-inflammatory activities**.**

Yu-Ping Fu, Cen-Yu Li, Xi Peng, Yuan-Feng Zou, Frode Rise, Berit Smestad Paulsen, Helle Wangensteen, Kari Tvete Inngjerdingen.

Carbohydrate Polymers 2022, 291, 119655.

Paper IV

Pectic polysaccharides from *Aconitum carmichaelii* leaves protects against DSS-induced ulcerative colitis in mice through modulations of metabolism and microbiota composition.

Yu-Ping Fu, Cen-Yu Li, Xi Peng, Helle Wangensteen, Kari Tvete Inngjerdingen, Yuan-Feng Zou.

Biomedicine & Pharmacotherapy 2022, 1551, 113767.

Paper V

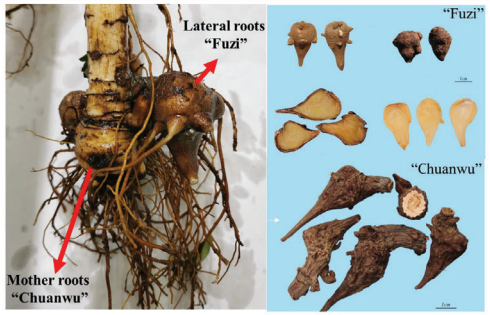
Bioactive phenolics and polysaccharides from the water extract of *Aconitum septentrionale* roots.

Yu-ping Fu, Karl Egil Malterud, Anne Grethe Harme, Kari Tvete Inngjerdingen, Helle Wangensteen.

Manuscript

Paper I

***Aconitum carmichaelii* Debeaux**



Water decoction

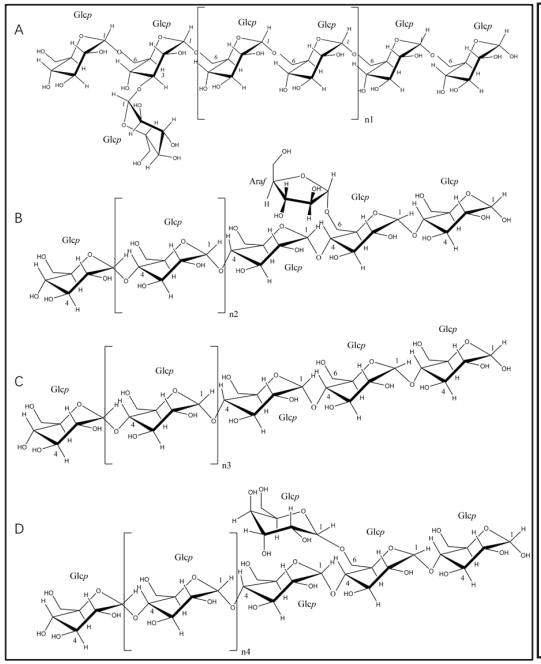
Traditional Use

"Fuzi"	**"Chuanwu"**
Rescue *Yang*	Dispel *Wind* and
Reverse collapse	*Dampness*
Dispel *Cold*	Disperse *Cold*
Relieve pain	Relieve pain

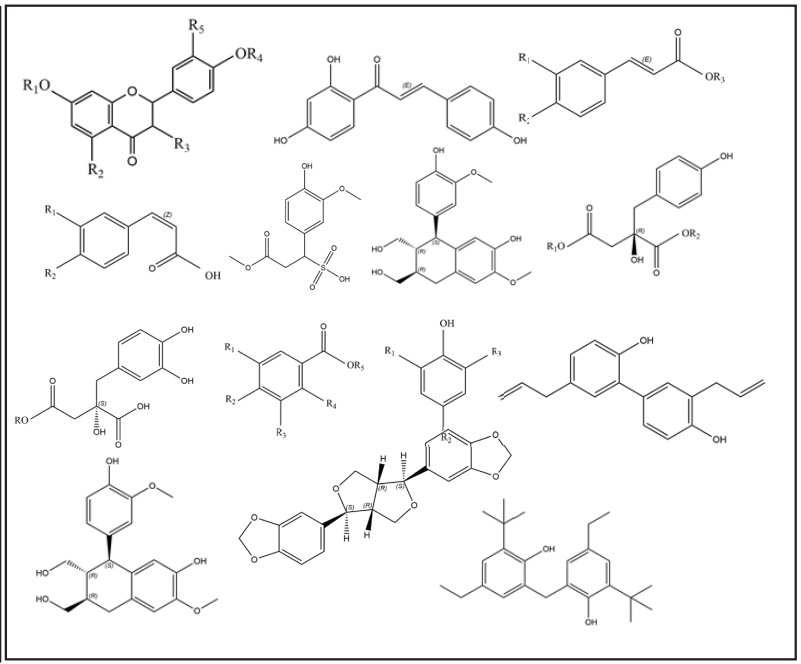
Alkaloids

Non-alkaloidal compounds

Polysaccharides



Phenolic compounds



Pharmacological Properties

Hypoglycemic
Hypolipidemic
Cardiovascular protection
Immunomodulatory
Antitumor
Neuropharmacological activities

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Aconitum carmichaelii Debeaux: A systematic review on traditional use, and the chemical structures and pharmacological properties of polysaccharides and phenolic compounds in the roots

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ABSTRACT

Ethnopharmacological relevance: Aconitum carmichaelii, belonging to the Ranunculaceae family, is a widely used traditional herbal plant in Asian countries, especially in China. The lateral ("Fuzi") and mother ("Chuanwu") roots are the two main plant parts used in Traditional Chinese Medicine (TCM), where they are used in the treatment of acute myocardial infarction, heart failure, rheumatoid arthritis, and as analgesics.

Aim of the study: In order to further guide the research direction and application of *A. carmichaelii*, this study aims to give a systematic and in-depth overview on the phytochemical and pharmacological studies of non-alkaloid natural products with focus on polysaccharides and phenolic compounds.

Materials and methods: A comprehensive search in the literature was conducted based on the databases Google Scholar, SciFinder (American Chemical Society), Springer Link, PubMed Science, Science Direct and China National Knowledge Internet, Wanfang Data, in addition to books, doctoral and master's dissertations, and official website. The main keywords were: "*Aconitum carmichaelii*", "Aconiti Lateralis Radix Praeparata", "Fuzi", "Chuanwu", "Aconiti Radix", "monkshood" and "Bushi".

Results: A. carmichaelii is known for the use of its different root parts, including "Fuzi" and "Chuanwu". Different types of polysaccharides, both neutral and acidic, and 39 phenolic compounds like flavonoids, phenylpropanoids, lignans, neolignans, and benzoic acid derivatives have been isolated and identified from the roots. Pharmacological studies of the isolated polysaccharides have demonstrated various biological effects such as hypoglycemic, hypolipidemic, cardiovascular, immunomodulatory, anti-tumor, and neuropharmacological activities. Studies on pharmacological effects of the phenolic compounds isolated from the roots are however limited.

Conclusions: This review shows that polysaccharides could be one of the active components in the roots of *A. carmichaelii*, and they are promising for future applications due to their pharmacological properties. In addition, polysaccharides are generally non-toxic, biocompatible, and biodegradable. This review also sheds light on new research directions for *A. carmichaelii*. A more detailed structural characterization of polysaccharides from different root parts of *A. carmichaelii*, and their structure-activity relationships are required. Additionally, their pharmacological properties as immunomodulators in the intestinal system should be investigated. Further, more knowledge about the pharmacological effects and molecular mechanisms of the phenolic compounds that have been identified are needed.

1. Introduction

Aconiti Lateralis Radix Praeparata (also known as "Fuzi", "Sheng Fuzi" or "Ni Fuzi") and Aconiti Radix (also known as "Chuanwu" or "Sheng Chuanwu") are the lateral and mother roots of *Aconitum carmichaelii* Debeaux (Ranunculaceae) respectively (Fig. 1). They are collected from late June to early August and are commonly used in Traditional Chinese Medicine (Chinese Pharmacopoeia Committee, 2020). "Fuzi" is more widely used than "Chuanwu", which is explained by the fact that each *A. carmichaelii* plant has two to fourteen lateral

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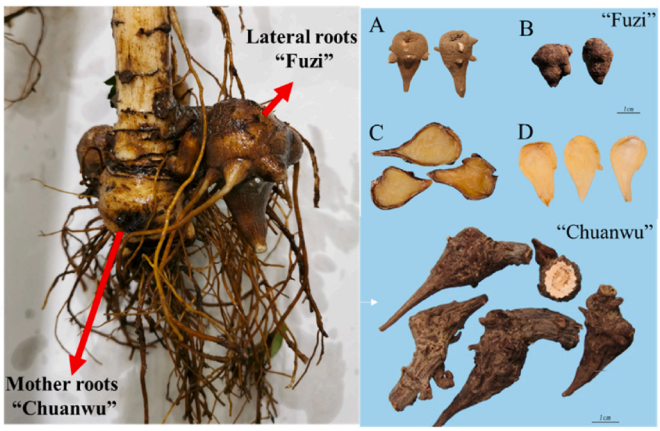


Fig. 1. The morphology of raw (left) and commercial (right) roots of *A. carmichaelii*; A, "Sheng Fuzi"; B, "Yan Fuzi"; C, "Hei Shun Pian"; D, "Bai Fu Pian". The left photo was taken in 2020 by the first author, and those on the right are from the Chinese Medicinal Material Images Database (School of Chinese Medicine, Hong Kong Baptist University, 2013).

roots and only one mother root (Wang, Y. et al., 2020). Commercial products of processed "Fuzi" and "Chuanwu" include "Yan Fuzi", "Hei Shun Pian", "Bai Fu Pian" and "Zhi Chuanwu", and are used in the treatment of shock caused by acute myocardial infarction, low blood pressure, coronary heart disease, chronic heart failure, rheumatoid arthritis and pain (Tang et al., 2017; Zhou et al., 2015). According to the Chinese Medicine Research CenterChina Academy of Chinese Medical Science (2017), the commercial trading of "Fuzi" occurs mostly in Sichuan and Shaanxi Province, with more than 200 tons of roots registered in Sichuan Province from November 2015 to April 2017.

Alkaloids have been regarded as the main constituents being responsible for the medical effects of "Fuzi" and "Chuanwu" decoctions used in clinical applications in Traditional Chinese Medicine (Wang and Chao, 2020; Zhang et al., 2019; Zhou et al., 2015). However, other water-soluble compounds such as polysaccharides and phenolic compounds have also been reported in *A. carmichaelii,* but they are less studied than alkaloids, as described in reviews of non-alkaloid components (Yin et al., 2019) and polysaccharides (Liu et al., 2019) from all *Aconitum* plants, as well as in reviews of *A. carmichaelii* (Wu et al., 2018; Zhou et al., 2015). Non-alkaloidal components in *Aconitum* plants are less toxic than the alkaloids (Yin et al., 2019), as shown for polysaccharides from "Fuzi" where toxic effects have been studied in Zebrafish embryos (Ding et al., 2019) and mice (Zhou et al., 2012). Polysaccharides have been reported to contribute to the anti-inflammatory, immunomodulatory (Yang et al., 2020), anti-oxidative (Huang et al., 2018; Zhang, 2016), anti-tumor (An et al., 2019), and myocardial cell protective activities (Wang et al., 2016) of "Fuzi" (Tang et al., 2017; Zhou et al., 2015), and anti-tumor effect of "Chuanwu" (Zhou et al., 2020a). Other phytochemicals also present in the root parts of *A. carmichaelii* include sterols, saponins, emodin, ceramides, and fatty acids (Li et al., 2013; Liang et al., 2018; Shim et al., 2005; Wang et al., 2014; Zhu et al., 2019).

Current reviews on pharmacological properties of polysaccharides derived from root parts of *A. carmichaelii* are restricted to "Fuzi", and they have mostly focused on the cardiovascular system (Huang et al., 2018; Liu and Wang, 2021; Ren et al., 2008; Xiong et al., 2013). An overview of polysaccharides or phenolic compounds from different root parts of *A. carmichaelii*, including extraction and isolation methods, chemical structures, and pharmacological effects, is still incomplete. Therefore, this review aims to provide a comprehensive and detailed overview of the non-alkaloid compounds found in different root parts by summarizing the phytochemistry and pharmacological effects of polysaccharides and phenolic compounds. In addition, the botany and traditional use of *A. carmichaelii* will be presented. This review will also provide a critical overview of current research and give some perspectives on future research that could lead to novel and less toxic applications of *A. carmicahelii*.

2. Materials and methods

The botanical information was collected from websites such as www.worldfloraonline.org (accessed February 1, 2021), www.worldplants.org (accessed March 2, 2021), and www.gbif.org (accessed February 24, 2021). Information about the traditional use was gathered from classical medical books and materia medica. The literature search was performed based on scientific databases, including Google Scholar, SciFinder (American Chemical Society), Springer Link, PubMed Science, Science Direct, China National Knowledge Internet (www.cnki.net, for Chinese literature) and Wanfang Data (http://www.wanfangdata.com.cn, for Chinese literature, doctoral and master's thesis) published from 1979 to May 2021. Official websites were also included. All the information was gathered using keywords like "Aconitum", "*Aconitum carmichaelii*", "Aconiti Lateralis Radix Praeparata", "Fuzi", "Chuanwu", "Aconiti Radix", "monkshood", "Bushi", "Prepared Aconite", "polysaccharide", "chemical substance", "phytochemistry", "pharmacology". Inclusion and exclusion criteria are described below. The Chemical Abstracts Service Registry Number (CAS RN) was checked in SciFinder, and ChemDraw Pro 16.0 was used to draw the chemical structures.

Inclusion criteria were studies concerning the traditional use of *A. carmichaelii* and studies reporting isolation, identification, and/or bioactivity studies of phenolic compounds and/or polysaccharides from the roots of *A. carmichaelii*. Exclusion criteria were dissertations, reviews, and other literature covering information of published articles with no original research results, conference abstracts, and studies with results restricted to alkaloids.

3. Botany and distribution

The genus *Aconitum* belonging to Ranunculaceae family consists of 337 species according to the World Flora Online (WFO) (2021). In China, over 200 *Aconitum* species exist, and 76 of them are used as medicinal herbs, *A. carmichaelii* being one of these (Yin et al., 2019). The plant is extensively cultivated at middle and low altitudes, in wet mountain grassland regions of southwestern China and northern Vietnam (Editorial Committee of Chinese FloraChinese Academy of Sciences, 1993; Yu et al., 2016). It is characterized with caudex obconical roots (2–4 cm long, 1–1.6 cm in diam), a 0.6–1.5 m stem branched with apically stems with sparsely retrorse pubescent, and pentagonal blade leaves (6–11 cm long and 9–15 cm wide) (Editorial Committee of Chinese FloraChinese Academy of Sciences, 1993). Five varieties (intraspecific taxon) are identified, and six synonyms are registered in the World Plants (Hassler, 2004–2021). The species names, synonyms of varieties, and their distributions are presented in Table 1.

A. carmichaelii is mainly distributed in 17 provinces in China, including Sichuan, Yunan, Guizhou, Chongqing, Anhui, Hubei, Liaoning, Nei Mongol, Hunan, Hebei, and Shaanxi Province (Yu et al., 2016). The oldest known preserved specimen can be tracked back to 1858 in Guizhou Province, China (Global Biodiversity Information Facility Secretariat, 2019). *A. carmichaelii* has been cultivated for about 1000 years in Sichuan Province (Jiangyou, Mianyang, Zhangming, Butuo, Pingwu, Qiongchuan, and Anxian City), and for more than 300 years in Shaanxi Province. During the last 70 years, cultivation areas have expanded to other Chinese provinces like Yunnan, Hebei, and Henan (Fang et al., 2020; Huang et al., 2011; Yu et al., 2016; Zhou et al., 2015). However, Sichuan is still the authentic area due to its mild climate, adequate rainfall, and the presence of well-developed technologies for cultivation (Dong et al., 2020; Huang et al., 2011). Today, *A. carmichaelii* is also found in Japan, the Netherlands, and the United States of America, where preserved specimens have been documented (Global Biodiversity Information Facility Secretariat, 2019), as well as in the Ha

Table 1
Morphological differences and distribution of *Aconitum carmichaelii* Debeaux varieties.

Plant species [a]	Distribution [a]	Synonyms [a]	Differences from *A. carmichaelii* Debeaux[b]
A. carmichaelii var. *hwangshanicum* (W.T.Wang & P. K.Hsiao) W.T. Wang & P.K. Hsiao	Anhui, Jiangxi, Zhejiang Province	*A. chinense* var. *hwangshanicum* W. T.Wang & P.K. Hsiao	The central lobe of leaves is longer and narrower, and the inflorescence rachis is often shorter.
A. carmichaelii var. *tripartitum* W.T. Wang	Jiangsu Province	/	The leaf division does not reach the base; the base of the leaf is broad heart-shaped or truncated heart-shaped.
A. carmichaelii var. *pubescens* W.T. Wang & P.K. Hsiao	Gansu and Shaanxi Province	/	The leaves are dasyphyllous.
A. carmichaelii var. *angustius* W.T. Wang & P.K. Hsiao	Henan Province	/	Not recorded.
A. carmichaelii var. *truppelianum* (Ulbr.) W.T.Wang & P.K.Hsiao	Jiangsu, Liaoning, Shandong and Zhejiang Province	= *A. carmichaeli* var. *fortunei* (Hemsl.) W.T. Wang & P.K.Hsiao = *A. fortunei* Hemsl. = *A. japonicum* var. *truppelianum* Ulbr. = *A. takahashii* Kitag. = *A. truppelianum* (Ulbr.)	The inflorescence rachis and pedicel, and the outside of the sepals have straight hairs spreading out.

[a] , Species names and distributions as recorded in World Plant (Hassler, 2004–2021).
[b] , Descriptions as recorded in Flora of China (Editorial Committee of Chinese FloraChinese Academy of Sciences, 1993).

Giang Province and high mountainous areas of northern parts of Vietnam (Do et al., 2019; Duc et al., 2015).

In Japan, the root parts of both *A. carmichaelii* and *A. japonicum* Thunb are used as the crude drug "Bushi" or "Aconiti Radix", for the same purpose as in China. Given the heterogeneous plant background of "Bushi" in Japanese applications (Hikino et al., 1982; Konno et al., 1979), only the studies indicating *A. carmichaelii* as the sole plant source are included in this review.

4. Traditional Chinese Medicine

In Traditional Chinese Medicine (TCM), all natural phenomena can be categorized into *Yin* and *Yang*, which are opposite, complementary, interdependent, and interchangeable aspects of nature. *Yin* refers basically to the material aspects of the organism, whereas *Yang* relates to its functions (Tang et al., 2008). The human body should have a healthy circulation of *Qi* (energy) and blood, as well as *Yin-Yang* equilibrium. Disease occurs when *Yin, Yang,* or *Qi* are disrupted in the body, or when it is attacked by six exogenous pathogenic evils (*Wind*, *Heat*, *Cold*, *Phlegm*, *Dampness*, and *Fire*). However, equilibrium can be restored by supplementation of Chinese herbs with specific natural capabilities (Liu and Liu, 2011). For instance, *Yang*-deficient patients with a sensitivity to heat and a preference for chilly environments require herbs possessing a *hot* nature (belonging to *Yang*) and the ability to withstand *Cold.* "Fuzi" and "Chuanwu" are examples of herbal drugs with such *hot* characteristics.

5. Traditional use of roots from *A. carmichaelii*

5.1. The lateral roots ("Fuzi" and "Cezi")

The lateral roots of *A. carmichelii* are frequently used in China (known as "Fuzi", Aconiti Lateralis Radix Praeparata, Prepared Common Monkshood Daughter Root, Carmichael's monkshood or Chinese wolfs bane), Japan (known as "Bushi", Aconiti Radix Processa or Processed Aconite Root), and Korea (known as Kyeong-Po Buja, Prepared Aconite) (Aboud et al., 2015; Zhou et al., 2015), and has been officially recorded in Chinese pharmacopoeias (since 1977), as well as in Japanese (15th-17th edition) and Korean pharmacopoeias (10th edition). In China, "Fuzi" and its processed products are pungent and sweet in flavor, hot in nature, toxic, and attributive to the heart, kidneys, and spleen meridians. They can rescue *Yang*, reverse collapse, augment *Fire* and *Yang*, dispel *Cold* and relieve pain. Therefore, it can be applied in cases of collapse with cold limbs and a faint pulse due to the prostration of *Yang* functions (Chinese Pharmacopoeia Committee, 2020; Zhou et al., 2015). This use of "Fuzi" was recorded in the earliest Chinese materia medica, *Shennong Bencao Jing*, during Han Dynasty (202 BC-220 AD). "Fuzi" was earlier recognized as a critical medicine because of its pure *Yang* and powerful effects, which was mentioned in *Xin Xiu Bencao*, the first officially issued Chinese pharmacopoeia in the Tang Dynasty (618 AD to 907 AD), and in *Zheng Lei Bencao* published in the Song Dynasty (960 AD–1279 AD). According to *Xin Xiu Bencao,* "Fuzi" should be used together with Zingiberis Rhizoma and Glycyrrhizae Radix et Rhizoma in order to minimize its poisonous effect, and these two medicines were also listed in the methods of commercial "Fuzi" processing techniques.

Both raw and processed "Fuzi" are utilized as cardiotonics in general. However, raw "Fuzi" has been applied more commonly in acute critical situations because of its fast and strong effect, while processed "Fuzi" has a slower onset of action but has a more prolonged effect (Yang, Y. et al., 2019). The production of various commercial products of "Fuzi" has been developed into safe and easy techniques, like soaking in salt-solution, boiling, peeling, slicing, and drying. Finally, they are known as "Yan Fuzi", "Bai Fu Pian", "Huang Pian", "Gua Pian" and "Hei Shun Pian" (see footnotes in Table 2) (Chinese Pharmacopoeia Committee, 2020; Liu et al., 2014, 2017). Only "Yan Fuzi", "Hei Shun Pian", and "Bai Fu Pian" are recorded in the latest edition of the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2020). "Fuzi" is currently utilized in 13.2% of 500 frequently used recipes and well-known prescriptions in clinical application in TCM (Song et al., 2013). It is also applied in moxibustion, brewing technology, agricultural production, locust control, religious ceremonies, and festival customs (Shen et al., 2020; Wang, Y. et al., 2020).

Formulas such as *"Sini Tang"* consisting of Radix Aconiti Lateralis Preparata, Zingiberis Rhizoma and Glycyrrhizae Radix et Rhizoma, "*Mahuang Xixin Fuzi Tang*" consisting of Ephedra Herba, Asari Radix et Rhizoma, and Radix Aconiti Lateralis Preparata, and "*Mahuang Fuzi Gancao Tang*" consisting of Ephedra Herba, Glycyrrhizae Radix et Rhizoma, and Radix Aconiti Lateralis Preparata, are increasingly used in clinical applications in order to reduce toxicity and enhance medicinal efficacy of "Fuzi" (Tai et al., 2015). The effect is most likely due to inhibition of certain side effects of alkaloids, increase of aconite metabolism in the liver, or chemical neutralization by compounds like isoliquiritigenin, liquiritin, and glycyrrhizic acid from the other herbs (Liu et al., 2017; Tai et al., 2015; Tang et al., 2018).

Furthermore, another lateral root called "Cezi", commonly identified as the bigger lateral roots, are less known and only used for the treatment of panneuritis epidemica. Information on "Cezi" is less recorded compared to "Fuzi" or "Chuanwu" in ancient herbalist's manuals, like *Wu Pu Bencao*, *Bencao Gang Mu*, *Zheng Lei Bencao*, and *Bencao Jing Ji Zhu*. It is rarely prescribed, but there are a few exceptions like the formulas *Cezi San*, *Shiwei Cezi Jiu*, *Cezi Tang*, and *Cezi Wan*.

5.2. The mother roots ("Chuanwu" and "Tianxiong")

The mother roots of *A. carmichaelii* are utilized in both China (known as "Chuanwu", Aconiti Radix/Common Monkshood Mother Root) and Japan (known as "Uzu"). Compared to "Fuzi" which is listed in nearly 3000 ancient Chinese descriptions, "Chuanwu" is mentioned in only approximately 670 traditional prescriptions (https://db.yaozh.com/). However, it has been known earlier than "Fuzi" and has been in use since the East Han Dynasty (25–220 AD) (Wang, Y. et al., 2020).

"Chuanwu" is characterized with a pungent and bitter flavor, hot in nature, toxic, and attributive to the heart, liver, kidneys, and spleen meridians. It has demonstrated abilities similar to "Fuzi", such as dispelling *Wind* and *Dampness*, dispersing *Cold*, and relieving pain. It is used to treat rheumatism, joint pain, cold, abdominal colic, and anesthesia in TCM theory (Chinese Pharmacopoeia Committee, 2020). "Wutou" was earlier a term deriving from both *A. carmichaelii* and *A. kusnezoffii* (Shi et al., 2018), which was gradually converted into "Chuanwu". Its original source was identified as *A. carmichaelii*, and the clinical use was separated by Shi-zhen Li in Ming Dynasty (1368–1644) (Zhou et al., 2015). "Chuanwu" was once used as a topical anesthetic in clinical surgery as recorded in the medicinal book *Huatuo Shenyi Mizhuan* and is now used in the treatment of stroke, sweating, and reducing appetite. It was also applied as a hallucination drug in ancient religious ceremonies (Li et al., 2020). In ancient China, "Zhi Chuanwu" (Aconiti Radix Cocta) was more commonly used as a processed medicine of "Chuanwu", as the use of "Fuzi". Today, the processing procedures have been simplified to include dipping into water, boiling/steaming, and drying, without the need of any additional materials (Chinese Pharmacopoeia Committee, 2020; Zhou et al., 2020b).

Apart from "Chuanwu", thin mother roots longer than 3 cuns (3 cuns equals to roughly 10 cm), called "Tianxiong", are also used and listed in *Shennong Bencao Jing* and *Bencao Jing Ji Zhu*, but its traditional use is not yet addressed in previous reviews. "Tianxiong" is probably the main root of *A. carmichaelii*, having lost the capacity to form lateral roots (Zhou et al., 2003). Besides its essential ability to eliminate dampness and relieve pain, which is the same as for "Fuzi" and "Chuanwu", "Tianxiong" is also used for strengthening sinews and bones, tonifying *Yang*, improving aspiration, and as essence-boosting. This is recorded in *Bencao Gang Mu, Mingyi Bie Lu*, and *Shang Han Lun*. A few non-classical formulas like *Bafeng Tang*, *Baidian Gao*, or *Baishen San* incorporate "Tianxiong" as the main ingredient (Cao et al., 2016).

These three root parts, "Fuzi", "Chuanwu" and "Tianxiong", produced in Sichuan Province (Jinzhou and Longzhou), have been considered to be of the highest quality, as recorded in *Xin Xiu Bencao* since Tang Dynasty. However, both "Tianxiong" and "Cezi", which are mainly used as local or folk herbs, are at the moment only sold in a few markets (Shi et al., 2018). Because of its uncommonness and limited applicability, published studies and other descriptions are rather limited.

6. Phytochemistry

6.1. Polysaccharides in A. carmichaelii

6.1.1. Preparation of different polysaccharide-containing fractions

Extraction and isolation of polysaccharides from "Fuzi" have been widely performed according to Table 2. The polysaccharide content in these roots has been reported to be from 3.3% (Zhao, X.S. et al., 2009) to 33.5% (Lv et al., 2011a). The main factors determining polysaccharide content are the geological locations of harvesting (Yue, 2015), followed by the growing period, storage environment for the plant material, and plant hormones (Shu and Hou, 2008; Shu et al., 2009; Zhao, X. et al., 2009). There are fewer reports on the extraction of polysaccharides from "Chuanwu" relatively to "Fuzi". A polysaccharide content of 20%–30% has been reported in "Chuanwu" (Lv et al., 2011a; Su and Liu, 1991a; Zhao et al., 2000), which is lower than what has been found in "Fuzi" (Gao et al., 2010; Lv et al., 2011a; Ou et al., 2013; Sun et al., 2018), as indicated in Table 2. It has further been demonstrated that the monosaccharide compositions of "Chuanwu" and "Fuzi" polysaccharides differ by one to three monosaccharides in both purified (Gao et al., 2010) and crude polysaccharides (Lv et al., 2011a). A content of 2% of polysaccharides has been reported in "Tianxiong" (Wang et al., 2019).

Different processing methods for "Fuzi" and "Chuanwu" could alter their polysaccharide contents (Gong et al., 2013; Guo et al., 2014; Kuang et al., 2014; Shu et al., 2010; Ye et al., 2019). It was demonstrated that the polysaccharide content in processed "Chuanwu" was reduced due to carbohydrate loss during boiling or steaming (Su and Liu, 1991a), and it has also been reported that the relative proportion of monomers in commercial "Hei Shun Pian" and "Bai Fu Pian" was slightly changed after processing. However, no detailed mechanism was discussed (Xu et al., 2014).

Pretreatments such as soaking and extraction in water have been employed in several studies (Ruan et al., 2000b; Ye et al., 2013; Zhu et al., 2015). Also, pre-extraction with 80%–96% ethanol has been used frequently before water extraction (WE) to remove lipids or hydrophobic substances (Lu and Niu, 2017; Lv et al., 2011a; Lv et al., 2011b; Shu et al., 2006; Su and Liu, 1991a; Xu et al., 2014; Yang et al., 2020; Zhao et al., 2006; Zhao, X.S. et al., 2009; Zhu et al., 2015), as well as petroleum ether (Fu et al., 2018). According to Table 2, the most common method for isolating crude polysaccharides from roots of *A. carmichaelii* is WE, which is generally combined with ethanol precipitation (EP). The extraction efficiency of "Fuzi" polysaccharide has been shown to be optimized by 0.8%–8% based on factors such as extraction temperature (90–100 °C), material/solvent ratio (1:10–1:40 g/mL), and extraction times (once to 3 times) (Shu et al., 2006; Yang et al., 2020; Ye et al., 2013). WE is occasionally assisted with other technologies like microwave and ultrasonic treatment, which resulted in around 5% (Zhu et al., 2015) and 15% (Lu and Niu, 2017) more polysaccharides from "Fuzi" respectively. Multi-enzyme-assisted extraction using a combination of amylase and cellulase is also reported to increase the polysaccharide content from 6.0% to 15.8% (Zhang, 2016). There are various methods used to purify polysaccharides from "Fuzi". Deproteinization, de-starching, and dialysis have been utilized in some studies (presented in Table 2) and have been optimized by Ye et al. (2013) and Ding et al. (2019). However, purification by ion-exchange chromatography and gel filtration to produce homogeneous fractions have only been performed in a few studies (Yang et al., 2020; Zhao et al., 2006), and hence less precise structural characterization except monosaccharide compositions have been reported so far.

6.1.2. Glucans

Starch is an energy storage polysaccharide produced by most green plants and commonly present in their roots, tuber, stems, and seeds. It is mainly composed of two constituents, including amylose, a linear α-D-(1 → 4)-linked glucose (Glc) chain, and amylopectin, a similar linear chain with α-(1 → 6)-branches (Mischnick and Momcilovic, 2010), as depicted in Fig. 2. It has been reported that there could be a high amount of starch present in the roots of *A. carmichaelii*, accounting for 73.9% of the total carbohydrate content in "Fuzi", and 55.0% of "Chuanwu" (Gao et al., 2010). More than 70% of starch in both raw and processed "Fuzi" was also reported after repeated depositing and re-suspending. The morphological features, particle size, amylose content, solubility, protein content, swelling power, water-binding capacity, crystallinity, and gelatinization of starch were changed during the processing phases (Xia et al., 2011; Yang, X. et al., 2019).

In addition to the starch found in "Fuzi" and "Chuanwu", four homogeneous polysaccharides from the product "Bushi" have been reported. One of these was named aconitan A and identified as a 1 → 6 linked α-D-glucan branched at *O*-3 (Konno et al., 1985; Masashi and Kazuyo, 1986) (Fig. 2). A water-soluble polysaccharide from dried "Fuzi" (FPS-1) has also been identified by Zhao et al. (2006) and is a similar α-glucan as aconitan A. In contrast, a β-glucan from "Bai Fu Pian" (FI) has been isolated (Ruan et al., 2000b), but no particular linkage type

Table 2
Polysaccharide-containing fractions isolated from different root parts of *Aconitum carmichaelii*.

Type of material	Root part[a]	Fraction(s) and yield[b]	Carbohydrate content	Extraction methods[c]	Purification methods	Mw/KDa	Structural characteristics (including monosaccharide compositions, linkage types, and sugar configuration)	Publication
"Bushi" cultivated in Hokkaido, Japan "Bushi"	R	Aconitan A	71.4/76.9/70.7/13.5% [d]	ME + WE	Cellulose (dH_2O or 50% MeOH); Sepharose 6B; DEAE-Toyopearl 650M (dH_2O and/or 0–1 M Nal); Sephacryl S-200	8.2	Glc with 0.9% peptide moieties; T-Glc*p*; 1,6-Glc*p*; 1,3,6-Glc*p*	Konno et al. (1985); Masashi and Kazuyo (1986)
		Aconitan B	51.1/52.2/43.0/16.3% [d]			210	Rha:Ara:Man:Gal:Glc = 0.6:0.1:2.3:1.0:1.2; with 6.5% *O*-acetyl groups and 5.5% peptide moieties	
		Aconitan C	55.6/58.2/42.8/29.9% [d]			430	Rha:Ara:Man:Gal:Glc = 1.1:1.0:0.2:1.0:0.8; with GlcA detected qualitatively, and 5.1% *O*-acetyl groups, and 4.4% peptide moieties	
		Aconitan D	40.1/40.5/34.7/18.0% [d]			42	Rha:Ara:Gal:Glc = 0.4:0.6:1.0:0.3; with 0.3% *O*-acetyl groups and 6.3% peptide moieties	
Processed "Fuzi" "Bai Fu Pian"[e]	LR	FI; 1.6%	97%	WE (hot); EP	Deproteinization (Sevag); DEAE-C32 (dH_2O); Sephadex G-200	260	Glc; β-configuration	Ruan et al. (2000b)
Processed "Fuzi"	LR	PS-I; 3%	/	WE (75 °C); EP (50%)	DEAE-C32 (dH_2O); Sephadex G-75	/	/	Ruan et al. (2000a)
"Huang Fu Pian"[f]		PS-II, 1%	/	WE (hot); EP	DEAE-C32 (0.2 M NaOH); Sephadex G-75	/	Contains 3% protein and 9% uronic acid	
Raw "Fuzi"	LR	FPS-1; 4.6%	97.1%	WE (100 °C); EP (80%);	Deproteinization (Sevag); DEAE-Cellulose (dH_2O); Sephadex G-75	~14	Glc; α-configuration; T-Glc*p*;1,6-Glc*p*;1,3,6-Glc*p*	Zhao et al. (2006)
Raw "Fuzi" (Radix Aconiti Lateralis)	LR	L-CWPS-N; 2.37%	99.9%	WE (4 °C and 95 °C); EP (80%)	Deproteinization (Sevag); de-starching (freeze-thawing) DEAE-Sepharose FF (dH_2O and 0.5 M NaCl)	10.2; 13.3	Man:Rha:GlcA:GalA:Glc:Gal:Ara = 0.53:0.82:1.50:2.54:90.71:3.41:0.49 GalA: Glc:Gal:Xyl:Ara = 2.04:78.27:5.66:0.72:13.32	Gao et al. (2010)
		L-HWPS-N; 0.30%	99.8%					
		L-CWPS-A; 0.29%	99.7%			160.2 and 5.8	Man:Rha:GlcA:GalA: Glc:Gal:Xyl: Ara = 1.25:3.67:6.60:11.14:31.14:19.04:1.51:25.65	
		L-HWPS-A; 0.61%	99.9%			≥1000, 207.3 and 10.2	Rha:GalA: Glc:Gal:Xyl:Ara = 8.30:20.02:3.78:12.87:5.37:49.68	
Raw "Chuanwu" (Radix Aconiti)	MR	A-CWPS-N; 1.42%	99.9%	WE (4 °C and 95 °C); EP (80%)	Deproteinization (Sevag); de-starching (freeze-thawing) DEAE-Sepharose FF (dH_2O and 0.5 M NaCl)	10.0	Glc:Gal:Ara = 95.71:4.01:0.28	Gao et al. (2010)
		A-HWPS-N; 0.97%	99.8%			302.9–10.8	Glc:Gal:Xyl:Ara = 96.96:1.71:0.18:1.20	
		A-CWPS-A; 0.21%	99.8%			160.2 and 13.0	Man:Rha:GlcA:GalA:Glc:Gal:Ara = 1.84:3.05:2.67:5.71:46.13:23.58:17.03	
		A-HWPS-A; 0.48%	99.8%			≥1000, 199.7 and 10.8	Rha:GlcA:GalA:Glc:Gal:Xyl:Ara = 4.82:0.92:33.49:6.50:11.48:2.91:39.87	
Raw roots	LR MR RL	33.5% 22% 6.1%	/	WE (100 °C); EP (80%);	Deproteinization (Sevag) only	/	Glc:Ara:GalA:Gal:Man = 100:5:7:3:1 Glc:GalA:Ara:Gal = 100:3:3:2 Glc:GalA:Ara:Gal:Xyl:Man:Rha = 100:137:59:24:20:3:3	Lv et al. (2011a)
Raw "Fuzi"	LR	/	/	WE (90 °C); EP (80%)	Deproteinization (Sevag) only	/	Glc:Gal = 16.55:28.72	Fu and Ye (2014)
Processed "Fuzi": "Sheng Fu Pian" (SFP)[g], "Hei Shun Pian" (HSP)[h], "Bai Fu Pian" (BFP)[e]	LR	SFP:4.77% HSP:4.53% BFP:4.91%	/	WE (90 °C); EP (80%)	No further purification	/	Rha:Ara:Man:Glc:Gal = 0.3:0.94:0.35:100:0.71 Rha:Ara:Man:Glc:Gal = 0.28:1.08:0.85:100:0.98 Rha:Ara:Man:Glc:Gal = 0.2:1.46:1.06:100:0.95	Xu et al. (2014)
Raw "Fuzi"	LR	/	70.24%	WE (100 °C); EP (80%)	Deproteinization (Sevag) only	/	Man, Glc (main), Rha, Gal, Xyl, Fuc, and Ara	Wang et al. (2016)
Processed Fuzi "Hei Shun Pian"[h]	LR	1.78%	/	WE (80 °C); EP (80%);	No further purification	/	D-Ara, D-Xyl, D-Glc, D-Gal	Fu et al. (2018)
Type of material	Root part[a]	Fraction(s) and yield[b]	Carbohydrate content	Extraction methods[c]	Purification methods	Mw/KDa	Structural characteristics (including monosaccharide compositions, linkage types, and sugar configuration)	Publication
	MR	PPTX; 2%	/			/	Glc:Man:GlcA:GalA:Rha:Gal:Xyl:Ara =	

(*continued on next page*)

Table 2 (*continued*)

Type of material	Root part[a]	Fraction(s) and yield[b]	Carbohydrate content	Extraction methods[c]	Purification methods	Mw/ KDa	Structural characteristics (including monosaccharide compositions, linkage types, and sugar configuration)	Publication
Processed "Tianxiong"				WE (100 °C); EP;	De-starching (α-amylase); deproteinization (Sevag)		65.28:4.30:0.94:2.47:5.79:4.36:0.71:5.08; small amount of talose, lactose, cellobiose, melibiose, trehalose	Wang et al. (2019)
Processed "Fuzi"	LR	FZPS-1; 5.17%	/	WE (90 °C); EP (80%);	Deproteinization (Sevag); de-starching (α-amylase); DEAE-C32 (dH_2O); Sephadex G-50	6290	Ara:Glc = 7.5:92.5; linkage units: →4)-α-D-Glc-(1→, →4)-β-D-Glc, →4,6)-α-D-Glc-(1→, →4)-α-D-Glc and α-L-Ara-(1→	Yang et al. (2020)

[a] LR, MR, R, and RL represent the lateral roots, mother roots, roots, and rootlets, respectively.

[b] Yields were calculated based on dried materials.

[c] Extraction methods include WE (water extraction), ME (methanol extraction), and precipitation with a certain concentration of ethanol (EP).

[d] Data determined with phenol-sulfuric acid, chromotropic acid-sulfuric acid, anthrone-sulfuric acid (calculated with Glc) and carbazole-sulfuric acid methods (calculated with GlcA) respectively. The detailed processing procedures of materials are (Chinese Pharmacopoeia Committee, 2020; Fan et al., 2019).

[e] "**Bai Fu Pian**", soaking in a salt solution for a few days, boiling with salt-solution until it penetrates into the central part, peeling off the skin, cutting into pieces about 0.3 cm thick, then steaming and drying under the sun.

[f] "**Huang Fu Pian**", cutting into pieces about 0.7 cm after soaking with salt-solution for several days and peeling, then soaking and washing with water, dying with brown sugar and tea solution, and drying under the sun.

[g] "**Sheng Fu Pian**", sliced "Sheng Fu Zi".

[h] "**Hei Shun Pian**", cutting into pieces about 0.5 cm after soaking with salt-solution and dying solution, then steaming and roasting over a charcoal fire; All plant materials used in research after 2000 were obtained in Sichuan Province, China.

was declared. The linkage types of these published glucans distinguish them from starch, and their Mw ranges from 8.2 kDa to 260 kDa, as can be seen in Table 2 and Fig. 2.

6.1.3. Heteropolysaccharides

According to the monosaccharide compositions of published polysaccharides in Table 2 (column 8), neutral heteropolysaccharides in different root parts of *A.carmichaelii* have been reported consisting of rhamnose (Rha), arabinose (Ara), galactose (Gal), Glc, mannose (Man), or xylose (Xyl). Konno et al. (1985) first isolated two neutral heteropolysaccharides aconitan B and D from "Bushi", and more were discovered afterwards. A neutral fraction from "Huang Fu Pian" (PS–I) was isolated in 2000, but no additional structural information was provided (Ruan et al., 2000a). Another two extracted with cold-water (L-CWPS-N) and hot water (L-HWPS-N) from "Fuzi", and two extracted with cold (A-CWPS-N) and hot water (A-HWPC-N) from "Chuanwu" were demonstrated to consist of various types and amounts of monosaccharides, as shown in Table 2 (Gao et al., 2010). These fractions were vaguely described as non-starch type α-glucans, while the presence of detectable uronic acids was not mentioned. Additionally, three more heteropolysaccharides from raw "Fuzi" (Fu and Ye, 2014; Lv et al., 2011b; Wang et al., 2016), and four more from processed "Fuzi" (Fu et al., 2018; Xu et al., 2014) have been reported, but only the monosaccharide compositions were described. Unlike the polymers mentioned above, the water-soluble polysaccharide fraction denominated FZPS-1 is the only neutral heteropolysaccharide where glycosidic linkages have been determined and identified as a 1,4 linked-glucan with Ara side chains (Yang et al., 2020) (Fig. 2).

Acidic heteropolysaccharides, like pectins, are essential structural polysaccharides in most primary cell walls. They may contain carboxyl groups and be methyl- or acetyl-esterified. Galacturonic acid (GalA) is the substantial component of pectins, along with Gal, Ara, Rha, and minor amounts of other monomers (Voragen et al., 2009). Glucuronic acid (GlcA) is not the main monosaccharide found in pectin (Voragen et al., 2009) but has been reported by Konno et al. (1985) to be present in aconitan C isolated from "Bushi".

As presented in Table 2, studies on acidic polysaccharides obtained from different roots of *A. carmichaelii* focus solely on monosaccharide compositions, and the determination of glycosidic linkages has not yet been performed. There are many studies describing the isolation and primary characterization of acidic polysaccharides from "Fuzi". For instance, the amounts of uronic acids in a fraction isolated from "Huang Fu Pian" (PS-II) were quantified (Ruan et al., 2000a). Further, two acidic polysaccharides extracted with cold-water (L-CWPS-A) and hot water (L-HWPS-A) were reported to contain GalA and/or GlcA (Gao et al., 2010). The acidic polysaccharides present in the mother roots are less studied than those in the lateral roots. Two fractions extracted with cold water (A-CWPS-A) and hot water (A-HWPS-A) consisting of GalA and GlcA were purified from "Chuanwu" by Gao et al. (2010), and an acidic polysaccharide isolated from "Tianxiong" (PPTX) has also been investigated (Wang et al., 2019). In addition, according to Lv et al. (2011a), identical acidic polysaccharides have been discovered in different root parts, with rootlets containing higher amounts of GalA in polysaccharides than found in lateral and mother roots (Table 2).

6.2. Phenols, phenolic acids and their derivatives in A. carmichaelii

The chemical structures and pharmacological activities of the diterpenoid alkaloids in *Aconitum* plants have been well studied and described in several reviews (Aslam and Ahmad, 2016; Nyirimigabo et al., 2015; Wang, H. et al., 2020). However, various other secondary metabolites, especially phenolic compounds, have also been reported to be present in these plants and may be involved in the medicinal functions of the *Aconitum* plants (Yin et al., 2019). At present, 39 phenolic compounds have been isolated and identified from the roots of *A. carmichaelii*, including four flavonoids (compounds **1**–**4**), eighteen

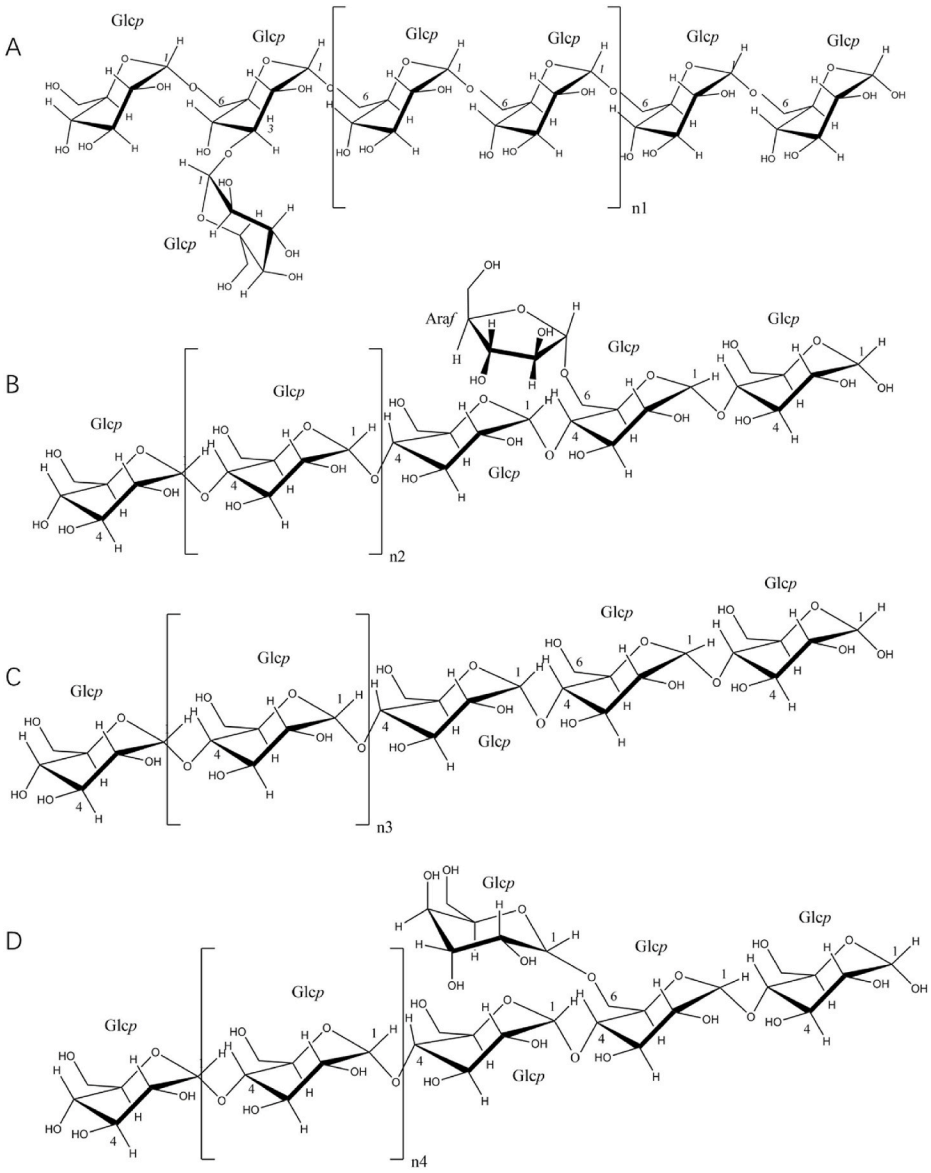


Fig. 2. Proposed structural features of polysaccharides from the roots of *Aconitum carmichaelii*. **A.** α-Glucan adapted from Masashi and Kazuyo (1986) and Zhao et al. (2006) **B**. Neutral heteropolysaccharide adapted from Yang et al. (2020). **C** and **D**. Starch consisting of amylose (C) and amylopectin (D) adapted from Gao et al. (2010), Xia et al. (2011), and Yang, X. et al. (2019). n1∼n4 represent the polymerization number of corresponding residues.

phenylpropanoids (compounds **5**–**22**), four lignans (compounds **23**–**26**), one neolignan (compound **27**), seven benzoic acid derivatives (compounds **28**–**34**), and five other phenolic compounds (compounds **35**–**39**), as listed in Table 3. CAS registry number, plant parts, and extraction methods are also presented. Their chemical structures are shown in Figs. 3–7. The isolation methods differed depending on the chemical nature of the compounds. Water, aqueous alcohol, or alcohol were used as extraction solvents to extract the water-soluble to less hydrophilic phenolic compounds. Common purification methods were silica gel column chromatography, the MCI-gel, Sephadex LH-20, reversed-phase high-performance liquid chromatography, and normal phase thin-layer chromatography. Usually, a combination of different chromatographic methods is employed to obtain pure substances. The separation methods are mainly based on adsorptive properties. However, Sephadex LH-20 separates molecules based on several principles, such as size exclusion and adsorption (Hostettmann et al., 1998). Structures were identified based on nuclear magnetic resonance spectroscopy (NMR), ultraviolet, infrared spectroscopy, and electrospray ionization mass spectrometry.

6.2.1. *Flavonoids*

Flavonoids are commonly found in the aerial parts of *Aconitum* plants and are considered as the chemotaxonomical biomarkers beside alkaloids (Yin et al., 2019). Anthocyanins are responsible for the purple-blue color of the flowers (Tatsuzawa et al., 2019). Flavonoids are known for their antioxidant, anti-inflammatory, cardiovascular, immunomodulatory, antibacterial, antiviral, and antifungal activities and are regarded as health beneficial (Jucá et al., 2020). They also have important functions in plants, like protection against ultraviolet radiation and microorganisms (Pourcel et al., 2007). However, from the root parts of *A. carmichaelii*, only four flavonoids, including three flavanones **(1**–**3)** and one chalcone (**4**), have been identified. Detailed chemical information is shown in Table 3 and Fig. 3.

6.2.1.1. Flavanones. The first flavonoids in *A. carmichaelii* were reported by Shim et al. (2005) from the methanol extract of processed *A. carmichaelii* tuber imported from Sichuan province, China. Liquiritigenin **(1)**, liquiritin **(2)**, and 6″-O-acetylliquiritin **(3)** were isolated and identified. In a later study, Lyu et al. (2008) reported compounds **1**

Table 3
Phenols, phenolic acids and their derivatives reported in root parts of *Aconitum carmichaelii*.

No.	Identified compounds	CAS RN	Material [a]	Extracts [b]	Publication
Flavanones					
1	Liquiritigenin	578-86-9	T-SC*	MeOH	Shim et al. (2005)
			T-K	80% EtOH	Lyu et al. (2008)
2	Liquiritin	551-15-5	T-SC*	MeOH	Shim et al. (2005)
			T-K	80% EtOH	Lyu et al. (2008)
3	6″-*O*-Acetylliquiritin	166531-17-5	T-SC*	MeOH	Shim et al. (2005)
Chalcone					
4	Isoliquiritigenin	961-29-5	T-SC*	MeOH	Shim et al. (2005)
Phenylpropanoids and derivatives					
5	*p*-Hydroxy-cinnamic acid	7400-08-0	LR-SC	90% EtOH	Zhang et al. (2014a)
6	Ferulic acid	1135-24-6	LR-SC	H_2O	Jiang et al. (2014)
7	Methyl ferulate	2309-07-1	LR-SC	H_2O	Jiang et al. (2014)
8	Butyl ferulate	4657-33-4	LR-SC	H_2O	Jiang et al. (2014)
9	Linocinnamarin	554-87-0	LR-SC	H_2O	Jiang et al. (2014)
10	*trans*-Feruloyl-4-β-glucoside	117405-51-3	LR-SC	EtOH	Li et al. (2013)
			LR-SC*	H_2O	Geng (2012)
			LR-SC*		Xue et al. (2018)
11	Methyl 4-β-D-allopyranosyl-ferulate	–	R–VN	MeOH	Do et al. (2019)
12	Methyl-4-β-D-gulopyranosyl cinnamate	–	R–VN	MeOH	Do et al. (2019)
13	Methyl 4-β-D-glucopyranosyl ferulate	951288-53-2	LR-SC	H_2O	Jiang et al. (2014)
			R–VN	MeOH	Do et al. (2019)
14	*cis*-Feruloyl-4-β-glucoside	94942-20-8	LR-SC	EtOH	Li et al. (2013)
15	*cis-p*-Coumaric acid 4-*O*-β-D-glucoside	117405-48-8	LR-SC	EtOH	Li et al. (2013)
16	(−)-(*R*)-eucomic acid	60449-48-1	LR-SC	H_2O	Jiang et al. (2014)
17	4-Methyl eucomate	446867-37-4	LR-SC	H_2O	Jiang et al. (2014)
18	Dimethyl eucomate	60449-49-2	LR-SC	H_2O	Jiang et al. (2014)
19	(−)-(*R*)-hydroxyeucomic acid	72619-98-8	LR-SC	H_2O	Jiang et al. (2014)
20	4-Methyl-(−)- (*R*)-hydroxyeucomate	1801857-55-5	LR-SC	H_2O	Jiang et al. (2014)
21	4-Butyl-(−)-(*R*)-hydroxyeucomate	1801857-56-6	LR-SC	H_2O	Jiang et al. (2014)
22	Methyl (±)-3-(4′-hydroxy-3′-methoxyphenyl)-3-sulfopropionate	1801857-61-3	LR-SC	H_2O	Jiang et al. (2014)
Lignans and neolignan					
23	Sesamin	607-80-7	LR-SC	95% EtOH	Zhang et al. (2014b)
24	Episesamin	133-03-9	LR-SC	95% EtOH	Zhang et al. (2014b)
25	Pinoresinol	487-36-5	LR-SC	90% EtOH	Zhang et al. (2014a)
26	Isolariciresinol	548-29-8	LR-SC	95% EtOH	Zhang et al. (2014b)
27	Honokiol	35354-74-6	LR-SC	90% EtOH	Zhang et al. (2014a)
Benzoic acid derivatives					
28	Salicylic acid	69-72-7	LR-SC	90% EtOH	Zhang et al. (2014a)
29	Isovanillic acid	645-08-9	R–VN	MeOH	Do et al. (2019)
30	4-Butyl-1-methyl (+)-(R)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl)malate	1801857-57-7	LR-SC	H_2O	Jiang et al. (2014)
31	1-Butyl-4-methyl (+)-(*R*)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl)malate	1801857-58-8	LR-SC	H_2O	Jiang et al. (2014)
32	Dimethyl (+)-(R)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl) malate	1801857-59-9	LR-SC	H_2O	Jiang et al. (2014)
33	Dimethyl-(+)-(*R*)-2-*O*-(4′-hydroxybenzoyl)malate	1801857-60-2	LR-SC	H_2O	Jiang et al. (2014)
34	2′-carbamoyl-4′-hydroxy benzyl-2-hydroxybenzoate		MR-HB	95% EtOH	Sun (2009)
Other phenolic compounds					
35	2,4-Di-*tert*-butylphenol	96-76-4	MR-LN	Volatile	Wang et al. (2014)
			R–SC	MeOH	Liang et al. (2018)
			MR-SC	Volatile	Chen (2011)
			MR-GZ	Volatile	Zhang and Zhao (2011)
36	Phenol, 2,6-di-tert-butyl-	128-39-2	RL-SC	Volatile	Chen (2011)
37	2,6-Di-tert-butyl-4- methylpheno	128-37-0	R–SC	MeOH	Liang et al. (2018)
38	Phenol, 2-(1,1-dimethylethyl) −4 (1,1,3,3-tetramethylbutyl)	5806-73-5	MR-LN	Volatile	Wang et al. (2014)
39	2,2′-Methylenebis [4-ethyl-6-*tert*-butylphenol]	88-24-4	R–SC	MeOH	Liang et al. (2018)

[a] , **T-SC**: tubers imported from Sichuan Province, China; **T-K**: tubers from material obtained from Korea; **R-GZ**, roots (no detailed information of which root parts) collected from Dafang, Guizhou Province, China; **LR-SC**: lateral roots cultivated in Sichuan Province, China; **R–VN**: roots from Vietnam; **R–SC**: roots (no detailed information of which root parts cultivated in Sichuan Province, China; **MR-GZ**: mother roots cultivated in Longli County, Guizhou Province, China; **MR-HB**, mother roots purchased from Anguo City, Hebei Province, China; **MR-LN:** mother roots purchased from a pharmacy in Shenyang City, Liaoning Province, China; **MR-SC**: mother roots cultivated in Sichuan Province, China; **RL-SC**: rootlets cultivated in Sichuan Province, China; **LR-YN**: lateral roots from Yunnan Province, China. Those marked with * are processed root parts.

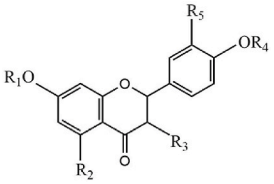
[b] , **MeOH**, methanol; **EtOH**, ethanol; **Volatile**, the volatile substances.

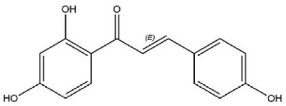
and **2** in an 80% ethanol extract of processed tuber materials from Korea.

6.2.1.2. Chalcone. Isoliquiritigenin (**3**) is the only chalcone isolated from *A. carmichaelii* (Shim et al., 2005). The chalcone is closely related to the flavanones biosynthetically. As shown in Fig. 3, compound **3** has the same substitution pattern as compound **1** but with the open C ring characteristic for chalcones.

6.2.2. Phenylpropanoids and derivatives

Phenylpropanoid glycosides are reported from the genus *Aconitum* by Li et al. (2013). Trans-feruloyl-4-β-glucoside **(10)**, cis-feruloyl-4-β-glucoside **(14),** and cis-*p*-coumaric acid 4-*O*-β-D-glucoside **(15)** were obtained and identified from the ethanol extract of raw "Fuzi". The predominant constituent was compound **10** (100 mg), followed by compound **14** (7 mg) and **15** (8 mg) from 5 kg dried root materials (Li et al., 2013). *p*-Hydroxycinnamic acid **(5)** was identified in a follow-up study from raw "Fuzi" (Zhang et al., 2014a), and a number of methyl- and glycosyl-derivatives of compound **5** have also been isolated from raw (Do et al., 2019; Jiang et al., 2014; Li et al., 2013; Zhang et al., 2014a) and processed "Fuzi" (Geng, 2012; Xue et al., 2018), including ferulic acid **(6)**, methyl ferulate **(7),** butyl ferulate **(8)**, linocinnamarin

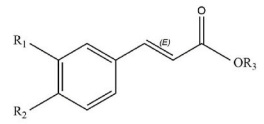




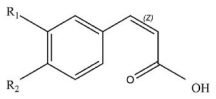
	R1	R2	R3	R4	R5
1	H	H	H	H	H
2	H	H	H	β-D-Glc*p*	H
3	H	H	H	6-*O*-Acetyl-β-D-Glc*p*	H

4

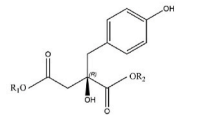
Fig. 3. Flavonoids obtained from the roots of *Aconitum carmichaelii*.



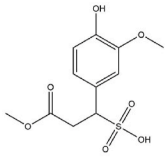
	R1	R2	R3
5	H	*O*H	H
6	*O*-Me	OH	H
7	*O*-Me	OH	Me
8	*O*-Me	OH	*n*-Bu
9	*O*-β-D-Glc*p*	H	Me
10	*O*-Me	*O*-β-D-Glc*p*	H
11	*O*-Me	*O*-β-D-All*p*	Me
12	H	*O*-β-D-Glc*p*	Me
13	*O*-Me	*O*-β-D-Glc*p*	Me



	R1	R2
14	*O*-Me	*O*-β-D-Glc*p*
15	H	*O*-β-D-Glc*p*



	R1	R2
16	H	H
17	Me	H
18	Me	Me



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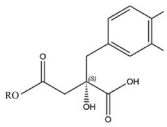
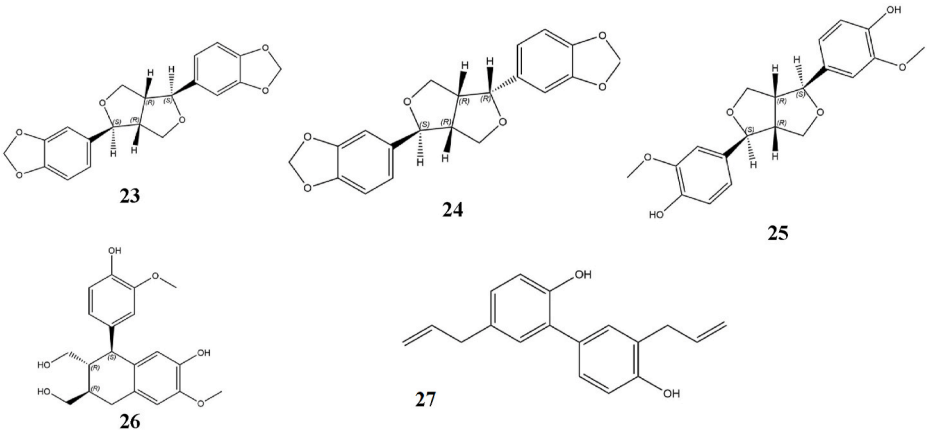


	R
19	H
20	Me
21	*n*-Bu

Fig. 4. Phenylpropanoids and derivatives obtained from the roots of *Aconitum carmichaelii*.



23 **24** **25** **26** **27**

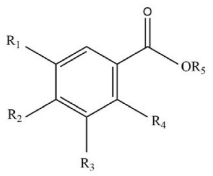
Fig. 5. Lignans and a neolignan obtained from the roots of *Aconitum carmichaelii*.

(9), and methyl 4-*O*-β-D-glucopyranosyl ferulate **(13)** from the water extracts (Jiang et al., 2014). Other phenylpropanoid derivatives including (−)-(*R*)-eucomic acid **(16)**, 4-methyl eucomate **(17)**, dimethyl eucomate **(18)**, (−)-(R)-hydroxyeucomic acid **(19)**, and three new derivatives, 4-methyl-(−)-(*R*)-hydroxyeucomate **(20)**, 4-butyl-(−)-(*R*)-hydroxyeucomate **(21)** and methyl (±)-3-(4′-hydroxy-3′-methoxyphenyl) -3-sulfopropionate **(22)** were also identified (Jiang et al., 2014). Compound **22** was a rare one containing a sulfonic acid group.

The majority of the hydroxycinnamic acids and derived compounds were obtained from the "Fuzi" from the Sichuan Province, as shown in Table 3 (Geng, 2012; Jiang et al., 2014; Li et al., 2013; Zhang et al., 2014a). The methyl 4-β-D-allopyranosyl-ferulate **(11)**, methyl-4--β-D-gulopyranosyl cinnamate **(12)**, and **13** were isolated from the materials cultivated in Vietnam in 2019 (Do et al., 2019), and the former two compounds were the new constituents isolated from this plant in the methanol extracts after alkalized with NH_4OH. Compounds **15** and two coumaric acid glycosides, (E)-4-*O*-(β-D-glucopyranosyl) coumaric acid and its (Z)-isomer were isolated from dried raw *Aconitum* roots and the processed roots, known as "Kako-Bushi-Matsu" (Matsui et al., 2002). However, they were not included in this review because of the heterogeneous plant source mentioned in the botany section. The chemical structures of compounds **5**–**22** are shown in Fig. 4.

6.2.3. *Lignans and neolignan*

Until now, four lignans (**23**–**26**) and one neoligan (**27**) have been identified in the ethanol extracts from raw "Fuzi", as shown in Table 3.



	R1	R2	R3	R4	R5
28	H	H	H	OH	H
29	OH	*O*-Me	H	H	H
30	*O*-Me	OH	H	H	4-Butyl-1-methyl (+)-(R)-2-*O*-malate
31	H	OH	*O*-Me	H	1-Butyl-4-methyl (+)-(R)-2-*O*-malate
32	H	OH	H	H	Dimethyl-(+)-(R)-2-*O*-malate
33	*O*-Me	OH	H	H	Dimethyl-(+)-(R)-2-*O*-malate
34	H	H	H	OH	2-carbamoyl-4-hydroxy benzyl

Fig. 6. Benzoic acids and derivatives obtained from the roots of *Aconitum carmichaelii*.

These lignans can be categorized as furofuran subtypes, including sesamin **(23)**, episesamin **(24)** and pinoresinol **(25)**, and aryltetralin lignan such as isolariciresinol **(26),** as illustrated in Fig. 5. Honokiol (**27)** is classified as a neolignan (Teponno et al., 2016), a biphenyl in which the two phenylpropen units are not coupled at the central carbon atom of the side chain as is usual for the lignans. All of them were found by Zhang et al. (2014a) and Zhang et al. (2014b) after purification with macroporous cation exchange resin, silica gel, and Sephadex LH-20 chromatography from the 90%–95% ethanol extracts. Moreover, compared to a review of non-alkaloidal constituents from the genus *Aconitum* (Yin et al., 2019), compound **26** and **27** were novel lignans discovered in *Aconitum* plants (Zhang et al., 2014a, 2014b). The chemical structures of compounds **23**–**27** are shown in Fig. 5.

6.2.4. Benzoic acid derivatives

Seven benzoic acid derivatives (compounds **28**–**34**) have been identified in the roots of *A. carmichaelii* cultivated in widespread places, including Sichuan (Fang, 2018; Lei et al., 2013; Liang et al., 2018) and Guizhou province in China (Zhang and Zhao, 2011), and Ha Giang province in Vietnam (Do et al., 2019). Detailed information and chemical structures are shown in Table 3 and Fig. 6.

Salicylic acid **(28)** in raw "Fuzi" from Sichuan (Zhang et al., 2014a) and its methyl derivative, isovanillic acid **(29)** in the roots from Vietnam (Do et al., 2019), have been identified. Jiang et al. (2014) isolated and identified four new (R)-2-*O*-benzoylmalate derivatives in the water extracts of raw "Fuzi", such as 4-butyl-1-methyl (+)-(R)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl)malate **(30)**, 1-butyl-4--methyl (+)-(R)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl)malate **(31)**, dimethyl (+)-(R)-2-*O*-(4′-hydroxy-3′-methoxybenzoyl) malate **(32)** and dimethyl-(+)-(R)-2-*O*-(4′-hydroxybenzoyl)malate **(33)**. A new natural product from dried raw "Chuanwu", 2′-carbamoyl-4′-hydroxy benzyl-2-hydroxybenzoate **(34),** was initially reported by Sun (2009).

6.2.5. Other phenolic compounds

There are additional six phenolic compounds confirmed in the roots of *A. carmichaelii*, which are presented in Table 3 and Fig. 7. Butyl phenols (compounds **35**–**36)** and those with methyl (**37**) and 1,1,3,3-tetramethylbutyl (**38**) substituents were extracted from different root parts (Chen, 2011; Liang et al., 2018; Wang et al., 2014; Zhang and Zhao, 2011). 2,2′-Methylenebis [4-ethyl-6-tert-butylphenol] (**39**) was also isolated in the same study of Liang et al. (2018).

7. Pharmacological properties of polysaccharides and phenolic compounds from *A. carmichaelii*

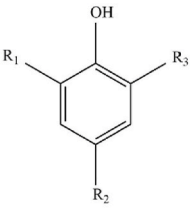
7.1. Pharmacological properties of polysaccharides

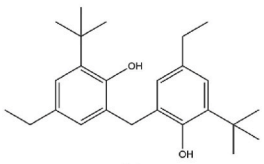
Polysaccharides isolated from *Aconitum* plants have been studied for a range of pharmacological effects, including immunomodulatory, antidiabetic, anti-tumor, anti-inflammatory, anti-oxidative, cholesterol-lowering, heart protective, and anti-HIV-1 reverse transcriptase activities (Liu et al., 2019; Wang, H. et al., 2020). Compared to a number of reviews on the pharmacological effects of crude extracts and alkaloids from "Fuzi" or "Chuanwu" (Aslam and Ahmad, 2016; Nyirimigabo et al., 2015; Wang, H. et al., 2020), only four Chinese reviews have summarized the biological effects of polysaccharides from "Fuzi" (Huang et al., 2018; Liu and Wang, 2021; Ren et al., 2008; Xiong et al., 2013), and these reviews have focused mainly on vascular protection (Liu and Wang, 2021) and delayed myocardial aging (Huang et al., 2018). Studies on polysaccharides from "Tian Xiong" are rare. One study demonstrated that a crude polysaccharide ameliorated chronic renal failure symptoms (Wang et al., 2019).

It has been proposed that the utilization of various processing procedures could be one of the factors causing the pharmacologically diverse activities of raw and commercial products, such as anti-inflammatory, analgesic, and immune-enhancing capabilities, as well as toxicological properties (Xiong et al., 2017; (Yang et al., 2019)). Therefore, the processing state of plant material is also covered in this review. The hypoglycemic, cholesterol lowering, cardiovascular modulatory, immunomodulatory, anti-tumor, and neuro-pharmacological activities of polysaccharides isolated from *A. carmichaelii* (raw or processed "Fuzi" and "Chuanwu") are the main focus of this review, which are listed in Table 4 and schematically represented in Fig. 8. The polysaccharides from "Fuzi" mentioned in these studies included crude and purified polysaccharides, which have also been emphasized in the text below and Table 4.

7.1.1. Hypoglycemic and hypolipidemic activities

An *in vivo* hypoglycemic effect of polysaccharides from the Japanese crude drug "Bushi" has been reported following intraperitoneal injection





39

	R1	R2	R3
35	*t*-Bu	*t*-Bu	H
36	*t*-Bu	H	*t*-Bu
37	*t*-Bu	Me	*t*-Bu
38	*t*-Bu	1,1,3,3-tetramethylbutyl	H

Fig. 7. Other phenolic compounds obtained from the roots of *Aconitum carmichaeli*.

Table 4
Pharmacological properties of polysaccharides isolated from different root parts *A. carmichaelii*.

Type of material	Polysaccharide fraction	Models/Test system	Dose range and administration	Pharmacological properties	Publication
Hypoglycemic and hypolipidemic activities					
Crude drug "Bushi" (Japan)	Aconitans A,B, C,D	Normal mice (*in vivo*)	10, 30 and 100 mg/kg/d by i.p.[a] for 7 and 24 h	Plasma glucose ↓	Konno et al. (1985)
	Aconitans A	Alloxan-produced hyperglycemic mice (*in vivo*)	10, 30 and 100 mg/kg/d by i.p. for 7 h	Plasma glucose ↓	
Raw and processed "Chuanwu"	/	Normal KM mice (*in vivo*)	500 mg/kg/d by i.g. [b] for 2, 10, 24 h	Plasma glucose ↓	Su and Liu (1991b)
		Normal KM mice deprived of oxygen (*in vivo*)	300 mg/kg/d by i.g. for 2, 10, 24 h	Survival time ↑	
Raw "Fuzi"	FPS [c]	High-fat diet-induced KM mice (*in vivo*)	40, 80, 160, 320 and 640 mg/kg/d by i.g. for 14 d	Serum total triglycerides ↓ Serum cholesterol and LDL ↓ LDL-receptor in liver ↑	Tang (2006)
		High-fat diet-induced KM mice (*in vivo*)	160 mg/kg/d by i.g. for 1 to 4 w	Triglycerides, total cholesterol and LDL ↓ Cholesterol in liver ↓	
		High cholesterol diet-induced hypercholesterolemia Wistar rat (male, *in vivo*)	224 mg/kg/d by i.g. for 1 to 4 w	Fecal total bile acid ↑ Cholesterol in liver ↓ LDL-receptor in liver ↑	
Raw "Fuzi"	FPS [c]	High cholesterol diet-induced hypercholesterolemia Wistar rat (male, *in vivo*)	224, 448 and 896 mg/kg/d by i.g. for 14 d	Serum cholesterol and LDL ↓ LDL-receptor in liver ↑ CYP7α −1 expression ↑ HMG-CoA reductase ↓	Huang et al. (2010)
				Serum total cholesterol ↓	Zhou et al. (2011a)
				Serum triglycerides ns Serum LDL-cholesterol ↓ High-density lipoprotein ns	Zhou et al. (2011b)
				HMG-CoA reductase ↓ CYP7α-1 expression ↑ Fecal total bile acid ↑	
Cardiovascular protection					
Raw "Fuzi"	FPS [d]	Exhaustive swimming KM mice (male, *in vivo*)	100 mg/kg/d by i.g. for 14 d	Forced swimming time ↑ SOD in serum, cardiomyocyte, liver, and lung ↑ MDA in serum, cardiomyocyte, liver, and lung ↓	Liu et al. (2008a).
				Forced swimming time ↑ Cardiomyocyte apoptosis index ↓ Cardiomyocyte SOD, CAT, GSH- Px ↑ Cardiomyocyte MDA content ↓ Cardiomyocyte Bcl-2 genes expression ↑ Cardiomyocyte caspase-3 genes expression ↓	Liu et al. (2008b).
Raw "Fuzi"	FPS [c]	Hypoxia-reoxygenation injured SD neonatal rat myocardial cell model (*in vitro*)	0.1, 1.0 and 10 μg/mL for 6 h	Cell apoptotic rate ↓ Mitochondrial membrane potential ↑ Bcl-2 expression ↑ MnSOD expression ↑	Liu and Ji (2011)
Raw "Fuzi"	FPS [c]	Hypoxia-reoxygenation injured SD neonatal rat myocardial cell model (*in vitro*)	10 μg/mL for 6 h	Cell survival rate ↑ Cell apoptotic rate ↓ LDH and creatine kinase ↓ Intracellular calcium concentration ↓ Apoptosis of cardiomyocytes ↓	Liu and Ji (2012)
				Cell apoptotic rate ↓ Mitochondrial membrane potential ↑ SOD activity ↑ MDA production ↓ AIF release ↓	Liu et al. (2012d)
				Cell apoptotic rate ↓ Bcl-xl expression ↑ Bcl-xc expressions ↓ Phosphorylation of STAT3 ↑ Cytochrome C ↓	Liu et al. (2012a)

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Table 4 (*continued*)

Type of material	Polysaccharide fraction	Models/Test system	Dose range and administration	Pharmacological properties	Publication
				Caspase-3 ↓	
			0.1, 1.0 and 10 μg/mL for 24 h	Synthesis of metallothionein ↑ Cell apoptotic rate ↓ LDH release ↓ MDA synthesis ↓	Liu et al. (2012c)
				Cell survival rate ↑ Cell apoptotic rate ↓ Bcl-2 expression ↑ Smac/Diablo ratio ↓	Ji and Liu (2012)
			0.1, 1, 10, 20 μg/mL for 24 h	Cell survival rate ↑ Cell apoptotic rate ↓ GRP78 expression ↓ CHOP expression ↓ Caspase 12 expression ↓	Liu et al. (2012b)
Raw "Fuzi"	FPS [c]	Glucose starvation-induced cytotoxicity in H9c2 cells (*in vitro*)	10, 100 and 1000 μg/mL for 6 h	Cell viability ↑ LC3-II/LC3-I ratio ↑ Number of GFP-LC3 dots/cell ↑ p-AMPK ↑ p-mTOR ↓ AMPK/mTOR activation ↑	Liao et al. (2013),
Raw "Fuzi"	FPS [c]	Ox-LDL-induced calcification of human VSMCs (*in vitro*)	0, 10, 100 and 1000 μg/mL for 14 d	Cell calcification ↓ Core binding factor α1 mRNA expression ↓ Smooth muscle 22α mRNA expression ↓ LC3-II/LC3-I ratio ↑ P62 expression ↓ Number of GFP-LC3 dots/cell ↑	Liao et al. (2018)
Processed "Fuzi"	FPS [d]	Modified Smith method induced MI male Sprague Dawley rats (*in vivo*)	1.6, 0.8, and 0.4 g/kg by i.g. at 10 mL/kg/d for 14 d	Left ventricular end-diastolic pressure ↓ Heart weight index ↓ Lung weight index ↓ Myocardial fibers damage, vascular lesions, and Inflammatory cell infiltration ↓ Ventricular hypertrophy ↓	Wu et al. (2019)
Raw "Fuzi"	PS	Propafenone hydrochloride induced acute heart failure SD mice (*in vivo*)	5 and 10 g crude drug/kg by the duodenum for 5, 10, 20, 30, and 60 min.	Heart rate (after 20 min) ↑ $+dp/dt_{max}$ ns $-dp/dt_{max}$ ns Atrial natriuretic peptide in serum ↓ Angiotensin-I, Angiotensin-II and TNF-α in serum ns	Chen et al. (2019)
Raw "Fuzi"	FPS	Ox-LDL-induced calcification of VSMCs (*in vitro*)	0.1, 0.5 and 1 mg/mL for 7 d	Cell calcification ↓ Alkaline phosphatase activity ↓ Msx2, Osterix, bone morphogenetic protein 2, and SMA expressions ↓ Cell apoptosis ↓ N-SMase activity and level of ceramide↓	Chen et al. (2020)
Raw "Fuzi"	FPS	LPS-induced VSMCs (*in vitro*)	2.5, 5 and 10 ng/mL for 6 h	Cell activity and migration ↓ Cell proliferation nuclear antigen Ki67 ↓ Neural cadherin (N-cadherin) ↓ Epithelial cadherin (E-cadherin) ↑ Cell in G0/G1 phase ↑ Cell in S phase ↓ miR-135b-5p expression ↓	Liu and Huang (2021)
Immunomodulatory activities					
Raw "Fuzi"	FPS-1 [c]	BALB/c (female, *in vivo*)	25, 50 and 100 mg/kg/d by i.p. for 6 d	Spleen lymphocyte proliferation ↑ Anti-sheep red blood cell antibody production ↑	Zhao et al. (2006)
		Normal mouse spleen lymphocytes (*in vitro*)	1, 10 and 100 μg/mL for 48 h	Spleen lymphocyte proliferation ↑	
Processed "Fuzi"	Acidic monkshood polysaccharide	Cy induced immunosuppressive BALB/c mice (half male and half female, *in vivo*)	10 and 20 mg/kg/d by i.p and i.g. for 15 d	Spleen and thymus indexes ↑ Phagocytosis index K (i.p. only) ↑ Phagocytosis coefficient α ↑ NK cell activity ↑ Lymphocyte differentiation ↑ White blood cell count ↑	Miao et al. (2007)
Raw "Fuzi" and "Chuanwu" (Radix Aconiti Lateralis, L; Radix Aconiti, A)	L-CWPS-N [e] L-CWPS-A L-HWPS-N L-HWPS-A A-CWPS-N A-CWPS-A A-HWPS-N A-HWPS-A	Cy-induced immunosuppressive ICR mice (half male and half female, *in vivo*)	100 mg/kg/d by i.p for 7 d	Phagocytosis index ↑ Phagocytosis coefficient α ↑ NK cell activity ↑ Lymphocyte proliferation ↑ Anti-SRBC antibody production ↑ DTH reaction ↑	Gao et al. (2010)
Processed "Fuzi", "Hei Shun Pian"	/	Cy-induced immunosuppressive mice (*in vivo*)	0.2 mL (1.5 mg/mL) by i.g. for 7 d	Spleen index ↑ Thymus index ns White blood cell and lymphocyte counts ↑	Zhao (2017)

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Table 4 (*continued*)

Type of material	Polysaccharide fraction	Models/Test system	Dose range and administration	Pharmacological properties	Publication
				$CD4^+$ FOXP3/$CD4^+$ in spleen ↑ IL-6 mRNA expression in spleen ↑ IFN-γ mRNA expression in spleen ↓ TNF-α mRNA expression in spleen ns COX2 mRNA expression in spleen ↓ CXCL and CXCR mRNA expression in spleen ↓	
Processed "Fuzi", "Hei Shun Pian"	/	Cy-induced immunosuppressive KM mice (*in vivo*)	50, 100 and 200 mg/kg/d by i.g. for 7 d	Spleen and thymus indexes ↑ Serum NO ↑ Serum IFN-γ ↑	Fu et al. (2018)
		Normal mouse spleen lymphocytes and isolated mouse macrophages (*in vitro*)	12.5, 25, 50 and 100 μg/mL for 44 h	Cell proliferation ↑	
Raw "Fuzi"	FZPS-1	Cy-induced immunosuppressive SD mice (male, *in vivo*)	100 and 200 mg/kg/d by i.g. for 14 d	Spleen and thymus indexes ↑ Carbon clearance index ↑ Swallow index ↑	Yang et al. (2020)
		Rhubarb decoction induced spleen deficiency (chronic diarrhea) KM mice (male, *in vivo*)	100 and 200 mg/kg/d by i.g. for 30 d	Diarrhea rate ↓ Intestinal propulsion rate ↑ Vasoactive intestinal peptide in the colon ↑ MDA, SOD, IL-6, IL-1, TNF-α and IFN-γ in colon ↓	
		RAW264.7 cells (*in vitro*)	6.25, 12.5, 25, 50 and 100 μg/mL for 24 h	Phagocytic activity ↑ NO production ↑ IL-6, IL-1β, and TNF-α ↑	
Anti-tumor activities					
Raw "Fuzi"	/	Human promyelocytic leukemia cells HL-60 (*in vitro*)	10, 100 and 1000 μg/mL for 5 d	Negative nitroblue tetrazolium ↑ Myeloperoxidase ↑ Neutrophil alkaline phosphatase ns CD_{11b} expression ↑ CD_{33} expression ↓	Peng et al. (2003)
Processed "Fuzi", "Hei Fu Pian"	Crude and acidic monkshood polysaccharide	Murine hepatoma H22 and S180 cell-induced KM mice (half male and half female, *in vivo*)	30 (crude monkshood polysaccharide and 20 (acidic monkshood polysaccharide) mg/mL by i.p. (0.2 mL each) and i.g. (0.4 mL each) for 5 d	Apoptotic rate of tumor tissue ↓ (45.3%~69.3%) Survival rate ↑ Spleen weight of mice ↑ Lymphocyte transformation capacity ↑ NK cell activity ↑ Tumor cells apoptotic rate ↑ Oncogenes p53 and Fas expressions ↑	Dong et al. (2003a)
Raw "Fuzi" and "Chuanwu" (Radix Aconiti Lateralis, L; Radix Aconiti, A)	L-CWPS-N [e] L-CWPS-A L-HWPS-N L-HWPS-A A-CWPS-N A-CWPS-A A-HWPS-N A-HWPS-A	Murine hepatoma H22 cell-induced ICR mice (*in vivo*)	100 mg/kg/d by i.g. for 7 d	Tumor growth inhibition ratios: 37.24–70.42% Number of the peripheral white blood cells ↓	Gao et al. (2010)
"Fuzi"	FPS [f]	Monocytes from peripheral blood of hepatocellular carcinoma patients (*in vitro*)	10, 100 and 1000 μg/mL for 72 h	Peripheral blood mononuclear cells differentiation into DC ↑ DC proliferation ↑ CD80, CD86 and CD83 expressions ↑	Gao et al. (2012)
Processed "Fuzi"	/	Murine hepatoma H22 cell-induced KM mice (half male and half female, *in vivo*)	1000 mg/kg/d by i.g. for 8 d	Tumor tissue weight ↓	Gao et al. (2016)
		Human hepatic adenocarcinoma cell SK-HEP-1 (*in vitro*)	5, 10, 25, 50 and 100 μg/mL for 48 h	β3GnT8 expression ↓ Polylactosamine expression ↓	
Processed "Fuzi" "Bai Fu Pian"	/	gastric cancer cell MFC induced nude mice xenograft BALB/c-nu mice (*in vivo*)	100 and 200 mg/kg/d by i.g. for 15 d	Inhibition rate: 40.57%, 52.83% Tumor tissue weight ↓ Serum TGF-β1 ↓ MMP-2 and MMP-14 expression in tumor ↓	An et al. (2019)
Neuropharmacological activities					
Raw "Fuzi"	FPS-1 [c]	Bromodeoxyuridine (BrdU+) labeled C57BL/6J mice (male, *in vivo*)	5, 10, 50, 100, 200 and 400 mg/kg/d by i.p. for 1 d	Total number of BrdU + cells in DG ↑	Yan et al. (2010)
		C57BL/6J mice (male, *in vivo*)	100 mg/kg/d by i.p. for 7 d	Number of neuronal nuclear (NeuN+)/BrdU + cells in DG ↑	

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Table 4 (*continued*)

Type of material	Polysaccharide fraction	Models/Test system	Dose range and administration	Pharmacological properties	Publication
				Ratio of NeuN+/BrdU + cells to total BrdU + cells ↑	
		C57BL/6J mice (male, *in vivo*)	50 and 100 mg/kg/d by i.p. for 30 min	Forced swimming time ↓ Spontaneous locomotor activity (open-field apparatus) ns	
		C57BL/6J mice (male, *in vivo*)	100 mg/kg/d by i.p. for 7 d	Novelty-suppressed feeding test ↓ Latency to feed ↓ Food consumption ns	
		Social defeat stress C57BL/6J mice (male, *in vivo*)	100 mg/kg/d by i.p. for 7, 14 and 28 d	Interaction time (after 14 d) ↑ Number of BrdU + cells in DG (after 14 d) ↑	
		C57BL/6J mice (male, *in vivo*)	50 and 100 mg/kg/d by i.p. for 45 min	Secretion of monoamine transmitters in frontal cortices, serotonin hydrochloride, dopamine hydrochloride, and norepinephrine hydrochloride ns	
		C57BL/6J mice (male, *in vivo*)	100 mg/kg/d by i.p. for 2, 6 and 12 h	Brain-derived neurotrophic factor in bilateral hippocampus ↑	
Raw "Fuzi"	FPS [d]	High glucose-stimulated RSC96 cells (*in vitro*)	1, 10 and 100 μg/mL for 48 h	Peroxide and superoxide anions ↓ Apoptotic ratio ↓ SOD and CAT expressions ↑ NADPH oxidase-1 expressions ↓ PGC-1α expressions and AMPK activation↑	Wang et al. (2016)
"Fuzi"	/	External environment stressed-depressive SD rat (male, *in vivo*)	100 mg/kg/d by i.p. for 21 d	Bodyweight ↑ Forced swimming time ↓ Hippocampus histomorphology ↑	Long et al. (2017)
Raw "Fuzi"	FPS [c]	C57BL/6J mice (male, *in vivo*)	0.5 mL of 2, 4 and 8 mg/mL by i.p. for 6 h	GSK-3β in hippocampus ns p-GSK-3β in hippocampus ↑	Xiao et al. (2017)

Note: ↓ indicates that the related pharmacological activity was reduced or suppressed; ↑ indicates that the related pharmacological activity was increased or stimulated; ns indicates that there was no significant change on the pharmacological activity; [a], the abbreviation of intraperitoneal injection; [b], the abbreviation of intragastrical administration; [c], the FPS or FPS-1 was isolated and characterized by Zhao et al. (2006) as an α-D-glucan with Mw 14 kDa and purity of 99.8%, see details in Table 2 and Fig. 2; [d], crude polysaccharides isolated from "Fuzi"; [e],-N means the neutral polysaccharide, -A means acidic polysaccharide (see details in Table 2); [f], polysaccharides offered by a medicinal company.

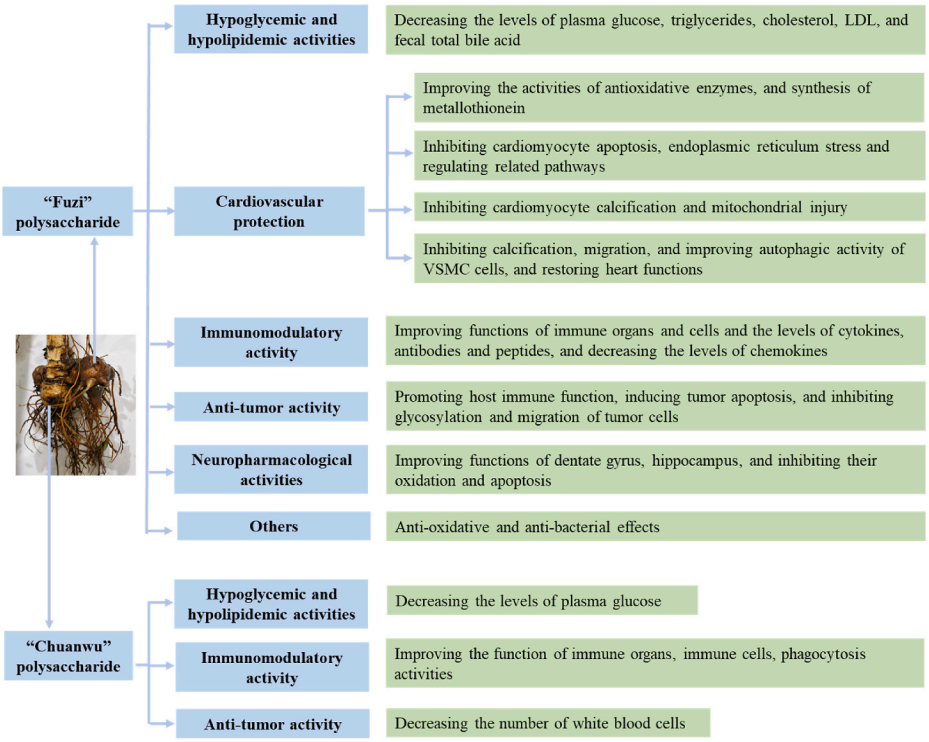

Fig. 8. Schematic diagram of pharmacological properties of polysaccharides from the roots of *A. carmichaelii.*

in mice. Aconitan A (an α-glucan mentioned in Table 2 and Fig. 2) was reported to reduce plasma glucose by Konno et al. (1985). This effect was mediated by increased glucose utilization in the liver and in peripheral tissues such as the small intestine, rather than modulating the insulin level or activities of liver enzymes (Hikino et al., 1989). Polysaccharides from raw and processed "Chuanwu" were likewise beneficial in lowering serum glucose and increasing survival rate in oxygen-deprived mice. Specific processing procedures like soaking and boiling minimized this effect (Su and Liu, 1991b). This was thought to be due to the loss of carbohydrates (Su and Liu, 1991a). However, there was no further discussion on the potential mechanism of the hypoglycemic effect. In addition, polysaccharides isolated from "Fuzi" have also been reported to promote the glucose consumption rate of 3T3-L1 adipocytes and the glucose intake rate of insulin-resistant adipocytes *in vitro* (Yu and Wu, 2009).

A glucan from "Fuzi" (FPS^c, see footnotes in Table 4) was reported to have a hypolipidemic effect in mice on a high-fat diet or high-cholesterol diet (Tang, 2006). A reduction in total triglycerides, total cholesterol, and low-density lipoprotein (LDL) levels in serum and liver was observed. FPS^c was suggested to act through a promotion of cholesterol metabolism by increasing the expressions of the hepatic LDL receptor and cytochrome P450 7α-1 (CYP7α-1) and decreasing 3-hydroxy-3--methyl glutaryl (HMG)-CoA expression, or through modulating endogenous cholesterol synthesis by down-regulating the expression of HMG-CoA reductase (Huang et al., 2010; Zhou et al., 2011a, 2011b).

7.1.2. Cardiovascular protection

"Fuzi", as "the first key medicine to restore depleted *Yang* and rescue the patient from danger" in TCM theory (recorded in *Bencao Jingdu*), possess healing functions for people suffering from myocardial infarction, low blood pressure, coronary heart disease, and heart failure (Tang et al., 2017). Two Chinese reviews summarize the protective effects on the cardiovascular system, where polysaccharides were reported as one of the active components in "Fuzi" decoction (Huang et al., 2018; Liu and Wang, 2021). However, no related studies on polysaccharides from "Chuanwu" have yet been performed.

To illustrate the protective effect of "Fuzi" polysaccharide (FPS^d, see footnotes in Table 4) *in vivo*, an exhaustive swimming exercise test in mice was employed as a cardiomyocyte injury model. Forced swimming time and activities of anti-oxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were increased, while the content of cardiomyocyte malondialdehyde (MDA) decreased (Liu et al., 2008a, b). These changes were suggested to be accomplished through upregulation of the gene expression of Bcl-2 and downregulation of the expression of caspase-3. Both are critical genes in anti-apoptosis pathways (Liu et al., 2008b).

Since 2011, *in vitro* studies on the protection of the cardiovascular system by an α-glucan polysaccharide from "Fuzi" (FPS^c) (mentioned in Table 2 and Fig. 2) have been conducted. A typical myocardial cell model induced by hypoxia-reoxygenation injury was used, as shown in Table 4. There are several ways that FPS^c may act, including maintenance of calcium concentration (Liu and Ji, 2012), anti-apoptosis, anti-oxidation, and the prevention of mitochondrial injury, as presented in Fig. 8 and discussed previously (Huang et al., 2018). The latter three effects have been studied more commonly and shown to be related to each other (Huang et al., 2018). In addition to the modulation on the cardiocellular calcium, the mitochondrial injury was reduced after FPS^c treatment (Liu and Ji, 2012), and the apoptosis-inducing factor (AIF)-related mitochondrial signaling pathway was suppressed as well (Liu et al., 2012d). It was also indicated that FPS^c promoted the expressions of the anti-apoptotic gene Bcl-2 (Ji and Liu, 2012; Liu and Ji, 2011) and Bcl-xl (Liu et al., 2012a), and reduced the release of mitochondria apoptosis-regulating protein Smac/Diablo (Ji and Liu, 2012). An inhibition on endoplasmic reticulum stress of FPS^c was indicated by down-regulating the expressions of CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, and glucose-regulated protein 78 (GRP78) (Liu et al., 2012b). Further, the activating signal transducers and activators of transcription 3 (STAT3) have also been considered as a probable mechanism of the protective ability of FPS^c on myocardial cells (Liu et al., 2012a). Also, promoting metallothionein synthesis by FPS^c was reported to be beneficial in order to defend against oxidative injuries and cell apoptosis by scavenging oxygen free radicals (Liu et al., 2012c). Recovery effects, including decreasing the release of MDA and lactate dehydrogenase (LDH), increasing the activity of SOD (Liu et al., 2012c, d), and promoting the manganese superoxide dismutase (MnSOD) expression (Liu and Ji, 2011), were also observed in FPS^c-treated cells. In addition, Liao et al. (2013) reported that FPS^c helped cardiomyocytes against starvation-induced cell death due to the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway. It has also been manifested that FPS^c could protect against oxidized low-density lipoprotein (Ox-LDL)-induced vascular calcification on human vascular smooth muscle cell (VSMC) via activating autophagy (Liao et al., 2018), reducing neutral sphingomyelinase (N-SMase) activity and ceramide levels (Chen et al., 2020), as summarized by Liu and Wang (2021). The lipopolysaccharide (LPS)-induced proliferation and migration of VSMCs were inhibited by FPS^c as well, through downregulation of miR-135b-5p expression (Liu and Huang, 2021).

An *in vivo* study performed by Wu et al. (2019) compared the potential anti-myocardial infarction effects of alkaloids, a water extract, and a polysaccharide from processed "Fuzi" (FPS^d). It was demonstrated that the polysaccharide fraction reduced the left ventricular end-diastolic pressure and limited myocardial damage after 14 days of the administration by stabilizing cardiac function and improving structural abnormalities. However, in this study, FPS^d was shown to be less effective than the alkaloids and the crude extract. Similarly, Chen et al. (2019) evaluated the curative capacity of different components on acute heart failure in rats. They suggested that 10 g/kg bodyweight of polysaccharides from raw "Fuzi" (PS) could raise heart rate and reduce the level of serum atrial natriuretic peptide, one of the neuro-humours, after 60 min of treatment.

7.1.3. Immunomodulatory activity

According to Table 4, polysaccharides isolated from *A. carmichaelii* have shown potent immunomodulatory effects both *in vitro* and *in vivo*.

The potential of neutral polysaccharides from raw "Fuzi" (FPS-1, see footnotes in Table 4) was first investigated by Zhao et al. (2006), showing that the glucan FPS-1 (Fig. 2) could act as an immunomodulator on normal mice and spleen lymphocytes. It was demonstrated to increase spleen lymphocyte proliferation both *in vivo* and *in vitro* and stimulate antibody production in response to sheep red blood cells (SRBC). Cyclophosphamide (Cy)-induced immunosuppressive mice were further employed by Gao et al. (2010), demonstrating that neutral fractions from "Fuzi" (L-CWPS-N and L-HWPS-N, Table 2) activated macrophages, restored phagocytosis activity, promoted anti-SRBC antibody production and delayed-type hypersensitivity (DTH) reaction. In addition, these neutral fractions had stronger abilities to augment T lymphocyte proliferation induced by either concanavalin A or LPS in Cy-induced mice, compared with acidic polysaccharide fractions. Furthermore, Yang et al. (2020) proposed that FZPS-1 (Table 2 and Fig. 2), a purified arabinoglucan, was able to ameliorate chronic diarrhea in spleen deficient mice by regulating the levels of the gut-related peptide vasoactive intestinal peptide, inhibiting oxidative reactions such as decreasing the activities of MDA and SOD and lowering the generations of the colonic pro-inflammatory cytokines IL-6, IL-1, TNF-α, and IFN-γ. It also displayed remarkable promotive effects on phagocytic activity, NO production, and secretion of pro-inflammatory cytokines in macrophage RAW264.7 cells in a dose-dependent manner.

Miao et al. (2007) evaluated the effect of an acidic polysaccharide from processed "Fuzi" on immunosuppressed mice for 15 days and compared intraperitoneal injection with oral administration. Its immunomodulatory function was revealed by increased immune organ

indexes, phagocytosis efficiency, natural killer (NK) cell activity, and white blood count (Table 4). A better effect was indicated by intraperitoneal injection compared to oral treatment, possibly because of the more direct influence on the host immune system (Miao et al., 2007). Immunomodulatory effects of pectic fractions were also observed in a study by Gao et al. (2010), which were shown to be more potent than neutral polysaccharides on B lymphocyte proliferation, as well as on the NK cell cytotoxicity. This was shown especially for fractions with higher contents of GalA and Ara and lower Mw.

Crude polysaccharides from processed "Fuzi" have also been studied by Zhao (2017) and Fu et al. (2018), as shown in Table 4. Immunomodulatory effects such as stimulating serum nitric oxide (NO) and interferon (IFN)-γ secretions (Fu et al., 2018), activating spleen gene expressions of pro-interleukin (IL)-6, IFN-γ and tumor necrosis factor (TNF)-α, and suppressing those of cyclooxygenase-2 (COX2), CXC chemokine ligand (CXCL), and CXC chemokine receptor (CXCR) (Zhao, 2017) were presented. A therapeutic action on rheumatoid arthritis by "Fuzi polysaccharide injection" has further been demonstrated by Zhao (2007). An inhibition of the feet edema, improvement of the immune organs' function, pathological changes of the synovium of joints, and elevation in the secretion of IL-2 and TNF-α were observed. These modulatory effects could be the result of T-lymphocyte activation.

Studies on polysaccharides from "Chuanwu" are more limited than "Fuzi". Only Gao et al. (2010) briefly unraveled both neutral and acidic polysaccharides displaying similar immunomodulatory properties to "Fuzi" on immunosuppressed mice, with the acidic ones outperforming the neutral ones.

7.1.4. Anti-tumor activity

Anti-tumor studies of *A. carmichaelii* extracts have been assessed against S180 sarcoma, hepatic carcinoma, and lung, colorectal, cervical, and breast cancer cells (Lin et al., 2017; Zhang et al., 2017; Zhou et al., 2020a). The alkaloids were considered as the main active constituents (Wada et al., 2015), while polysaccharides received less attention. However, both *in vivo* and *in vitro* studies have shown that numerous types of polysaccharides from medicinal plants have significant anti-tumor properties, and they are less toxic or have fewer side effects than chemotherapeutic drugs (Yu et al., 2018), which is therefore of great interest to be further studied.

As shown in Table 4, most studies have focused on polysaccharides from "Fuzi", and the studies on the treatment of hepatic carcinoma are relatively comprehensive. Inducing apoptosis of tumor cells and promoting host immune functions were demonstrated as active mechanisms by several of the following studies. Dong et al. (2003a) evaluated the anti-tumor effects of a crude monkshood polysaccharide and an acidic monkshood polysaccharide from "Hei Fu Pian" on mice transplanted with murine hepatoma H22 and S180 tumor cells. It was found that the activities of immune cells like lymphocyte and NK cells were elevated, and tumor cells were induced into apoptosis by up-regulating the expressions of oncogenes p53 and Fas. Dong et al. (2003b) and Dong et al. (2006) also manifested a synergy effect of polysaccharides from "Fuzi" combined with adriamycin magnetic albumin microsphere or long-circulating liposome therapies on tumors induced by H22 or S180 cells. This was suggested to be via increased NK cell activity, enhanced gene expressions of IL-2 and IL-12 in spleen lymphocytes, and an up-regulation of the expressions of apoptosis trigger genes Fas and Fas-l in tumor cells. Gao et al. (2012) further revealed that FPS[e] (see footnotes in Table 4) could trigger the differentiation of peripheral blood mononuclear cells into dendritic cells (DC) and promote DC proliferation and expression of mature surface molecules. A polysaccharide from processed "Fuzi" was similarly efficient in decreasing tumor weight of hepatoma H22 cell-induced tumor in mice, as well as reducing glycosyltransferase level in human hepatic adenocarcinoma cells, through dose-dependently suppressing the β3GnT8 and polylactosamine expressions (Gao et al., 2016). Another related study was carried out by Gao et al. (2010), in which significant inhibition on tumor growth and a reduced number of peripheral white blood cells were observed by both neutral and acidic fractions from "Fuzi" and "Chuanwu" after seven days of oral administration. It was also demonstrated that the polysaccharide with the highest content of GalA and Ara possessed better anti-tumor effects. Conversely, Qian (2015) reported that FPS[d] had no direct inhibitory effect on hepatocellular carcinoma but was capable of improving immune function, such as increasing the ability of spleen lymphocytes to strengthen the anti-cancer effect and restoring the immunological injury caused by aconite in Hepal-6 tumor-bearing mice (Qian, 2015). Lately, Yao et al. (2021) indicated an anti-tumor synergy effect of crude monkshood polysaccharide combined with aconite, showing that the ability of the immunocyte to kill the tumor cell *in vitro* was strengthened and had an additive effect on anti-hepatocellular carcinoma *in vivo*. Immune organ indexes, $CD4^+$ T and $CD8^+$ T cells and macrophages in the spleen, and serum TNF-α and IFN-γ levels of hepatocellular carcinoma mice were enhanced, and the level of IL-6 in serum was decreased.

In addition, anti-leukemia effects of FPS[d] have been reported by increasing the negative nitroblue tetrazolium reduction, myeloperoxidase concentration, and differentiating the leukemia cell HL-60 into CD11 types after five days of co-incubation (Peng et al., 2003). An et al. (2019) investigated the anti-gastric cancer potential of polysaccharides from processed "Fuzi" on tumor-bearing mice after 15 days of administration. This study found that restricting the expressions of matrix metalloproteinase (MMP)-2 and MMP-14, which disrupt tumor cell invasion and adhesion, resulted in a 40%–52% suppression and decreased tumor weight and TGF-β1.

7.1.5. Neuropharmacological activities

Neuropharmacological activities of "Fuzi" like anti-depressive, analgesic, anti-epileptic, and anticonvulsive properties could be attributed to a variety of components, including aconitine, mesaconitine, hypaconitine, total alkaloid, and polysaccharides (Zhao et al., 2020). The contribution of polysaccharides has rarely been mentioned besides its anti-depressive effect. According to Yan et al. (2010), a polysaccharide fraction from raw "Fuzi" (FPS-1) induced newborn neuro-cells in the dentate gyrus (DG) to differentiate into neurons, decreased mice appetite, and activated the brain-derived neurotrophic factor signal pathway. Long et al. (2017) also reported that FPS[d] improved the condition of depressive mice stressed by external environment changes, such as pain, cage shaking, fasting feeding, day and night inversion, coldness, and immobility. FPS[d] also has been shown to protect RSC96 cells, the myelin cells in the peripheral nervous system, against high glucose stimulation through lowering the peroxide and superoxide anions' level and apoptotic ratio, as well as suppressing the expressions of anti-oxidant enzymes via AMPK-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) pathway (Wang et al., 2016). A promotive effect on the expression of glycogen synthase kinase (GSK)-3β in the hippocampus, which is associated with the growth and structure of the neuronal cells, has also been observed (Xiao et al., 2017).

7.1.6. Other activities

In vitro anti-oxidative effects of polysaccharides from processed "Fuzi" (Zhang, 2016) and the purified neutral fraction FZPS-1 (Yang et al., 2020) have been reported with scavenging abilities of hydroxyl radical, superoxide radical, $K_3Fe(CN)_6$, ABTS + free radicals and nitrite (Yang et al., 2020; Zhang, 2016). These protective effects were also seen on cardiomyocyte (Liu et al., 2008b, 2012d), colon tissue (Yang et al., 2020), and Schwann cells (Wang et al., 2016), and has been demonstrated as one of the active mechanisms of the protective effect against hepatic ischemia-reperfusion injury in rats, by increasing liver SOD and CAT contents, and attenuating necrotic states (Lin et al., 2009).

An isolated crude polysaccharide from "Fuzi" also displayed an antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* in a time- and concentration-dependent relationship (Lin et al., 2011).

According to Zhao (2017), who compared the change in the intestinal microbiota by a water extract and crude polysaccharide from "Hei Shun Pian" (processed "Fuzi"), the abundance of *Lactobacilli* and *Helicobacter* genus in mice treated with a polysaccharide component after Cy injection were significantly elevated, and that of *Bacteroides* genus was reduced. In addition, stereoselective synthesis of the FPS^{c} (α-glucan) to glycoclusters was introduced, which could be applied as human vaccine adjuvants (Zhou, 2020).

7.2. Pharmacological properties of phenolic compounds

The phytopharmacological research on phenolic compounds obtained from *A. carmichaelii* is limited compared to polysaccharides. One published study has reported that intragastric administration of transferulic acid-4-β-glucoside (**10**) for six days was shown to alleviate the oxidative stress and cold tolerance in the liver induced by cold environment in C57BL/6 mice. The reduction of oxidative stress in the liver was achieved by increased levels of the anti-oxidant enzymes (GSH-PX and SOD). To combat coldness, the liver heat production was promoted by increasing ATP levels, as well as gene expressions related to mitochondrial biogenesis (nrf1, tfam, and tfb2m), lipid metabolism (acox1 and acadm), and thermogenesis in brown adipose tissue (uncoupled protein 1) (Xue et al., 2018).

8. Conclusions and perspectives

This review provides an overview of non-alkaloid compounds from different root parts of *A. carmichaelii*, focusing on polysaccharides and phenolic compounds, which are common compounds present in root decoctions of the plant. The chemical characteristics and pharmacological effects of polysaccharides and phenolic compounds are herein comprehensively summarized. In addition, an overview of the botany and traditional use of *A. carmichaelii* is included. There are evident gaps in the research on *A. carmichaelii* and its water-soluble polysaccharides and phenolic compounds, and these will be discussed below.

Firstly, different approaches for extraction and purification of polysaccharides from roots of *A. carmicahelii* have been performed (see section 6.1.1), but additional pretreatment such as pre-soaking in water could be beneficial for increasing extraction yield. The types of polysaccharides isolated from different root parts are various, consisting of glucans, and neutral and acidic heteropolysaccharides, as shown in Table 2. However, the polysaccharide fractions are not comprehensively studied, and essential information is still unclear. For instance, the reported content of uronic acids in some published studies on polysaccharides lacks explanation (Gao et al., 2010; Yang et al., 2020). And compared to the clear structures of glucans given in several papers, the elucidation of glycosidic linkage units of acidic polysaccharides is still absent based on Table 2. Therefore, the description of pectin as part of the polysaccharides in *A. carmichaelii* is theoretically incorrect, unless typical subunits such as 1,4 linked GalA and 1,2 linked Rha are determined (Voragen et al., 2009). Further, the quality of structural characterization is insufficient in many studies. A notable example is a study by Miao et al. (2007), where the Molish reaction was the only method used to identify the acidic polysaccharide, and the methods, extraction yield, or structural information were not described. Precise methodology, such as two-dimensional NMR spectroscopy, is highly recommended, especially for the identification of monomer configurations, like cases reported by Yang et al. (2020) and Zhao et al. (2006). Thus, considerable effort will be required to provide a more comprehensive structural characterization of isolated polysaccharides.

Secondly, Table 4 and Fig. 8 illustrate that the pharmacological properties of polysaccharides are not sufficiently studied. On the one hand, the traditional use of "Fuzi" and "Chuanwu" are documented independently, which indicates potential pharmacological distinctions. The variation in the contents of different compounds could be an explanation. Additionally, the pharmacological effects of polysaccharides from "Chuanwu" are significantly less studied than "Fuzi", both *in vivo* and *in vitro* experiments. More relevant studies on "Chuanwu" polysaccharides would be valuable.

Moreover, many natural polysaccharides are not digested by human gastrointestinal enzymes (Huang et al., 2017; Xu et al., 2013). The published *in vivo* studies of isolated polysaccharides from *A. carmichaelii* are consequently insufficient to explain how certain polysaccharides act. Studies on the modulatory effects on gut microbiota, intestinal barrier functions, mucosal immune system, and anti-inflammatory activities, could be promising approaches for comprehensively understanding the biological activities of polysaccharides from *A. carmichaelii*, as seen for other natural polysaccharides (Huang et al., 2017; Song et al., 2021; Tang et al., 2019). Further molecular and cellular studies on the immunomodulatory effects of polysaccharides should also be performed, like those that have already been done in cardiovascular studies. Besides, few pharmacological studies have reached definitive conclusions. A study reported an increase in the abundance of *Lactobacillus* spp. and *Helicobacter* spp. in mice by orally administered polysaccharides from processed "Fuzi" (Zhao, 2017). However, bacteria in the *Helicobacter* genus, particularly *H. pylori,* are generally associated with several inflammatory diseases and exhibit carcinogenic potential on animals or humans (Jr and Crabtree, 2005).

On the other hand, information on the polysaccharides used in some of these pharmacological studies is missing. In three studies, polysaccharides were obtained from only ethanol-precipitation, and fundamental chemical compositions such as carbohydrate content were not provided to demonstrate the polysaccharide purity (Chen et al., 2019; Gao et al., 2016; Wu et al., 2019). The polysaccharides used in some of the studies were either a product from a medicinal company (Gao et al., 2012), or a gift from other research groups without any specifics (Peng et al., 2003). Further, the exploration of the structure-activity relationship is crucial for understanding biological activities, but this remains a limitation in most studies. The anti-tumor and immunomodulatory effects have been suggested with relation to GalA and Ara units of polysaccharides from "Fuzi" (Gao et al., 2010). Hence, it is important to control the quality of the polysaccharide substances before performing more profound pharmacological research, and the polysaccharide needs to be pure and accompanied with structural details. More attention should be attracted to enzymatic degradation or modification for better structural characterization.

Thirdly, phenolic compounds, especially flavonoids, which commonly exist in plants with broad biological activities (Jucá et al., 2020), have been proposed as chemotaxonomic markers in *Aconitum* plants to facilitate taxonomic discrimination (Yin et al., 2019). 55 flavonoid glycosides, followed by 22 phenylpropionic acids, have been isolated and identified in *Aconitum* plants (Yin et al., 2019). However, only four flavonoids from *A. carmichaelii* have been reported so far (Table 3), including three flavanones and one chalcone. Therefore, more systematic and in-depth studies are needed to explore the composition of phenolic compounds in *A. carmichaelii*, which can form the basis for future taxonomic discrimination. Meanwhile, more effort is also needed to study the pharmacological activity of phenolic compounds and other non-alkaloid secondary metabolites in the water extracts, as around 40 identified constituents have been reported (Table 3), with few pharmacological studies performed. The aerial parts together with rootlets of *A. carmichaelii* are valuable plant sources that should be explored further. Then, toxicological studies of polysaccharides and phenolic compounds should be considered due to their poisonous plant source.

In conclusion, this review presents the pharmacological values of *A. carmichaelii* as a medicinal material and shows that both polysaccharides and phenolic compounds are promising bioactive non-alkaloid constituents with potent medicinal value. Additional investigations need to be performed to provide a solid foundation for further exploration of *A. carmichaelii* and hopefully break the limitations due to the presence of toxic alkaloids.

CRediT authorship contribution statement

Yu-Ping Fu: Conceptualization, Data curation, Writing – original draft, Visualization. **Yuan-Feng Zou:** Conceptualization, Resources, Writing – review & editing, Supervision. **Fei-Yi Lei:** Data curation, Visualization, Resources. **Helle Wangensteen:** Conceptualization, Writing – review & editing, Supervision. **Kari Tvete Inngjerdingen:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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List of Abbreviations

AIF	Apoptosis-inducing factor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
CAS	Chemical Abstracts Service
CAT	Catalase
CHOP	CCAAT/enhancer-binding protein homologous protein
COX2	Cyclooxygenase 2
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CYP7α-1	Cytochrome P450 7α −1
Cy	Cyclophosphamide
DC	Dendritic cells
DG	Dentate gyrus
DTH	Delayed type hypersensitivity
EP	Ethanol precipitation
FPS	“Fuzi” polysaccharide
GRP78	Glucose-regulated protein 78
GSH- Px	Glutathione peroxidase
GSK-3β	Glycogen synthase kinase 3β
HMG	3-hydroxy-3-methyl glutaryl
IFN-γ	Interferon γ
IL-1/2/6/12	Interleukine 1/2/6/12
LDL	Low-density lipoprotein
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MMP-2/14	Matrix metalloproteinase 2/14
MnSOD	Manganese Superoxide Dismutase
mTOR	Mammalian target of rapamycin
NK	Natural killer
NO	Nitric oxide
N-SMase	Neutral sphingomyelinase
Ox-LDL	Oxidized low-density lipoprotein
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
SOD	Superoxide dismutase
SRBC	Sheep red blood cells
STAT3	Signal transducers and activators of transcription 3
TCM	Traditional Chinese Medicine
TNF-α	Tumor necrosis factor α
VSMC	Vascular smooth muscle cell
WE	Water extraction

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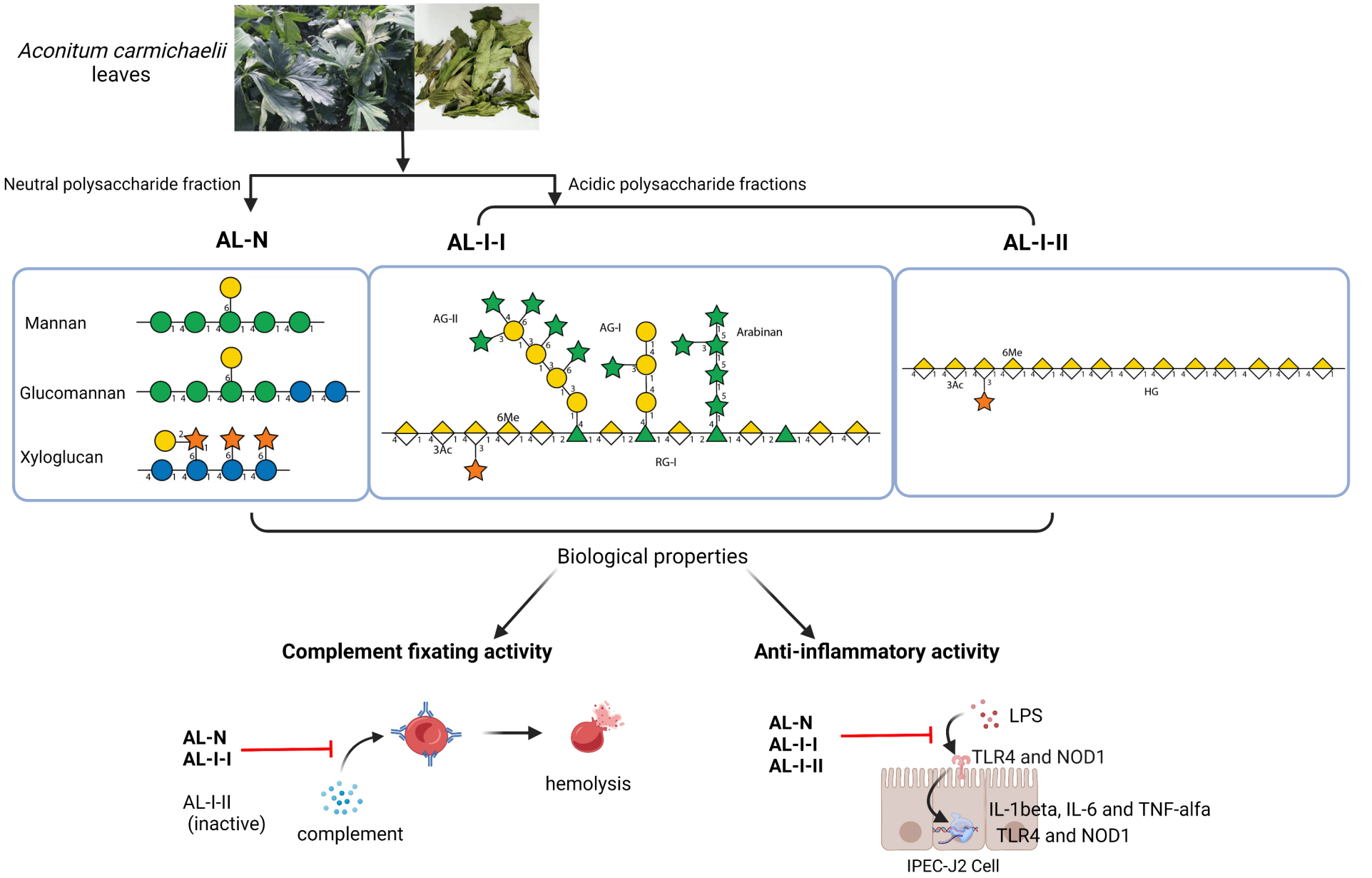
Paper II

Paper III


Aconitum carmichaelii
leaves
Neutral polysaccharide fraction
Acidic polysaccharide fractions
AL-N
AL-I-I
AL-I-II
Mannan
Glucomannan
Xyloglucan
AG-II
AG-I
Arabinan
6Me
3Ac
RG-I
HG
Biological properties
Complement fixating activity
Anti-inflammatory activity
AL-N
AL-I-I
AL-I-II
(inactive)
complement
hemolysis
LPS
TLR4 and NOD1
IL-1beta, IL-6 and TNF-alfa
TLR4 and NOD1
IPEC-J2 Cell

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Polysaccharides from *Aconitum carmichaelii* leaves: Structure, immunomodulatory and anti-inflammatory activities

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ABSTRACT

Roots of *Aconitum carmichaelii* are used in Asian countries due to its content of bioactive alkaloids. In the production of root preparations, tons of leaves are usually discarded, leading to a huge waste of herbal material. The aim of this study is to investigate the polysaccharides in these unutilized leaves. A neutral polysaccharide (AL-N) appeared to be a mixture of heteromannans, and two purified acidic polysaccharides (AL-I-I and AL-I-II) were shown to be pectins containing a homogalacturonan backbone substituted with terminal β-Xyl*p*-units. AL-I-I consisted of a type-I rhamnogalacturonan core, with arabinan and type-II arabinogalactan domains while AL-I-II was less branched. AL-N and AL-I-I were able to modulate the complement system, while AL-I-II was inactive. Interestingly, AL-N, AL-I-I and AL-I-II were shown to exert anti-inflammatory effects on porcine enterocyte IPEC-J2 cells. AL-I-I and AL-I-II were able to down-regulate the expression of toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain 1 (NOD1).

1. Introduction

Aconitum carmichaelii Debeaux (Ranunculaceae) is indigenous mainly to China, but can be found in other Asian countries, and also in Europe (Fu et al., 2022). It is a perennial herb, 60–150 cm high, with pentagonal leaves 6–11 cm long and 9–15 cm wide (Committee for the flora of China, 2004). In China, the lateral and mother roots of *A. carmichaelii*, known as "Fuzi" and "Chuanwu", are used in Traditional Chinese Medicine (TCM) in the treatment of acute myocardial infarction, rheumatoid arthritis, and coronary heart disease, as well as for analgesic use (Chinese Pharmacopoeia Committee, 2020; Fu et al., 2022). Currently, the plant is commercially grown in Sichuan Province, where most of the trading of "Fuzi" and "Chuanwu" exist. More than 200 tons of dried roots were traded within the two year period from 2015 to 2017 (China Academy of Chinese Medical Science, 2017).

The market of TCM is attractive, but a great amount of unutilized parts of medicinal plants is generated from the industry, such as stems and leaves for TCM based on roots. A better utilization of bio-resources is highly required, and these residues should be recycled and converted into valuable products such as phytochemicals (Huang, Li, et al., 2021; Huang, Peng, et al., 2021; Saha & Basak, 2020). The aerial parts of *A. carmichaelii*, making up 40% of the biomass of the whole plant, are normally discarded after the roots are harvested, and a vast amount of waste of this plant source is consequently generated. To date, the aerial parts of *A. carmichaelii* have shown similar analgesic and anti-inflammatory activities as for the roots (He et al., 2018). Alkaloids, flavonoids, lignin (Duc et al., 2015; Zhang, Yang, et al., 2020), fatty acids (Chen, 2011; Ni et al., 2002), sterols (Guo, 2012; Yang et al., 2011) and polysaccharides (Ou et al., 2013) have been identified in the leaves. A content of approximately 5% (on dry basis) polysaccharides has been determined in *A. carmichaelii* leaves (Ou et al., 2013), but further studies on structural characterization and pharmacology have not been performed.

Many natural polysaccharides are unable to be digested by mammalian enzymes in the gastrointestinal tract, and act as dietary fiber. These have attracted increasing attention due to their positive health effects, such as immunoregulatory, anti-tumor, anti-viral, anti-oxidative, and hypoglycemic activities, and low toxicity (Yang et al., 2022; Yu et al., 2018). Pectins, for instance, have been shown to exert potent immunomodulatory effects on the complement system,

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macrophages, T cells, natural killer cells, and the intestinal immune system (Beukema et al., 2020; Zaitseva et al., 2020). It has been suggested that pectic polysaccharides could interact with plasma complement proteins *via* the alternative and/or the classical pathways. This could lead to either activation of the complement system, which contributes to inflammatory responses in addition to host defense reactions, or inhibition of complement cascade which would be a good therapeutic strategy for treating inflammatory diseases (Yamada & Kiyohara, 2007). Pectins have also attracted growing attention for their role in the preservation of epithelial integrity, and might directly interact with pattern recognition receptors, such as Toll-like receptors 2 (TLR2) and 4 (TLR4) or Galectin-3 (Beukema et al., 2020), inhibit inflammation and oxidative responses, or modulate the levels of cytokines and chemotactic factors (Huang et al., 2017; Tang et al., 2019). Therefore, we hypothesized that the unutilized leaves of *A. carmichaelii* could be a potential medicinal source due to the presence of polysaccharides with possible immunomodulatory and anti-inflammatory activities.

The aim of this study was to isolate and characterize polysaccharides present in the leaves of *A. carmichaelii* and to determine their complement fixation activity and intestinal anti-inflammatory effects on lipopolysaccharide (LPS)-induced inflammatory intestinal epithelial cells (IPEC-J2).

2. Materials and methods

2.1. Materials

The whole plant of *A. carmichaelii* Debeaux was collected in Wudu Village, Jiangyou City, Sichuan Province, P.R. China in June 2019 (31°50′24.0″N/ 104°47′24.0″E, 517.11 m), and was identified by Yuan-Feng Zou, Sichuan Agricultural University. A voucher specimen with number 2019-06-342 is deposited in the Department of Pharmacy, Sichuan Agricultural University. The fresh leaves were separated from the rest of the plant immediately after collection, and then dried in a drying oven at 40 °C with flowing air.

2.2. Isolation and purification of polysaccharides from *A. carmichaelii* leaves

Polysaccharides from *A. carmichaelii* leaves were isolated and purified as depicted in Fig. 1. Fifty grams of dried leaves of *A. carmichaelii* were pre-extracted with 96% ethanol (500 mL, 1 h × 4) under reflux in order to remove small molecular weight and other lipophilic compounds. The dried residues were further extracted with boiling water (1 L, 1 h × 2) under reflux. The combined aqueous extracts were filtered, evaporated at 50 °C, added 4-fold volumes of ethanol and kept at 4 °C for 24 h for precipitation of the polysaccharides. The precipitant was redissolved in distilled water, dialyzed with cut-off 3500 Da, and freeze-dried, giving a crude polysaccharide fraction, named ALP (*A. carmichaelii* Leaves Polysaccharide).

ALP (2.1 g) was fractioned by anion exchange chromatography using a column packed with ANX Sepharose™ 4 Fast Flow (high sub) material (GE Healthcare, 5 × 40 cm). A neutral fraction (AL-N) was first eluted with distilled water (600 mL) with flow rate 1 mL/min, while an acidic fraction (AL-I) was eluted with a linear gradient of NaCl (0–1.5 M, 1200 mL) with flow rate 2 mL/min. 10 mL fractions were collected and

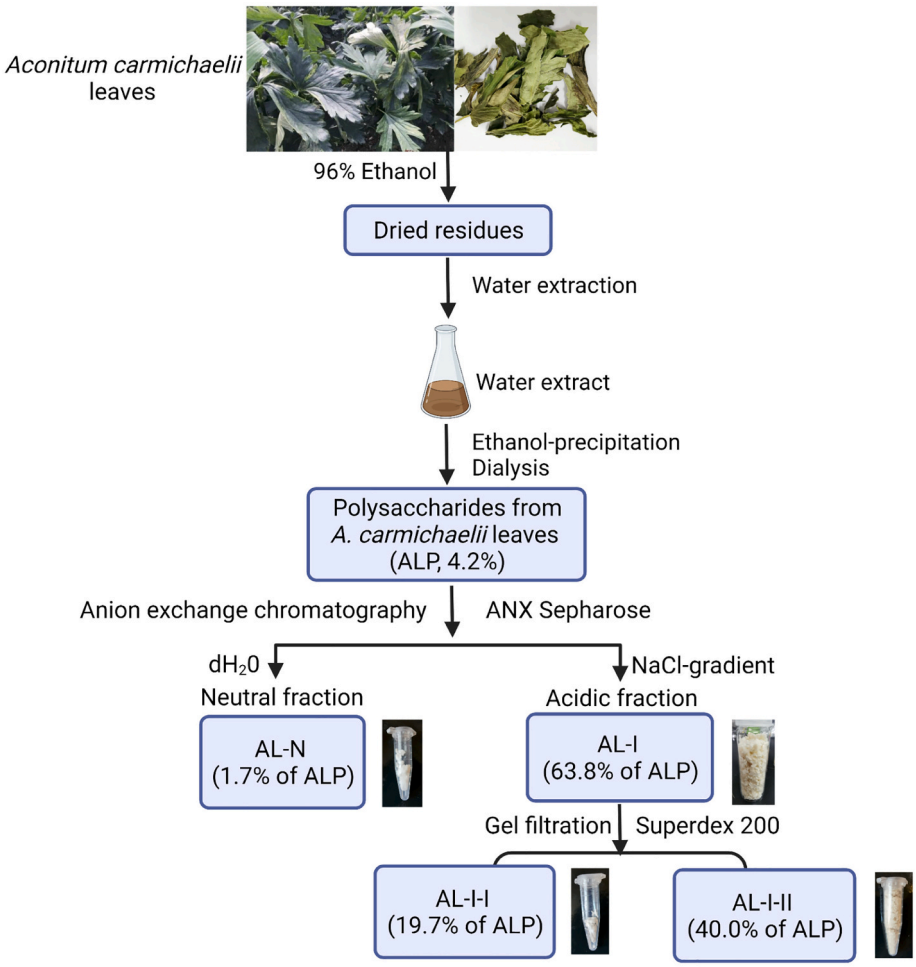

Fig. 1. Work flow of isolation and purification of polysaccharides from *A. carmichaelii* leaves.

monitored by phenol-sulfuric acid assay to locate the polysaccharides (Dubois et al., 1956). The related fractions were combined and dialyzed at cut-off 3500 Da for removal of NaCl, and lyophilized.

AL-I (20 mg) was further separated by size exclusion chromatography (SEC) based on differences in molecular size. 2 mL sample (10 mg/mL in 10 mM NaCl) was applied onto an Hiload™ 16/60 Superdex 200 prep grade column (GE Healthcare) using the Äkta FPLC system (Pharmacia Äkta, Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with 10 mM NaCl, 0.5 mL/min (2 mL per tube). Fractions were combined based on their elution profiles after phenol-sulfuric acid assay (Dubois et al., 1956), then dialyzed and lyophilized.

2.3. Determination of the chemical composition and monosaccharide composition

The total amounts of phenolic compounds and proteins per fraction were quantitatively determined by Folin-Ciocalteu (Singleton & Rossi, 1965) and Bio-Rad protein assay (Bradford, 1976) respectively. Standard curves were prepared using gallic acid (0–50 μg/mL) for determination of phenolic compounds, and bovine serum albumin for protein determination (BSA, 1.5–25 μg/mL).

The monosaccharide composition of the fractions were determined as described by Chambers and Clamp (1971) with modifications as described before (Wold et al., 2018). In short, samples were subjected to methanolysis using 3 M hydrochloric acid in water-free methanol for 24 h at 80 °C, then trimethylsilylated (TMS) before they were analyzed using capillary gas chromatography (GC) on a Trace™ 1300 GC (Thermo Scientific™, Milan, Italy). Mannitol was used as an internal standard, and calibration curves were prepared by TMS-derived standards, including arabinose (Ara), rhamnose (Rha), fucose (Fuc), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc), glucuronic acid (GlcA) and galacturonic acid (GalA). The Chromelion Software v.6.80 (Dionex Corporation, Sunnyvale, CA, USA) was used for GC data analysis.

2.4. Glycosidic linkage determination by methylation and GC/MS

Determination of glycosidic linkages of the different monosaccharides was performed after permethylation of the reduced polymers or native not containing uronic acid. Briefly, 2 mg of samples with uronic acids was reduced to their corresponding neutral sugars with sodium borodeuteride ($NaBD_4$) after activation by carbodiimide, which led to dideuteration in position 6 ($-CD_2-$). This gives an increased mass of related ion fragments ($M^+ + 2$) and helped to distinguish uronic acid from the neutral sugar. Then methylation, hydrolysis, reduction, and acetylation were performed according to previously published methods (Ciucanu & Kerek, 1984; Pettolino et al., 2012; Wold et al., 2018). These derivatives were extracted with dichloromethane, and the partially methylated alditol acetates were analyzed by GC–MS using a GCMS-QP2010 (Shimadzu) as earlier described (Braünlich et al., 2018), in which a Restek Rxi-5MS capillary column (30 m; 0.25 mm i.d.; 0.25 μm film) was attached. The estimation of relative amounts of each linkage type was related to the total mol percent of monosaccharides as determined by methanolysis as described above, and the effective carbon-response factors were considered for quantification of separated fragments based on integration of GC chromatograms (Sweet et al., 1975; Zou et al., 2017).

2.5. Molecular weight determination

The homogeneity and the weight-average molecular weight (*Mw*) of samples (2 mg/mL, 0.5 μL) were determined by SEC on Superose™ 6 (Amersham Biosciences, 10 × 300 mm) combined with the Äkta FPLC system. A calibration curve was prepared using dextran polymers with different *Mw* (5.6, 19, 50, 80, 150, 233, and 475 kDa, Pharmacia). Standards and samples were eluted with 10 mM NaCl, and 0.5 mL fractions were collected. The retention volume was converted to molecular weight based on the calibration curve provided by standards above.

2.6. NMR spectroscopy

^1H NMR (with continuous-wave presaturation, pulse program "zgpr"), ^{13}C NMR (pulse program "zrestse.dp.jcm800"), HMBC (pulse program "awhmbcgplpndqfpr" and "awshmbcctetgpl2nd.m"), HSQC (pulse program "awhsqcedetgpsisp2.3-135pr" and "awshsqc135pr") and COSY (pulse program "cosygpprqf") spectra of purified polysaccharides dissolved in 600 μL D_2O (99.9%, Sigma) were acquired on a Bruker Advance III HD 800 MHz spectrometer equipped with a 5-mm cryogenic CP-TCI z-gradient probe at 60 °C (Bruker, Rheinstetten, Germany). Spectra were analyzed by MestReNova software (Ver.6.0.2, Mestrelab Research S.L., Spain) and calibrated relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate at 0 ppm.

2.7. Complement fixation assay

The complement fixating activity of plant-derived polysaccharides has been used as an indicator for their potential effect on the immune system, which is measured based on inhibitory effects of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera (Michaelsen et al., 2000) (Method A). A published highly active pectin from the aerial parts of *Biophytum petersianum* Klotzsch (Grønhaug et al., 2011), BPII, was used as the positive control. The 50% inhibition of hemolysis (ICH_{50}) of tested samples are obtained according to dose-response curves. A lower ICH_{50} value means a higher complement fixation activity. All samples were analyzed in duplicates in three separate experiments.

2.8. Anti-inflammatory effects on porcine jejunum epithelial cells (IPEC-J2)

2.8.1. Cell culture

IPEC-J2 cells were obtained from the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and were cultured in DMEM/F-12 medium (Beijing Solarbio Science & Technology Co., Ltd.), containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific (China) Co., Ltd) and 1% penicillin-streptomycin (100 U/mL, Beijing Solarbio Science & Technology Co., Ltd.). They were maintained in a cell incubator with 5% CO_2 at 37 °C.

2.8.2. Cell viability and treatment

Cells were plated in 96-well cell plates (5×10^3 cells per well), and final concentrations of 20 μg/mL of AL-N, AL-I, AL-I-I and AL-I-II were added and co-cultivated for 24 h for the measurement of cell viability. The cytotoxic effects of all samples were assessed by Cell Counting Kit-8 reagent (CCK-8, Dojindo, CK04-11, Minato-ku, Tokyo, Japan) according to the manufacturer's instruction.

20 μg/mL LPS (Sigma-Aldrich, USA, purity ≥99%) was employed to induce inflammation on IPEC-J2 in a 6-well plate (5×10^3 cells per well) for 12 h. Then all samples were supplemented at final concentrations of 20 μg/mL in medium for the screening of the anti-inflammatory activity. High-yield acidic polysaccharides were further tested for a comprehensive comparison of anti-inflammatory activities among different fractions. Cells treated with LPS and medium were set as control cells, and those with only medium were negative control. After another 12 h of co-cultivation, all wells were rinsed with PBS, and total RNA was collected with Trizol Reagent (Biomed, RA101-12, China) for further analysis.

2.8.3. qRT-PCR

Total RNA of all collected cells was isolated using Trizol Reagent, and reverse transcribed into cDNA using M-MLV 4 First-Strand cDNA Synthesis Kit (Biomed, RA101-12, China). All real-time PCR analysis were

performed by SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Mei5Bio, China), and the gene expressions were quantified as relative regulation fold compared with *β-actin* (normalizing reference). Primers of all genes were shown in Table S1.

2.9. Statistical analysis

All experimental data were expressed as the mean ± S.D., and analyzed using one-way analysis of variance and Duncan test (IBM SPSS Statistics version 24, IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Isolation and purification of polysaccharide fractions from *A. carmichaelii* leaves

A crude polysaccharide, ALP, extracted from the dried leaves of *A. carmichaelii* was obtained, making up approximately 4.2% of the dried plant mass (2.1 g/50 g). This is in accordance with a previous study, reporting the presence of 4.9% polysaccharide in leaves of *A. carmichaelii* (Ou et al., 2013). As shown in Fig. 1 and by elution profiles in Fig. 2, one neutral fraction, AL-N (Fig. 2A), and one acidic fraction, AL-I (Fig. 2B), were obtained after anion exchange chromatography, with yields of 1.7% and 63.8% of ALP, respectively. The remaining amount of ALP might consist of undissolved compounds left in the filter before applying to IEC and colored compounds bound in the ANX Sepharose matrix. AL-I was further fractionated by SEC based on *Mw* difference, and two purified polysaccharides, named AL-I-I and AL-I-II, were obtained (Fig. 2C). Extraction yields are shown in Table 1. There was no detectable phenolic content in these fractions as assessed by the Folin-Ciocalteu test (Singleton & Rossi, 1965), and less than 1% of protein was detected (Table 1).

Table 1
Carbohydrate yields, weight-average *Mw*, and contents of protein in polysaccharide fractions isolated from *Aconitum carmichaelii* leaves.

	AL-N	AL-I-I	AL-I-II
Yields[a]	1.7%	19.7%	40.0%
Mw/kDa[b]	10.2	169.1	41.6
Total protein[c]	0.6%	1.0%	0.9%

[a] Yields related to the weight of the crude polysaccharide fraction ALP.
[b] Determined by SEC with a calibration curve of dextran standards (Section 2.5).
[c] Determined by Bio-Rad protein assay (Bradford, 1976).

3.2. Molecular weights of polysaccharide fractions

Homogeneity and weight-average molecular weight *Mw* of AL-N, AL-I-I and AL-I-II were determined by gel filtration (Fig. 2D), and is shown in Table 1. AL-N was considered a homogeneous fraction with lowest *Mw* among all fractions, as shown after applying on both Superose 6 (*Mw* range 5×10^3 to 5×10^6 Da, Fig. 2D) and Sephacryl S-100 High Resolution (*Mw* range 1×10^3 to 1×10^5 Da, Fig. 2E) columns. AL-I-I with a *Mw* of 169.1 kDa was the fraction with highest *Mw*. A huge *Mw* variation was also observed in acidic heteropolysaccharides isolated from the roots of *A. carmichaelii*, with *Mw* ranging from 5.8 kDa to more than 1000 kDa (Gao, Bia, et al., 2010).

3.3. Monosaccharide composition of polysaccharide fractions from *A. carmichaelii* leaves

The monosaccharide composition of AL-N, AL-I-I and AL-I-II were analyzed by GC as TMS derivatives of methylated monomers, and are presented in Table 2. The GC chromatograms are shown in Fig. S1. In AL-N, Glc (37.2 mol%) and Man (25.0 mol%) were the predominant monosaccharides, followed by Ara, Xyl, Gal and Fuc. A minor amount of GalA was detected in AL-N, and this could be due to methyl esterification of the uronic acid. The acidic heteropolysaccharides, AL-I-I and AL-

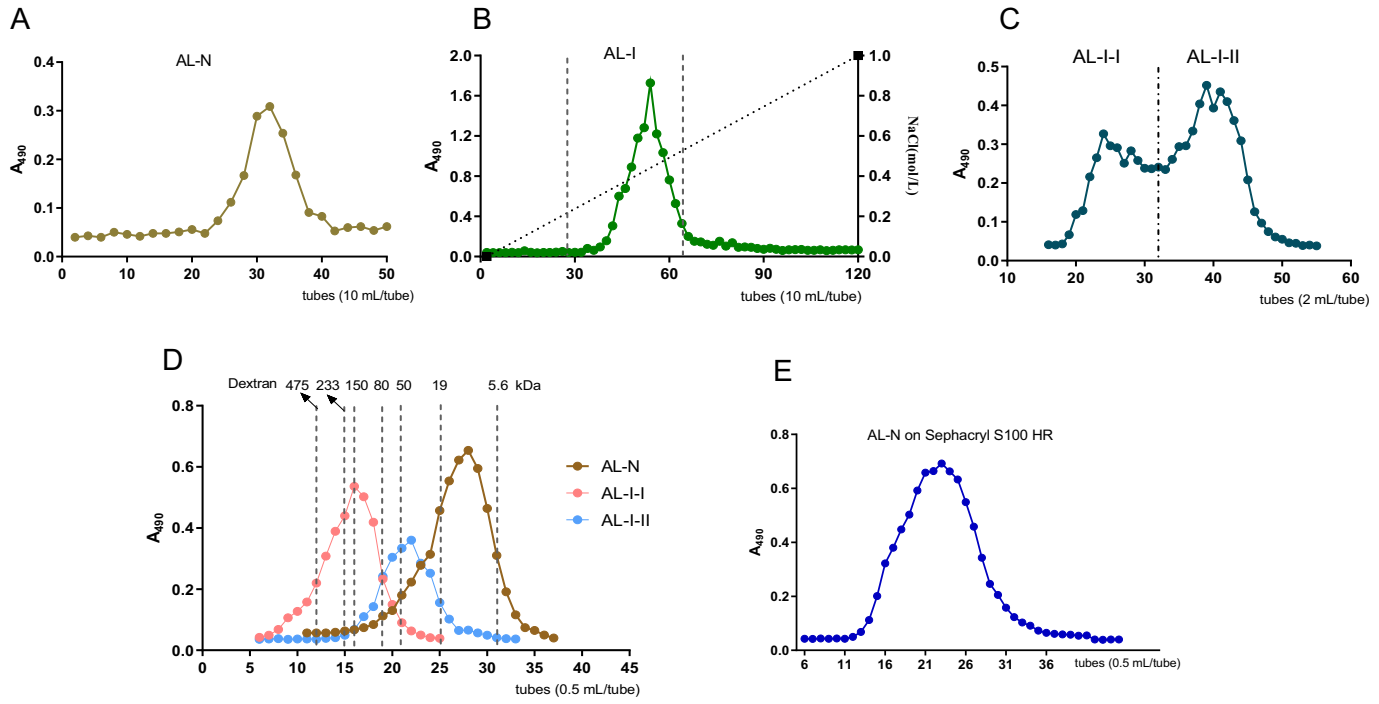

Fig. 2. The elution profiles of polysaccharides fractions AL-N, AL-I, AL-I-I and AL-I-II from *A. carmichaelii* leaves. Anion exchange chromatography elution profile of AL-N (A) and AL-I (B) on ANX Sepharose; Size exclusive chromatography elution profile of AL-I-I and AL-I-II on Superdex 200 (C), of AL-N, AL-I-I and AL-I-II on Superose 6 (D), and of AL-N on Sephacryl S100 HR (E).

Table 2
The monosaccharide composition (mol%) of polysaccharide fractions from *Aconitum carmichaelii* leaves.

	AL-N	AL-I-I	AL-I-II
Ara	12.7	28.0	5.9
Rha	0.4	7.2	5.2
Fuc	2.2	0.6	1.3
Xyl	12.7	5.2	5.4
Man	25.0	0.5	0.3
Gal	9.0	21.4	3.8
Glc	37.2	2.6	3.0
GlcA	n.d.	1.3	0.8
GalA	1.0	33.2	74.3

Note: mol% related to total content of the monosaccharides Ara, Rha, Fuc, Xyl, Man, Gal, Glc, GlcA, and GalA. n.d. = not determined.

I-II were composed of almost the same monosaccharides, but in different ratios. Both of them had a high proportion of GalA, but also neutral monosaccharides. Ara, Gal and Rha were the main monomers in addition to GalA in AL-I-I, while AL-I-II mostly consisted of GalA with lesser amounts of the neutral ones. These compositions are typical of pectic polysaccharides (Kaczmarska et al., 2022; Zaitseva et al., 2020).

As the first study on the structural characterization of polysaccharides from *A. carmichaelii* leaves, this study shows differences in the polysaccharide composition in leaves compared to those isolated from roots. Glucans and other neutral heteropolysaccharides mainly composed of Glc have been reported from roots of *A. carmichaelii* (Gao, Bia, et al., 2010; Wang et al., 2016; Yang et al., 2020; Zhao et al., 2006), but no polysaccharides consisting mainly of Man, Ara and/or Xyl have been reported so far. A possible pectin containing mainly Glc, Ara, Gal, and 5.7–33.5% of GalA have been reported in the roots by Gao, Bia, et al. (2010). However, no detailed structural analysis that can give evidence for the presence of pectin in any plant parts of *A. carmichaelii* have been performed.

3.4. Structural characterization of polysaccharides from leaves of *A. carmichaelii*

3.4.1. Glycosidic linkages

Based on monosaccharide compositions, the glycosidic linkage types of AL-N, AL-I-I, and AL-I-II were determined by GC–MS after permethylation, and are shown in Table 3. The GC chromatograms of fragments and MS spectra of each corresponding fragment are shown in Fig. S2.

The major linkage patterns of AL-N were 1,4-linked Man*p* (22.4 mol %) and 1,4-linked Glc*p* (22.8 mol%), both monomers also having 1,4,6-linkages. Ara*f* was present mainly as terminal and 1,5-linked units, in addition to 1,3,5-linked residues. Xyl*p* and Gal*p* were present as terminal units and as linear chains, 1,2-linked and 1,3-linked respectively. As reported previously, hemicellulose or storage polysaccharides in primary plant cell wall (Fry, 2011; Hayashi & Kaida, 2011; Nishinari et al., 2007) includes mannans (a backbone rich in or entirely composed of 1,4-linked β-Man*p* and occasionally carrying terminal β-Gal*p* at *O*-6 as side chains), glucomannans (mannans with 1,4-linked β-Glc*p* within the backbone and/or terminal β-Gal*p* at *O*-6 of Man*p*) and xyloglucans (composed of 1,4-linked β-Glc*p* as backbone and branched at *O*-6 with terminal α-Xyl*p*, and/or 1,2-linked Xyl*p* connected with terminal Gal*p*). According the xyloglucan models described by Fry et al. (1993), the specific structure of the xyloglucan in AL-N could be XXLG (X, α-$_D$-Xyl*p*-(1 → 6)-β-$_D$-Glc*p*; L, β-$_D$-Gal*p*-(1 → 2)-α-$_D$-Xyl*p*-(1 → 6)-β-$_D$-Glc*p*; G, β-$_D$-Glc*p*) or XLXG model due to the ratio of relative amounts of T-α-Xyl and1,2-linked α-Xyl (7.7:4.7, Table 3). Given the homogenous composition observed in Fig. 2D and Fig. 2E, AL-N might be a mixture of mannans, xyloglucans and/or glucomannans and minor amounts of arabinogalactan with similar *Mw*, as depicted in Fig. 4. The rather low yield of this fraction compared to the high yield of AL-I (Table 1) was the reason for not perform in further studies on AL-N.

Table 3
Glycosidic linkage types (mol%) present in polysaccharide fractions from leaves of *Aconitum carmichaelii*.

Linkage types	Rt/min	Primary fragments	AL-N	AL-I-I	AL-I-II
Ara*f*					
T-	12.41	45, 118, 161, 162	4.1	21.6	4.8
1,3-	14.76	45, 118, 233	trace	1.1	trace
1,5-	15.53	118, 162, 189	4.8	3.3	trace
1,3,5-	17.55	118, 261	2.6	1.8	trace
Rha*p*					
T-	13.31	118, 131, 162, 175	n.d	trace	3.7
1,2-	15.53	131, 190	n.d	3.9	trace
1,2,4-	17.91	190, 203	n.d	2.8	trace
Fuc*p*					
T-	14.04	118, 131, 162, 175	2.2	trace	1.3
Xyl*p*					
T-	13.31	117, 118, 162	7.7	5.2	4.2
1,2-	15.71	117, 130, 190	4.7	n.d	n.d
1,4-	15.71	118, 162, 189	n.d	n.d	1.2
Man*p*					
1,4-	19.05	45, 118, 162, 233	22.4	n.d	n.d
1,4,6-	21.60	118, 162, 261	1.5	n.d	n.d
Gal*p*					
T-	17.17	45, 118, 162, 205	3.2	1.6	1.2
1,3-	19.42	118, 161, 234, 277	2.4	2.3	trace
1,4-	19.03	45, 118, 162, 233	n.d	1.0	trace
1,6-	20.41	118, 162, 189, 233	trace	1.7	trace
1,3,6-	22.63	118, 189, 234, 305	trace	7.1	trace
1,3,4-	20.71	45, 118, 305	n.d	1.0	n.d
1,4,6-	22.00	118, 162, 261	trace	1.4	trace
1,3,4,6-	23.4	118, 333	1.1	5.2	trace
Glc*p*					
T-	16.62	45, 118, 161, 162, 205	1.1	n.d	1.4
1,3-	18.93	45, 118, 161, 234, 277	2.3	trace	n.d
1,4-	19.22	45, 118, 162, 233	22.8	1.9	1.5
1,4,6-	21.80	118, 162, 261	10.4	trace	trace
Glc*p*A					
T-	16.62	47, 118, 161, 162, 207	n.d	1.1	trace
Gal*p*A					
T-	17.17	47, 118, 162, 207	trace	trace	2.3
1,4-	19.03	47, 118, 162, 235	trace	27.9	62.6
1,2,4-	21.19	47, 190, 235	n.d	trace	1.7
1,3,4-	20.71	47, 118, 307	n.d	4.6	8.0

Note: trace, relative amount less than 1.0%, n.d, not detected.

The acidic polysaccharides AL-I-I and AL-I-II consists of monomers and glycosidic linkages typically found in pectic polysaccharides. The main linkage types for both AL-I-I and AL-I-II was 1,4-linked Gal*p*A, most probably coming from a homogalacturonan (HG) domain that is often present in intercellular tissues as part of plant cell wall (Voragen et al., 2009). The HG region can be substituted by terminal Xyl*p*, as xylogalacturonan (XGA) (Patova et al., 2021; Wang et al., 2019), as well as by terminal Fuc*p* at position C-3 of 4)-Gal*p*A-(1 → (Braünlich et al., 2018), which also can be the case in both AL-I-I and AL-I-II. The HG region is longer in AL-I-II than AL-I-I, as it contains 35 mol% more of 1,4-linked Gal*p*A (Table 3).

Further, several types of neutral monosaccharides were found in AL-I-I, such as 1,2- and 1,2,4-linked Rha*p*, terminal- (T-), 1,5- and 1,3,5-linked Ara*f*, and 1,3- and 1,3,6-Gal*p*. These linkage patterns indicate a possible presence of type I rhamnogalacturonan (RG-I), arabinan and arabinogalactan (AG) domains, respectively (Kaczmarska et al., 2022; Voragen et al., 2009). 1,3,4,6-linked Gal*p* (5.2 mol%) detected in AL-I-I could be terminated with Ara*f*, as has been described in other pectic polysaccharides (Braünlich et al., 2018; Shen et al., 2021; Zhang, Li, et al., 2020). More than 20 mol% of terminal Ara*f* was found in AL-I-I, which might be due to arabinan and AG domains, as the total amount (20.3 mol%) of branched monomers including 1,3,5-Ara*f*, 1,3,4-Gal*p*, 1,3,6-Gal*p* and 1,3,4,6-Gal*p* (connected with two Ara*f*) was close to the amount of terminal Ara*f*. Both AG type II (AG-II) moieties, 1,3 linked Gal*p* units branched at C-6 (7.1 mol%), and AG type I (AG-I) moieties, 1,4-linked Gal*p* blocks branched at C-3 (1.0 mol%), were present in AL-I-

I (Table 3). The ratio of AG-II: AG-I: arabinan could be approximate 7:1:1 according to the relative amounts of these branching units. These results illustrated a highly branched structure of AL-I-I. For AL-I-II, a longer HG backbone was found, and therefore more moieties would be attached to C-3 of Gal*p*A compared to AL-I-I. Few neutral side chains were shown for AL-I-II, as only trace amounts of 2,4)-Rha*p*-(1 → units were detected, and consequently, less amount of arabinan or AG domains were revealed. Terminal Glc*p*A could be located on the end of arabinogalactan side chains (Makarova et al., 2016; Zhang, Li, et al., 2020).

3.4.2. NMR analysis

The structure of AL-I-I and AL-I-II were further analyzed by NMR. The data were interpreted by comparing and matching chemical shift

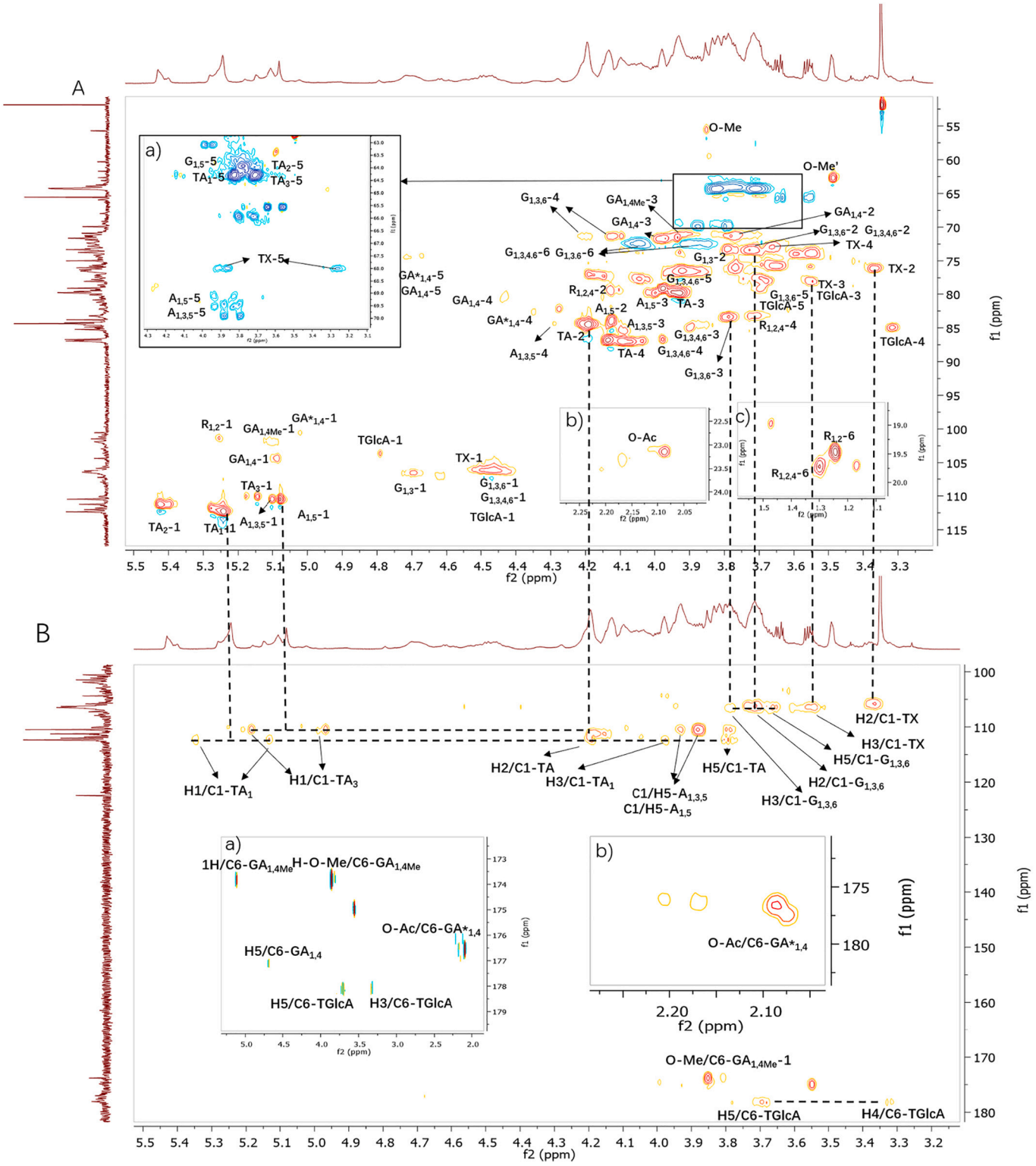

Fig. 3. 2D NMR spectra of pectic polysaccharides from leaves of *A. carmichaelii*. HSQC (A) and HMBC spectra (B) of AL-I-I, and HSQC (C) and HMBC spectra (D) of AL-I-II. Inserted plots were selective HSQC or HMBC spectra zooming in specific chemical shift range.

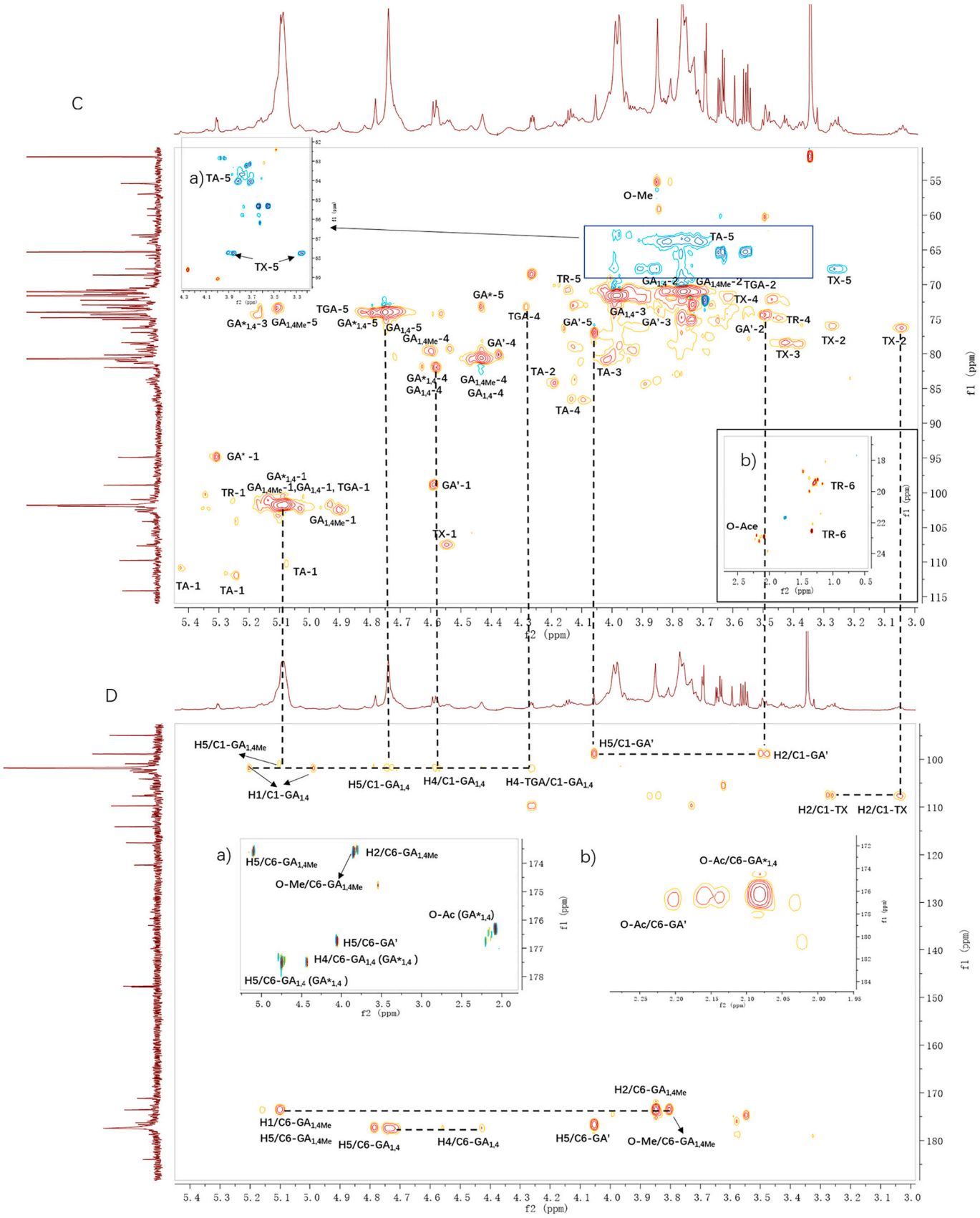

Fig. 3. (*continued*).

values from the 1D spectra ^{1}H and ^{13}C (Fig. S3A and B, Fig. S4A and B,), and the 2D spectra COSY (Fig. S3C and Fig. S4C), HSQC and HMBC (Fig. 3). Space correlation of AL-I-I including ROESY and NOESY are presented in Fig. S3D and Fig. S3E respectively, but only a few correlations of AL-I-II were detected. Typical residues were assigned based on the methylation analysis and previously reported literature (Huang, Li, et al., 2021; Huang, Peng, et al., 2021; Makarova et al., 2016; Patova et al., 2021; Patova et al., 2019; Shakhmatov et al., 2019; Shakhmatov et al., 2015; Zhang, Li, et al., 2020; Zou et al., 2021; Zou et al., 2020), and the values of the chemical shifts are presented in Table 4. However, signals from trace residues and bound correlations between monomers are hard to be recorded.

The anomeric region between δ 5.1 to δ 5.8 in ^{1}H NMR and δ 98 to δ 103 in ^{13}C NMR are signals of sugar residues with α-configuration, while those in β-configuration commonly appear in δ 4.4 to 4.8 and δ 103 to 106 (Yao et al., 2021). Peaks in the region δ 1.1 to 1.4 in ^{1}H NMR and δ 16 to 18 ^{13}C NMR indicated the presence of —CH_3 of Rha, while those at δ 2.0 to 2.2 and δ 18 to 22, and δ 3.3 to 3.8 and δ 55 to 61 suggested the presence of acetyl (CH_3CO—) and methyl units (—OCH_3) respectively (Yao et al., 2021). The rest of the high-intense peaks could be assigned to protons and carbons from C-2 to C-5 or C-6 of monomers, and their chemical shifts change if they are in different chemical environment.

Many signals and cross peaks from Ara*f* can be detected due to its high concentration in **AL-I-I** based on results of methylation, therefore signals of anomeric carbon (C-1) at 103 to 112 ppm derived from furanose should be assigned to α-Ara*f* (Yao et al., 2021). As shown in Table 4 and Fig. 3A, the intense signals of H/C-atoms at δ 5.24/112.3 (**TA_1-1**), δ 5.42/111.2 (**TA_2-1**), and δ 5.14/110.1 (**TA_3-1**), belong to α-Ara*f*-(1 → residues (Makarova et al., 2016; Shakhmatov et al., 2015). They might differ in terms of their appendences to Gal*p*, or various substituted α-Ara*f* (Zhang, Li, et al., 2020). However, it was hard to distinguish these in this case, as correlations between H-1 of terminal Ara*f* and H-3/4/6 of substituted Gal*p* or H-3/5 of substituted Ara*f* were highly overlapped. In the current HSQC pulse program, a multiplicity edited with Distortionless Enhancement by Polarization Transfer (DEPT)-135 carbon experiment was set, in which the intensity of all protonated carbons depends on the magnitude of the flip angle and the number of protons attached to a carbon. As a result, after polarization transform, carbon signals from methine (CH) and methyl (CH_3) groups are generally positive, but those from methylene (CH_2) groups are negative. For Ara*f*, signals of C-5 and H-5 (CH_2-OH) were detected as negative (blue) cross points at 64 to 70 ppm (Fig. 3A). The cross peaks related to C-1 of Ara*f* in HMBC helped to assign the protons located at other carbons in the same sugar ring, such as H-2 and H-3. For example, protons at 4.19, 3.98 and 3.82 ppm correlated to C-1 at 112.3 ppm in HMBC were assigned to H-2, H-3 and H-5 of TA_1 respectively (Fig. 3B), and correlations among them were also observed as cross peaks in COSY and space correlations in ROESY (Fig. S3D) and NOESY (Fig. S3E). However, protons correlated to C-1 at 110.5 ppm in HMBC (residues at δ 110.5/3.88, δ 110.5/3.80 and δ 110.5/3.93, Fig. 3B) should be assigned to H-5 of *O*-5-substituted Ara*f*, due to the downfield chemical shifts of their attached carbons at 69.9 (δ 3.80, 3.88/69.9, **$A_{1,5}$-5**) and 69.5 ppm (δ 3.83, 3.93/69.5, **$A_{1,3,5}$-5**) in HSQC compared to the carbons of terminal Ara*f* at 63–64 ppm (Fig. 3A) (Shakhmatov et al., 2015; Zhang, Li, et al., 2020; Zou et al., 2021), which were also proved by the H/C correlations at δ 5.08/69.9 in HMBC (Fig. S3F, a).

Highly branched arabinogalactans were further confirmed by the residues of →3,4,6)-β-Gal*p*-(1 → (**$G_{1,3,4,6}$**), →3,6)-β-Gal*p*-(1 → (**$G_{1,3,6}$**) and →3)-β-Gal*p*-(1 → (**$G_{1,3}$**) according to high intense H/C correlations of typical β-pyranose at δ 4.49/106.3 (**$G_{1,3,4,6}$-1**), δ 4.46/105.9 (**$G_{1,3,4,6}$-1**), and a weak one at δ 4.69/106.5 (**$G_{1,3}$-1**) in HSQC spectrum (Fig. 3A), and those between H-2/3/5 and C-1 in HMBC (Fig. 3B), as well as proton-proton correlations between H-1 and H-2 in COSY (Fig. 3SC), and between H-1 and H-2/3/6 in ROESY (Fig. 3SD) and NOESY (Fig. S3E), which were in line with earlier reported values (Shakhmatov et al., 2018; Shakhmatov et al., 2015; Zhang, Li, et al., 2020). A downfield chemical shift of H/C-atoms of *O*-4 substituted Gal*p* was also observed at δ 3.98/86.7 in HSQC (Fig. 3A, **$G_{1,3,4,6}$-4**) (Zhang, Li, et al., 2020).

Furthermore, the anomeric spin systems H-1/C-1 at δ 5.26/101.4 was assigned to 1,2-α-Rha*p* (**$R_{1,2}$**), and the signal of H-2 were assigned due to the proton-proton correlations in COSY (Fig. 3C) and NOESY (Fig. S3E). Signals of C-4 and C-5 of Rha*p* were appointed according to H-6/C-4 correlations at δ 1.24/75.0 and δ 1.30/83.2 and H-6/C-5 correlations at δ 1.24/71.8 and δ 1.30/71.2 in HMBC (Fig. S3F, b), based on values reported in previous studies (Shakhmatov et al., 2018; Shakhmatov et al., 2019). Due to the relative low amounts of Rha*p* residues in AL-I-I, some proton signals were not able to detected. Regarding the signals of H/C-atoms at δ 5.09/104.3, and weak ones at δ 5.11/101.8 and δ 5.02/100.6 in HSQC, they belong to anomeric H/C atoms of 1,4-α-Gal*p*A (**$GA_{1,4}$**), 1,4-α-Gal*p*A-6-*O*-Me (**$GA_{1,4Me}$**) and 4-α-3-*O*-Ac-Gal*p*A (**$GA^{*}_{1,4}$**) respectively (Patova et al., 2019; Shakhmatov et al., 2019; Zou et al., 2020). Peaks in the downfield region in ^{13}C NMR at 173.8, 177.1 and 177.6 ppm should be assigned to C-6 of Gal*p*A. Other protons related to C-6 of Gal*p*A in HMBC were assigned to H-3/4/5 (Fig. 3B). The ROESY spectrum also shows cross peaks among H-1, H-2 of 1,2-linked Rha*p* and H-1 and H-3 of 1,4-linked Gal*p*A, indicating the presence of RG-I backbone moiety →4-α-Gal*p*A-(1,2)-α-Rha*p*-(1 → (Fig. S3E) (Shakhmatov et al., 2016). Besides the cross peak of residue ***O*-Ac** in HSQC, the presence of acetyl esterified Gal*p*A was evidenced by the carbon signal of carboxyl in acetyl units due to the cross peak at δ 2.09/176.3 in HMBC (Fig. 3B) (Patova et al., 2019). According to linkage analysis 1,3,4-linked Gal*p*A was found in AL-I-I (Table 3), which could indicate a substitution of an acetyl-group at *O*-3 of Gal*p*A (4-α-3-*O*-Ac-Gal*p*A). However, due to the relative low amount of 1,3,4-linked Gal*p*A, which would give the same PMAA fragments during permethylation as 4-α-3-*O*-Ac-Gal*p*A, the downfield shifts of proton H-3/C-3 was not detected (Kostálová et al., 2013). The existence of methyl esterified Gal*p*A (1,4-α-Gal*p*A-6-*O*-Me) was illustrated by cross peaks at δ 3.85/55.6 in the HSQC spectra (**O-Me**, Fig. 3A). However, the spin system reported for Gal*p*A methyl ester residues with downfield shifts of H-5 from about 4.7 to about 5.10 was not detected. But the shift of C-6 was observed at 173.8 ppm compared to those of non-esterified Gal*p*A at around 177 ppm, as well as correlation between *O*-Me and carboxyl group in HMBC at δ 3.85/173.8 (**H-O-Me/C6-$GA_{1,4Me}$**) (Fig. 3B) (Rosenbohm et al., 2003; Shakhmatov et al., 2016; Zou et al., 2020).

The position of the anomeric proton and carbon for terminal Xyl*p* (**TX-1**) was identified due to the signals at δ 3.37/105.8 (**H2/C1-TX**), δ 3.55/106.1 (**H3/C1-TX**) in HSQC (Fig. 3A) as earlier described (Patova et al., 2021), and strong correlations at δ 4.49/3.37 and δ 4.53/3.04 in COSY (Fig. S3C). The terminal Xyl could be attached to the HG region at position 3 of Gal*p*A (Patova et al., 2021; Wang et al., 2019) or to galactan domains at position 6 of Gal*p* (Zhang et al., 2019). Similarly, the assignment of methyl esterified Glc*p*A was deduced by spin systems at δ 3.49/62.7 (**O-Me′**) and δ 3.32/84.9 (**TGlcA-4**) in HSQC (Fig. 3A), residues at δ 3.32/178.01 (**H4/C6-TGlcA**), δ 3.69/178.1 (**H5/C6-TGlcA**), δ 3.49/84.9 (*O*-Me/C4-TGlcA, Fig. S3F, c) and δ 3.32/78.0 (H4/C3-TGlcA, Fig. S3F, c) in HMBC spectra (Fig. 3B), and proton-proton correlations in COSY (**H1/H2-TGlcA**), which were in agreement with values of chemical shifts published by Makarova et al. (2016) and Zhang, Li, et al. (2020), as terminal units of galactans or arabinogalactans.

The assignment of **AL-I-II** is easier than for AL-I-I as it consisted of more than 60 mol% of Gal*p*A. Briefly, C-1 and C-6 of α-Gal*p*A gave intense signals in anomeric regions in HSQC (such as residues **$GA_{1,4Me}$-1, $GA_{1,4}$-1** and **TGA-1** in Fig. 3C), and cross peaks in the anomeric (such as residues **H5/C1-$GA_{1,4}$** and **H4/C1-$GA_{1,4}$** in Fig. 3D) and downfield areas (such as residues **H1/C6-$GA_{1,4Me}$, H5/C6-$GA_{1,4Me}$** and **H5/C6-$GA_{1,4}$** in Fig. 3D) in HMBC. Most proton signals correlated with H-1 of Gal*p*A were appointed to H-2 by cross peaks in COSY (Fig. S2C), and their correlations to C-1 of Gal*p*A in HMBC (Fig. 3D). Carbon signals correlated to H-1 were assigned to C-2/3/4 of Gal*p*A (Fig. S4D, a). Some of the 1,4-α-Gal*p*A residues were *O*-6 methyl esterified. Because of the downfield shifts of H-5 from about 4.7 ppm to about 5.10 ppm and the

Table 4
^{1}H and ^{13}C NMR chemical shifts (ppm[a]) assignment of AL-I-I and AL-I-II.

Residues (Abb.)		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	O-Me/O-Me′/O-$\underline{C}H_3$	O-Ac/$\underline{C}H_3CO$ ($CH_3\underline{C}O$)	Ref.
AL-I-I										
α-Ara*f*-(1→	(**TA$_1$**)	5.24/112.3	4.19/84.5	3.98/79.1	4.14/86.8	3.71, 3.82/64.3				
α-Ara*f*-(1→	(**TA$_2$**)	5.42/111.2	4.19/84.5	3.98/79.1	4.14/86.8	3.82/63.7				
α-Ara*f*-(1 →	(**TA$_3$**)	5.14/110.1 5.18/110.1	4.14/84.6	3.93/79.8	4.04/87.0 4.10/87.0	3.78/63.9 3.71, 3.82/64.3				(Makarova et al., 2016) (Shakhmatov et al., 2015) (Zou et al., 2021)
→5)-α-Ara*f*-(1→	(**A$_{1,5}$**)	5.08/110.5	4.12/83.9	4.00/79.8	4.20/85.2	3.80, 3.88/69.9				
→3,5)-α-Ara*f*-(1→	(**A$_{1,3,5}$**)	5.10/110.5	4.14/n.d.	4.10/85.4	4.29/84.4	3.80, 3.88/69.4 3.83, 3.93/69.5				
→2)-α-Rha*p*-(1→	(**R$_{1,2}$**)	5.26/101.4	4.10/79.4	3.93/73.7	3.43/75.0	n.d./71.8	1.24/19.5			(Shakhmatov et al., 2018; Shakhmatov et al., 2019)
→2,4)-α-Rha*p*-(1→	(**R$_{1,2,4}$**)	n.d.	4.10/79.4	4.10/73.3	3.71/83.2	n.d./71.2	1.30/19.7			
β-Xyl*p*-(1→	(**TX**)	4.49/105.8 4.53/106.1	3.37/76.1 3.04/n.d.	3.55/78.0	3.66/72.9	3.26, 3.87/68.0				(Patova et al., 2021)
→3)-β-Gal*p*-(1→	(**G$_{1,3}$**)	4.69/106.5	3.79/73.2	3.87/84.6	4.21/71.3	n.d.	3.82/63.7			(Shakhmatov et al., 2015; Shakhmatov et al., 2018)
→3,6)-β-Gal*p*-(1→	(**G$_{1,3,6}$**)	4.49/106.3	3.73/73.4	3.79/83.4	4.12/71.34.10/71.4	3.92/76.5	3.92, 4.04/72.4			
→3,4,6)-β-Gal*p*-(1→	(**G$_{1,3,4,6}$**)	4.46/105.9	3.73/73.4	3.90/85.0	3.98/86.7	3.69/77.5 3.65/75.7	3.92, 4.04/72.4			(Zhang, Li, et al., 2020).
→4)-α-Gal*p*A-6-*O*-Me-(1→	(**GA$_{1,4Me}$**)	5.11/101.8	3.83/71.0	3.93/71.5	4.43/80.2	n.d.	173.8	3.85/55.6		(Patova et al., 2019; Shakhmatov et al., 2018)
→4)-α-Gal*p*A-(1→	(**GA$_{1,4}$**)	5.09/104.3	3.78/71.3	3.97/71.7	4.44/80.7	4.67/74.2	177.0 177.1			
→4)-α-3-*O*-Ac-Gal*p*A-(1→	(**GA*$_{1,4}$**)	5.02/100.6	n.d.	n.d.	4.44/80.7	4.72/74.4	177.6		2.09/23.2 2.17/23.3 (176.3)	(Patova et al., 2019)
β-Glc*p*A-4-*O*-Me-(1→	(**TGlcA**)	4.46/n.d.	3.37/n.d.	3.55/78.0	3.32/84.9	3.69/78.9	178.1	3.49/62.7		(Makarova et al., 2016)
AL-I-II										
α-Ara*f*-(1→	(**TA**)	5.08/110.2 5.24/111.9 5.43/110.9	4.19/84.2 4.12/83.7	4.01/80.8 4.02/81.0	4.13/86.5 4.10/86.6	3.71, 3.81/64.0				(Makarova et al., 2016)
α-Rha*p*-(1→	(**TR**)	4.93/101.6	3.91/71.9	3.70/71.1	3.44/74.8	3.90/71.9 n.d./71.6	1.29/19.4 1.24/19.2			(Makarova et al., 2016)
β-Xyl*p*-(1→	(**TX**)	4.55/107.5 n.d./107.7	3.27/76.1 3.04/76.3	3.38/78.6 3.43/78.4	3.61/71.8 3.73/72.9	3.26, 3.86/67.8 3.90/67.7				(Patova et al., 2021)
α-Gal*p*A-(1→	(**TGA**)	5.03/102.3	3.77/71.0	3.98/71.5	4.28/73.2	4.75/74.0	177.4			(Shakhmatov et al., 2018) (Patova et al., 2021)
→4)-α-Gal*p*A-6-*O*-Me-(1→	(**GA$_{1,4Me}$**)	4.90/102.4 5.10/101.8 5.16/102.1	3.77/71.0 3.77/70.9 4.00/n.d. 3.83/71.0	3.80/72.7 3.98/71.5 3.61/71.8 3.91/72.0	4.43/80.6 4.60/79.6	5.11/73.4 5.16/74.1	173.5	3.85/55.3 3.85/59.1		(Shakhmatov et al., 2016)
→4)-α-3-*O*-Ac-Gal*p*A-(1→	(**GA*$_{1,4}$**)	5.08/101.7	4.06/n.d.	5.17/74.4	4.58/81.9 4.43/80.6	4.79/74.0	177.4		2.08/22.9 2.16/23.2 2.14/22.9 (176.3)	(Patova et al., 2019)
	(**GA$_{1,4}$**)				4.43/80.6		177.4			(Patova et al., 2021)

(*continued on next page*)

Table 4 (*continued*)

Residues (Abb.)		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	O-Me/O-Me′/O-$\underline{C}H_3$	O-Ac/$\underline{C}H_3CO$ ($CH_3\underline{C}O$)	Ref.
→4)-α-Gal*p*A-(1→		5.08/101.7	3.77/71.0	3.91/72.0		4.79/74.0 4.75/74.0				
→4)-α-Gal*p*A	(**GA***)	5.31/94.8	3.83/71.0	3.98/71.5	4.46/80.9	4.43/73.2	n.d.			
	→4)-β-Gal*p*A	(**GA′**)	4.59/98.8	3.49/74.3 3.45/74.8	3.77/74.1 3.73/75.0	4.38/80.1	4.06/77.0 3.92/76.2	176.6		

[a] Values of the chemical shifts were determined from the HSQC spectra of each sample (solvent: D_2O). n.d., not detected.

shifted signal of C-6 at 173.8 ppm for Gal*p*A methyl ester residues (Rosenbohm et al., 2003; Shakhmatov et al., 2016), the →4)-α-Gal*p*A-6-*O*-Me-(1 → residue was further identified by cross peaks at δ 3.85/55.3, δ 3.85/59.1 (**O-Me**) and δ 5.11/73.4, 5.17/74.4 (**GA$_{1,4Me}$-5**) in HSQC, and δ 3.85/173.5 (**O-Me/C6-GA$_{1,4Me}$**), δ 3.77/173.5 (**H2/C6-GA$_{1,4Me}$**) and δ 5.10/173.5 (**H1/C6-GA$_{1,4Me}$**) in HMBC. Some of 1,4-α-Gal*p*A of AL-I-II were acetyl esterified at *O*-3 of Gal*p*A according to cross peaks at δ 2.08/22.9, δ 2.14/23.2 and δ 2.14/22.9 in HSQC (**O-Ac**, Fig. 3C), δ 2.08/176.3 in HMBC (**O-Ac/C6-GA***$_{1,4}$, Fig. 3D), as well as downfield shifts of H/C-3 at δ 5.17/74.4 (Table 3). This is equivalent to results of previous studies (Kostálová et al., 2013; Patova et al., 2019). Particularly, a 4 → β-Gal*p*A was found in AL-I-II, since cross peaks of H/C at δ 4.59/98.8 (**GA′-1**), δ 4.38/80.1 (**GA′-4**) and δ 3.49/74.4 ppm (**GA′-2**) in HSQC, δ 4.06/98.8 (**H5/C1-GA′**), δ 3.49/98.8 (**H2/C1-GA′**), δ 4.06/176.7 (**H5/C6-GA′**) in HMBC (Fig. 3D) and H1/H2 and H2/H3 correlations in COSY (Fig. S4C) were detected, which also has been shown in other studies (Patova et al., 2019; Patova et al., 2021; Zou et al., 2020). The β-linkage was detected in AL-I-II due to the high-resolution 800 MHz NMR instrument, and it might be the reason that this structure has not been highly mentioned in most papers related to pectins. The signals of terminal β-Xyl*p* were also found in AL-I-II by similar cross peaks as described above in AL-I-I. However, few signals of *O*-5-substituted Ara*f* and *O*-6-substituted Gal*p* were found due to the low amounts of these linkage types in AL-I-II (Table 4), which was why less —CH_2— signals at around 68–72 ppm were observed in the inserted plot in HSQC (Fig. 3C). In addition, the residues **TR-1**, **TR-2**, and **TR-4** in HSQC demonstrated the presence of terminal α-Rha*p*, as well as H/C cross peaks at δ 1.29/71.9, δ 1.29/74.8 and δ 1.24/71.6 in HMBC (Fig. S4D, b) and H/H cross peak at δ 1.29/3.90 in COSY spectra (not shown), as described in earlier published studies (Cui et al., 2007; Makarova et al., 2016). Likewise, the terminal α-Rha*p* residue might be located at the end of Glc*p*A, Gal*p*, or Ara*f* containing side chains, since around 3 mol% in total of all trace linkages belonging to Ara*f* and Gal*p* were measured in methylation analysis, such as 1,2-, 1,3-, 1,3,5-linked Ara*f* and 1,6-, 1,3,6- and 1,4,6-linked Gal*p*.

Thus, according to the aforementioned results and NMR elucidation, both AL-I-I and AL-I-II could be typical pectin polysaccharides with both methyl- and acetyl-esterified α-GalA units, as depicted in Fig. 4. According to the known structure of plant-derived pectic polysaccharides (Kaczmarska et al., 2022; Zaitseva et al., 2020) and the results of glycosidic linkages and NMR analysis above, AL-I-I was probably mainly composed of AG-II and arabinan as side chains of a RG-I core chain besides a HG backbone. The correlations in NMR were however too weak to indicate how the side chains were connected to the RG-I core and HG backbone. AL-I-II consisted of a longer HG backbone with substituents at α-3-*O*-Gal*p*A.

So far, no structural characterization of pectins in any plant part of *A. carmichaelii* has been reported, besides the description of a possible

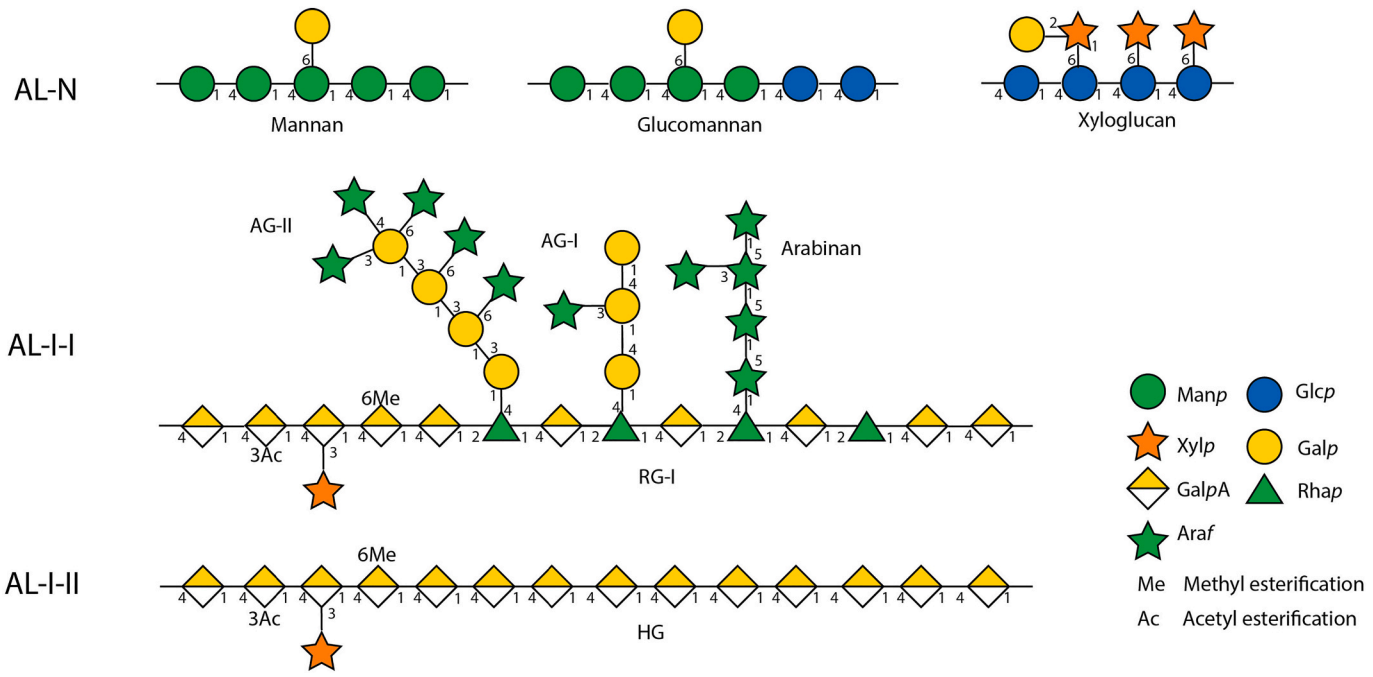

Fig. 4. Proposed structures of polysaccharides from *A. carmichaelii* leaves. HG, homogalacturonan; RG-I, type I rhamnogalacturonan; AG-II, type II arabinogalactan; AG-I, type I arabinogalactan. Graphical symbols are depicted according to the symbol nomenclature for glycans (SNFG) (Varki et al., 2015).

pectin by Gao, Bia, et al. (2010) due to a detectable amount of GalA in polysaccharides from the roots. In other *Aconitum* plants, various types of polysaccharides have been identified in the roots of *A. coreanum*, including a type II rhamnogalacturonan (RG-II) polysaccharide (Li, Jiang, Shi, Bligh, et al., 2014), an arabinoglucan (Song et al., 2020), glucans (Gao, Bi, et al., 2010; Li, Jiang, Shi, Su, et al., 2014; Zhao et al., 2006), and glucomannan (Zhang et al., 2017). Comparatively, this study could be the first one giving clear evidence of the presence of pectin with HG backbone and RG-I domains in *Aconitum* plants. The roots and other plant parts of *A. carmichaelii* will be further explored for the presence of bioactive polysaccharides.

3.5. Complement fixation of polysaccharides from A. carmichaelii leaves

The complement fixation assay has been shown to be a good indicator for effects in the immune system by plant polysaccharides (Inngjerdingen et al., 2012; Zaitseva et al., 2020). As can be seen from Table 5, all isolated polysaccharide fractions from *A. carmichaelii* leaves except AL-I-II showed strong human complement fixating activities *in vitro*, and have higher activities than the positive control BP-II. The acidic fraction AL-I and one of its purified fractions, AL-I-I, were shown to be more potent than the neutral fraction AL-N ($p < 0.05$).

Complement fixating activity observed in the hemolysis assay could include activation and/or inhibition of the complement system, and these modulatory effects are related to structural difference of polysaccharides (Yamada & Kiyohara, 2007). Pectins with high *Mw* tend to be more active in the complement fixating assay (Togola et al., 2008; Zou et al., 2017). AL-I-I with a *Mw* of 169.1 kDa (Table 1) was shown to be more active than AL-I-II, which had a 4-fold lower *Mw* (41.6 kDa) and was found to be inactive. AL-N with an even lower *Mw*, on the other hand, did not follow this trend and was determined to be effective in complement fixation. This is most likely due to the various types of monosaccharide linkages. Effects of glucomannans on the complement system have not been much studied previously, but have shown to be inactive, except for highly heterogenous glucomannans mostly composed of 1,4-linked Glc, in addition to 1,3-linked Gal, 1,3-linked Fuc, 1,3-linked Man, and 1,3- or 1,6-linked Glc (Yamada & Kiyohara, 1999). As shown in Tables 2, 22.8 mol% of 1,4-linkded Glc, and minor amounts of 1,3-linked Gal (2.4 mol%) and 1,3-linked Glc (2.3 mol%) were all detected in AL-N. A comparable neutral polysaccharide primarily composed of Glc and Man from the African mushroom *Podaxon aegyptiacus* was reported with efficacy in the complement fixation assay as well (Diallo et al., 2002). In addition, the RG-I region in pectin has been reported to have high complement fixating activities, whereas the oligogalacturonides (HG domain) have weaker or negligible activities. Most arabinogalactans acting on the complement system are characterized as AG-II (Ferreira et al., 2015; Yamada & Kiyohara, 2007). These structure-activity relationships consequently explain the strongest complement fixating effect of AL-I-I among these fractions. Furthermore, an α-3,5-arabinofuranan have also demonstrated moderate complementary fixation in earlier studies (Yamada & Kiyohara, 2007), which is consistent with the current results that the active AL-I-I contains 1 mol% more 1,3,5-linked Ara units than the inactive AL-I-II, and partially explains the activity of AL-N which contained 2.6 mol% of 1,3,5-linked Ara units. Thus, AL-N and the branched pectic polysaccharide AL-I-I from *A. carmichaelii* leaves were found to have complement fixating activities and might be potential immunomodulatory substances.

Table 5
The inhibition of serum-induced hemolysis of sheep erythrocytes by polysaccharides from *Aconitum carmichaelii* leaves.

Sample name	ICH_{50} μg/mL
AL-N	18.3 ± 9.0^{b}
AL-I	8.1 ± 0.7^{a}
AL-I-I	6.6 ± 1.7^{a}
AL-I-II	>500
BP-II (positive control)	50.8 ± 3.6^{c}

Note: The different superscripted letters mean the statistical differences with $p < 0.05$ after Duncan's test.

3.6. Anti-inflammatory effects of polysaccharides from A. carmichaelii leaves on LPS-treated IPEC-J2 cells

All samples including AL-N, AL-I, AL-I-I and AL-I-II were tested for anti-inflammatory activities. As shown in Fig. 5A, cell viability of IPEC-J2 cells was not affected by 20 μg/mL of LPS treatment, but an inflammatory injury was caused by LPS according to the statistical upregulation of mRNA transcription of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Fig. 5B, $p < 0.05$). Cell viability of cells co-cultivated with AL-N, AL-I-I or AL-I-II were shown to increase significantly ($p < 0.001$) compared with untreated cells (negative control), as shown in Fig. 5A. It was manifested that the possible glucomannan and pectic polysaccharides had no cytotoxic effect on IPEC-J2 cells, and could affect the proliferation of intestinal epithelial cells, as previously concluded by Huang et al. (2017). All polysaccharide fractions at a final concentration of 20 μg/mL were shown to inhibit the LPS-promoted gene expression of pro-inflammatory cytokines on IPEC-J2 cells at transcription level, including IL-1β, IL-6, and TNF-α (Fig. 5B). There was no statistically difference among the different fractions except that AL-N exerted a more potent effect in the inhibition of gene expression of IL-1β. AL-N is the first reported polysaccharide mainly consisting of mannans in *A. carmichaelii*. Its anti-inflammatory activity might be achieved through a direct contact with a cell surface mannose receptor, or mannose-binding lectins to prompt inflammatory response through cytokine expressions, as has been illustrated for most natural mannans (Tiwari et al., 2020). However, the rather low yield of AL-N compared to the high yield of AL-I resulted in the end of further in-depth biological studies of AL-N. Consequently, AL-I, and its purified fractions, AL-I-I and AL-I-II, were chosen as the main substances for further intestinal anti-inflammatory studies, which is also conductive to understand their effects in a microbiota-independent way.

As exhibited in Fig. 5C to F, the inflammatory injury caused by LPS was finally mitigated by all pectic polysaccharides in a light dose-dependent manner, by down-regulating mRNA transcriptions of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IL-18 ($p < 0.001$). AL-I led to a decrease in expression of IL-1β and IL-6, and a moderate suppression of the relative expressions of TNF-α and IL-18. The purified fractions AL-I-I and AL-I-II acted effectively on the inhibition of all inflammatory markers ($p < 0.05$), and no significant difference between AL-I-I and AL-I-II ($p > 0.05$) was observed, except a considerably higher efficacy of AL-I-II in reducing IL-6 expression ($p < 0.05$, Fig. 5D). The involvement of inflammatory pattern recognition receptors (PRR) was studied in order to further investigate the underlying mechanism of their anti-inflammatory effects. mRNA expressions of nucleotide-binding and oligomerization domain (NOD)-like receptor 1 (NOD1), NOD2, and TLR4 were upregulated by LPS, as shown in Fig. 5G, H, I. They were attenuated in all treated groups except NOD2, and a significant improvement was manifested in cells treated with AL-I-II compared with AL-I or AL-I-I ($p < 0.05$). Hence, the current study suggest that the pectic polysaccharides from *A. carmichaelii* leaves, AL-I-I and AL-I-II, possess promising anti-inflammatory activities on intestinal epithelial cells by inhibiting the expression of NOD1 and TLR4, but not by regulating NOD2. Further studies would be needed to determine how these pectic polysaccharides control the downstream proteins in TLR4 and NOD1 signal pathways using western-blot, and cells with depletion or silencing of TLR4 or NOD1would be included to confirm the regulatory effects at the same time. It is also of interest to investigate how AL-I-II exerted anti-inflammatory effects and through which receptor it works. The current results uncovered a promising medicinal use of these leaves in the treatment of intestinal inflammatory disease.

Similar effects of pectin consisting of a HG backbone with various

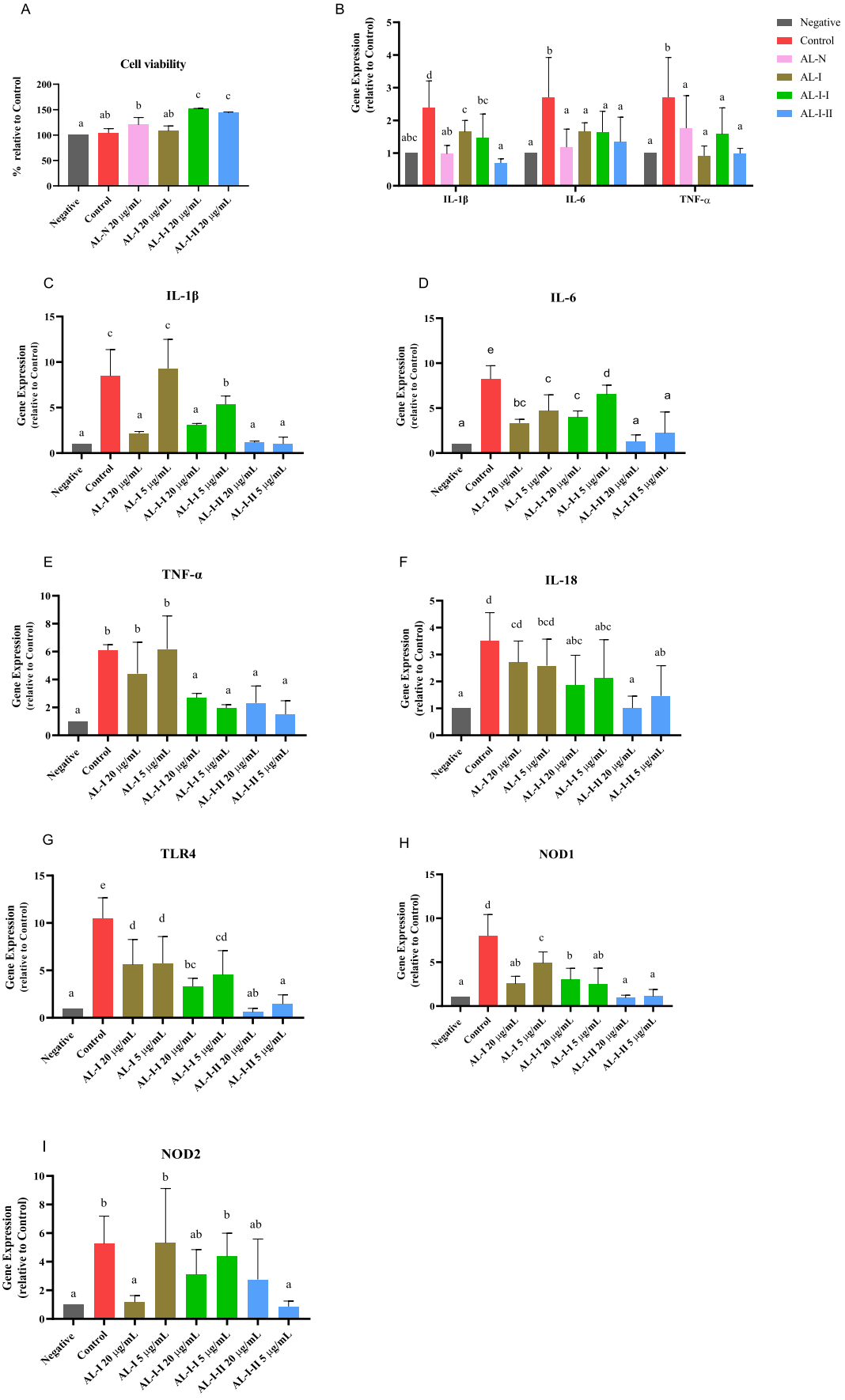

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Fig. 5. Cell viability and anti-inflammatory effects of polysaccharides from *A. carmichaelii* leaves on IPEC-J2 cells. Cells were pre-treated with LPS for 12 h and then supplemented with samples at the final concentration of 20 and 5 μg/mL for a further 12 h. (A) Cell viability of cells after 12 h of co-culture determined by CCK-8. (B) Relative mRNA expressions of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α after the treatment of AL-N, AL-I-I and AL-I-II at final concentration of 20 μg/mL quantified by qRT-PCR. Relative mRNA of pro-inflammatory cytokines IL-1β (C), IL-6 (D), TNF-α (E), and IL-18 (F), as well as inflammation-related receptors TLR4 (G), NOD1 (H), and NOD2 (I) were quantified by qRT-PCR. All values are presented as the means ± SD (n = 3). The different lowercase letters (a, b, c and d) labeled above the column indicate that the mean values are significantly different among groups in each plot ($p < 0.05$) according to the Duncan's multiple range test, but those columns labeled with the same lowercase letter are not ($p > 0.05$).

amounts of RG-I core chain, and neutral side chains have been reported previously (Wu et al., 2021; Zou et al., 2020; Zou et al., 2021). Specific relationships between pectin structures and immune responses on dendritic or macrophage cells have been demonstrated, and the degree of methylation, acetylation, RG-I, and RG-II of pectin are all crucial for anti-inflammatory properties on the immune barrier *via* immune cells, mucus layer, or PRRs (Beukema et al., 2020; Wu et al., 2021; Yang et al., 2022). Moreover, pectin has been highly reported to act indirectly on the intestinal immune system after being fermented in the colon, and chemical differences, like the degree of methylation, acetylation, and branch conditions would affect their activities (Wu et al., 2021). However, *in vitro* assays to determine the direct impact of pectin on intestinal epithelium in spite of bacteria, are not extensively studied, as well as the corresponding structure-activity relations. In the current study, both AL-I-I and AL-I-II performed similarly in most of the inhibitory effects of intestinal inflammation, but AL-I-II containing a longer HG backbone, β-Gal*p*A (Shen et al., 2021; Zhang, Li, et al., 2020), and terminal Rha*p* regions, was more potent. It is unclear whether these structural domains are dominant on anti-inflammatory effects compared to typical pectin with 1,4-linked α-Gal*p*A and RG-I domains. A further comprehensive evaluation of pectin with unexplored regions is still required. Moreover, the structure-activity relationship of different polysaccharides varies with the biological evaluation system. In the complement fixation assay, the linear AL-I-II with minor amounts of side chains were shown to be inactive, whereas it had potent anti-inflammatory activities. More comparative studies on the bioactivities of polysaccharides on multiple evaluations systems are needed to expand the structure-activity relationships of natural polysaccharides.

4. Conclusion

In the current study, a neutral polysaccharide fraction, AL-N, and two purified acidic polysaccharides fractions, AL-I-I and AL-I-II, were isolated and characterized. AL-N is possibly a fraction of heteromannans, mainly consisting of 1,4-linked Man*p* and 1,4-linked Glc*p*, in addition to a xyloglucan. AL-I-I and AL-I-II are pectic polysaccharides. AL-I-I is highly branched with RG-I regions containing arabinans, AG-II, and minor amounts of AG-I side chains, while, AL-I-II contains predominantly a linear HG backbone, with few side chains. All neutral and pectic polysaccharides from *A. carmichaelii* leaves were shown to have potent complement fixating activity. They also exerted intestinal anti-inflammatory effects on IPEC-J2 cells, and the high-yield pectins AL-I-I and AL-I-II were further shown to act through inhibiting expression of TLR4 and NOD1. This study presents a comprehensive chemical characterization of polysaccharides from *A. carmichaelii* leaves, and unravels their promising medicinal use as natural immunomodulatory and anti-inflammatory substances. Further, the use of this plant part would lead to less waste of biomaterial in the industrial processing of *A. carmichaelii* roots.

CRediT authorship contribution statement

Yu-Ping Fu: Data curation, Investigation, Methodology, Visualization, Roles/Writing - original draft. **Cen-Yu Li**: Data curation, Investigation, Methodology, Visualization. **Xi-Peng**: Data curation, Software. **Yuan-Feng Zou**: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **Frode Rise**: Funding acquisition, Methodology. **Berit Smestad Paulsen**: Project administration, Writing - review & editing. **Helle Wangensteen**: Project administration, Supervision, Writing - review & editing. **Kari Tvete Inngjerdingen**: Methodology, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119655.

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Supplementary data for

"Polysaccharides from *Aconitum carmichaelii* leaves: structure, immunomodulatory and anti-inflammatory activities"

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Supplementary Tables

Table S1. Primer sequences for qRT-PCR

Gene	Primer sequence	NCBI no.	bp
IL-6	F: TAACCCCACCACAAATGCC R: CCCCAGTACATTATCCGAA	NM_001252429.1	70
IL-1β	F: ATGGCTAACTACGGTGACAAC R:CCATCAGCCTCAAATAACAGGT	NM_214055.1	65
TNF-α	F: TTGAGCATCAACCCTCTGGC R: GGCATACCCACTCTGCCATT	NM_001124357.1	134
IL-18	F:TGAACCGGAAGACAATTGCAT R:TGCCAAAGTAATCCGATTCCA	NM_213997.1	91
TLR4	F: CACTTTATTCAGAGCCGTTG R: CCTTAGCTGATTTGGTCGAA	NM_001113039.2	115
NOD1	F: ACCAGCAGTCCTACGAG R: CACTGTCATCCACCACGAG	NM_001114277.1	87
NOD2	F: CCTCCTTCCTTGCTCCC R: GGCCATCAAATGTTCCCT	NM_001105295.1	66
β-actin	F: GATGAGATTGGCATGGCTTT R: CACCTTCACCGTTCCAGTTT	XM_003124280	122

Supplementary Figures

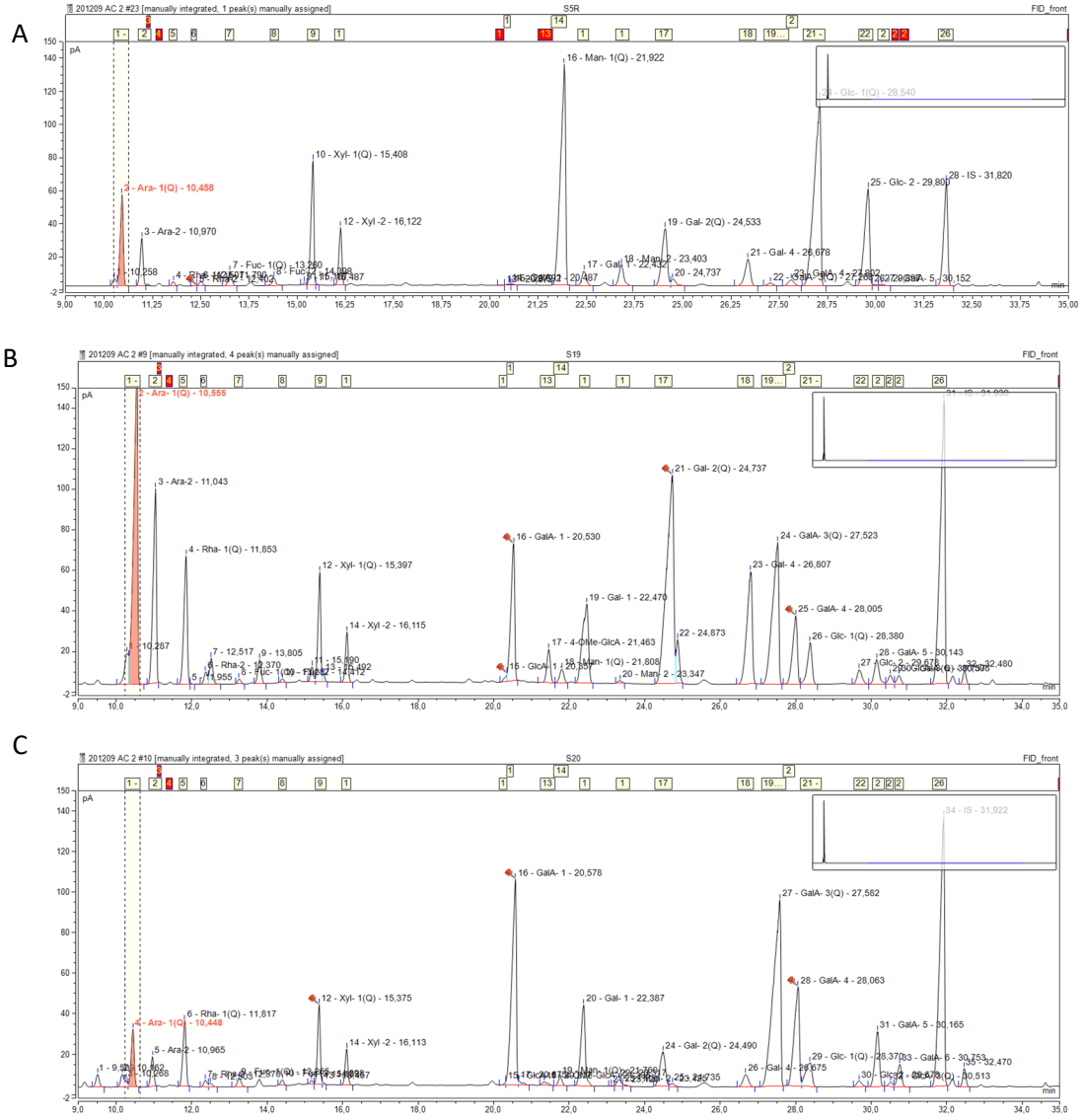
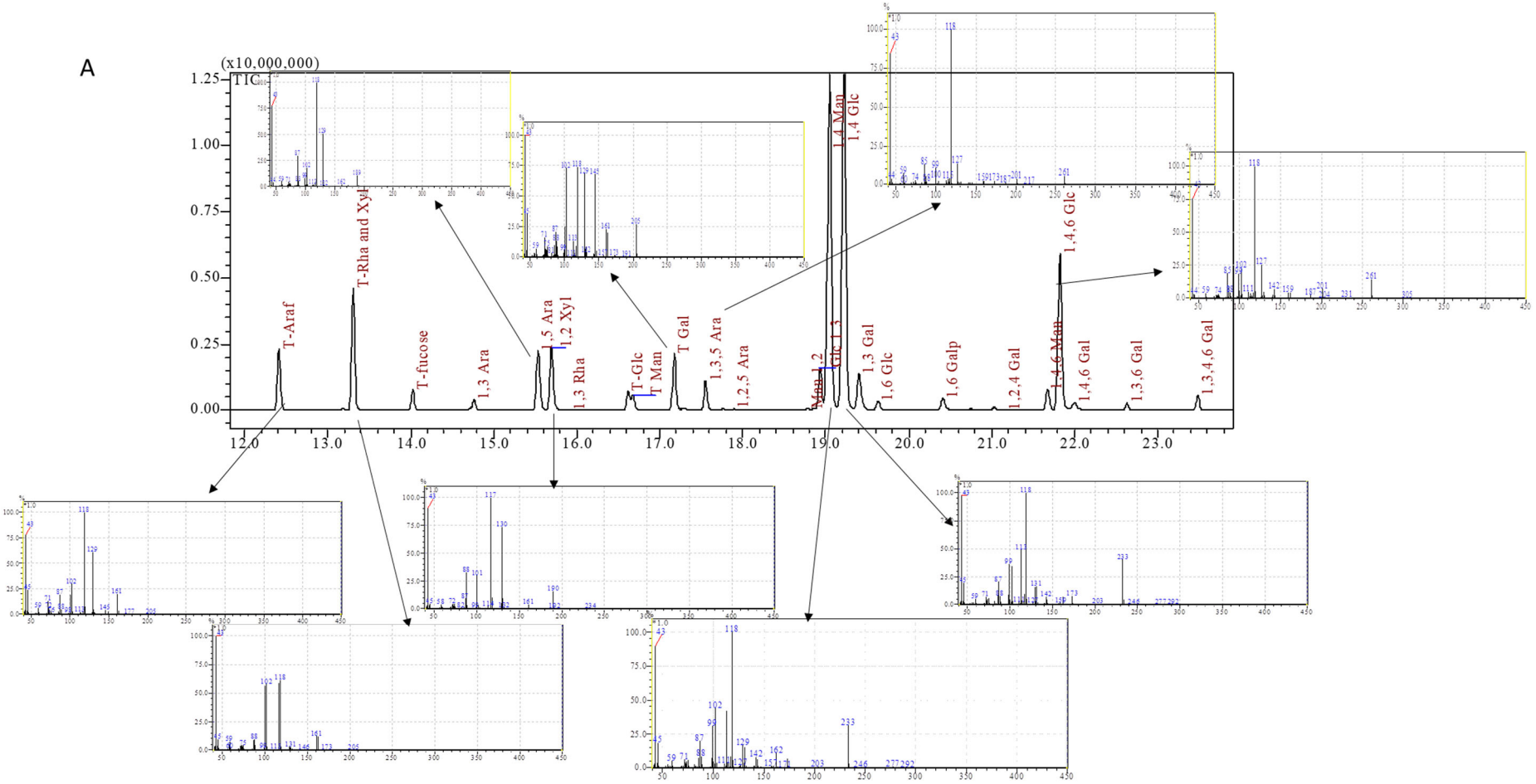


Fig. S1. GC chromatograms of monosaccharide composition of AL-N (A), AL-I-I (B) and AL-I-II (C).


A
(x10,000,000)
TIC
1.25
1.00
0.75
0.50
0.25
0.00
12.0
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
23.0
T-Araf
T-Rha and Xyl
T-fucose
1,3 Ara
1,5 Ara
1,2 Xyl
1,3 Rha
T-Glc
T Man
T Gal
1,3,5 Ara
1,2,5 Ara
Man 1,2
Glc 1,3
1,4 Man
1,4 Glc
1,3 Gal
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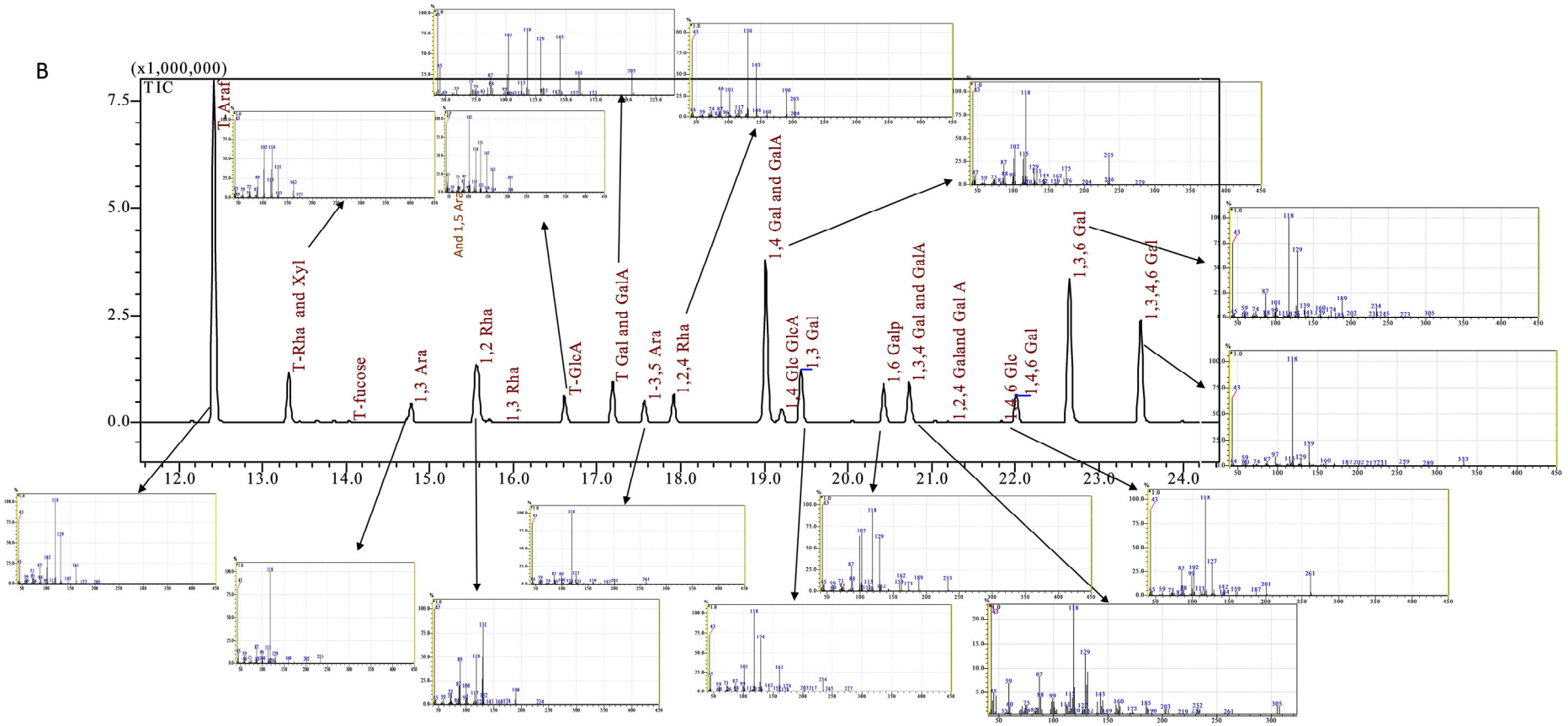
1,3,6 Gal
1,3,4,6 Gal


B
(x1,000,000)
TIC
7.5
5.0
2.5
0.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
23.0
24.0
T-Araf
T-Rha and Xyl
T-fucose
1,3 Ara
1,2 Rha
1,3 Rha
T-GlcA
T Gal and GalA
1-3,5 Ara
1,2,4 Rha
1,4 Gal and GalA
1,4 Glc GlcA
1,3 Gal
1,6 Galp
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1,3,4,6 Gal

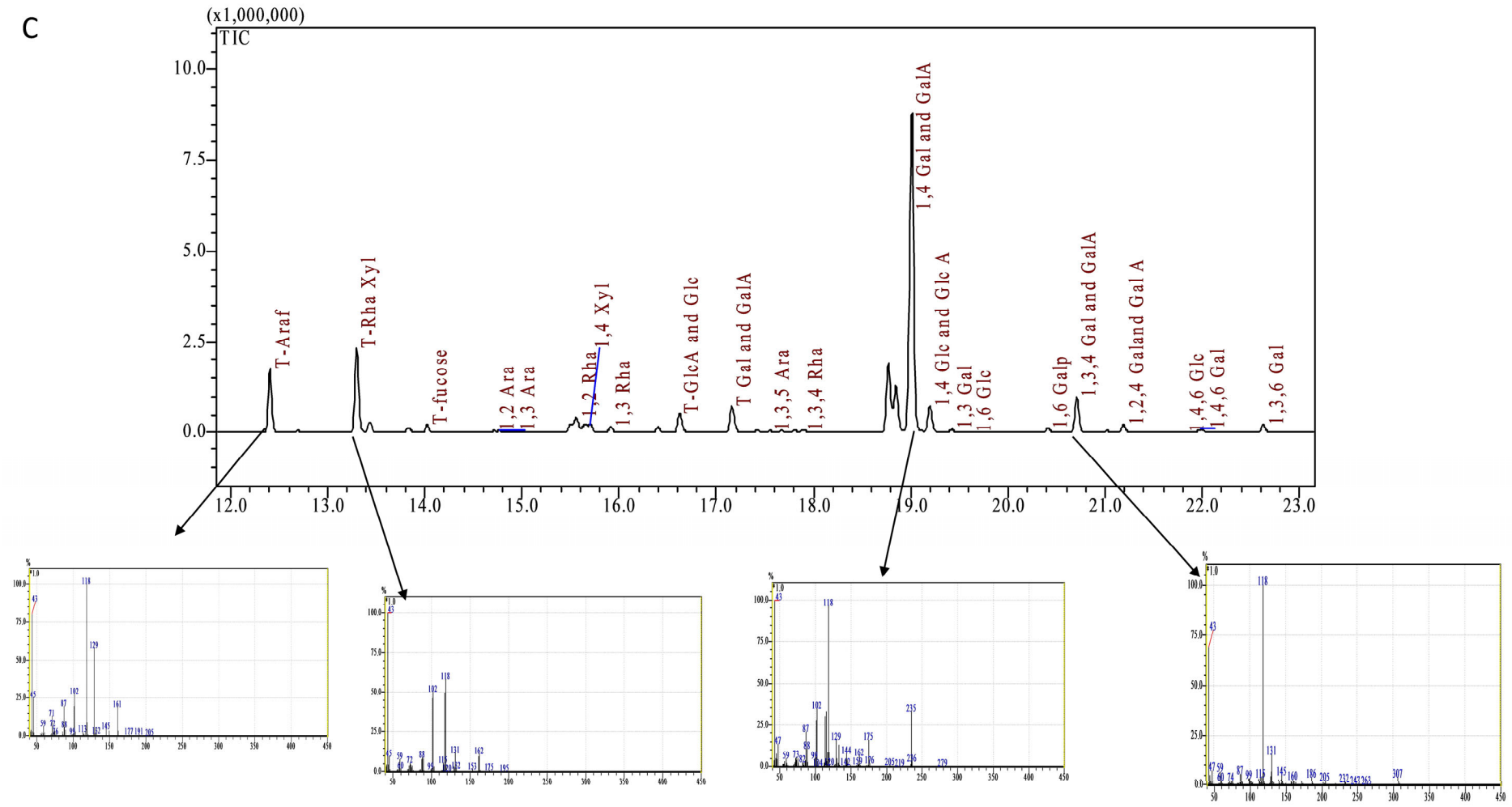
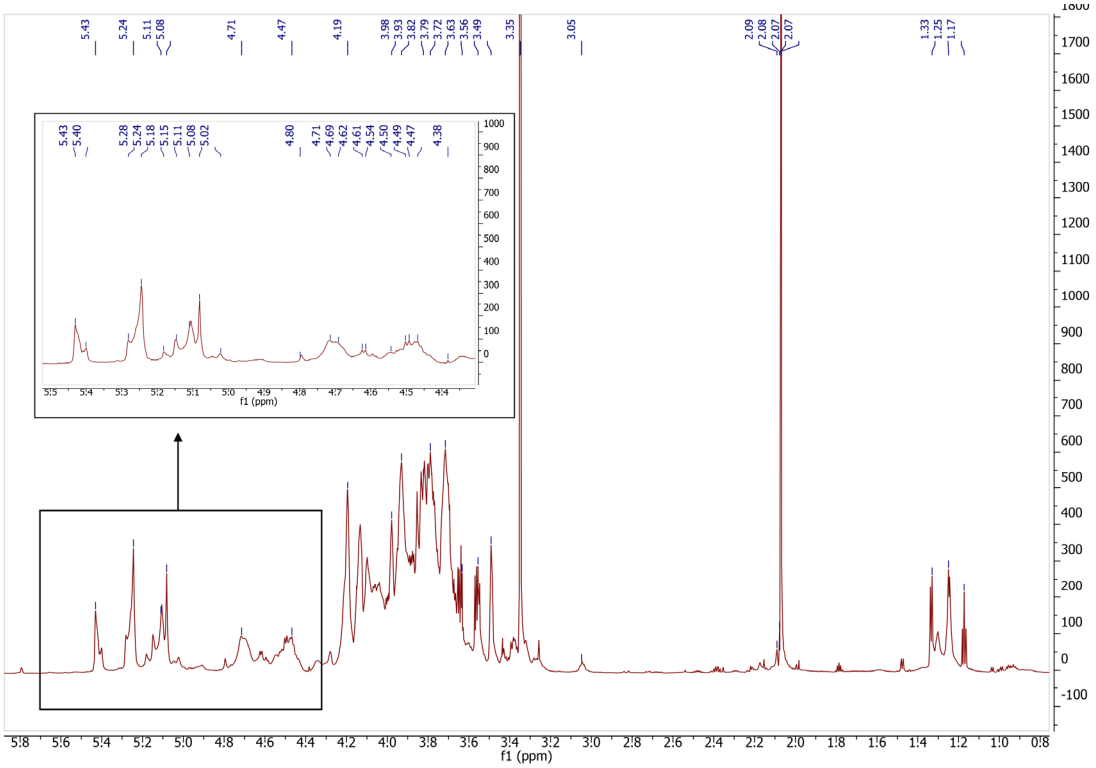
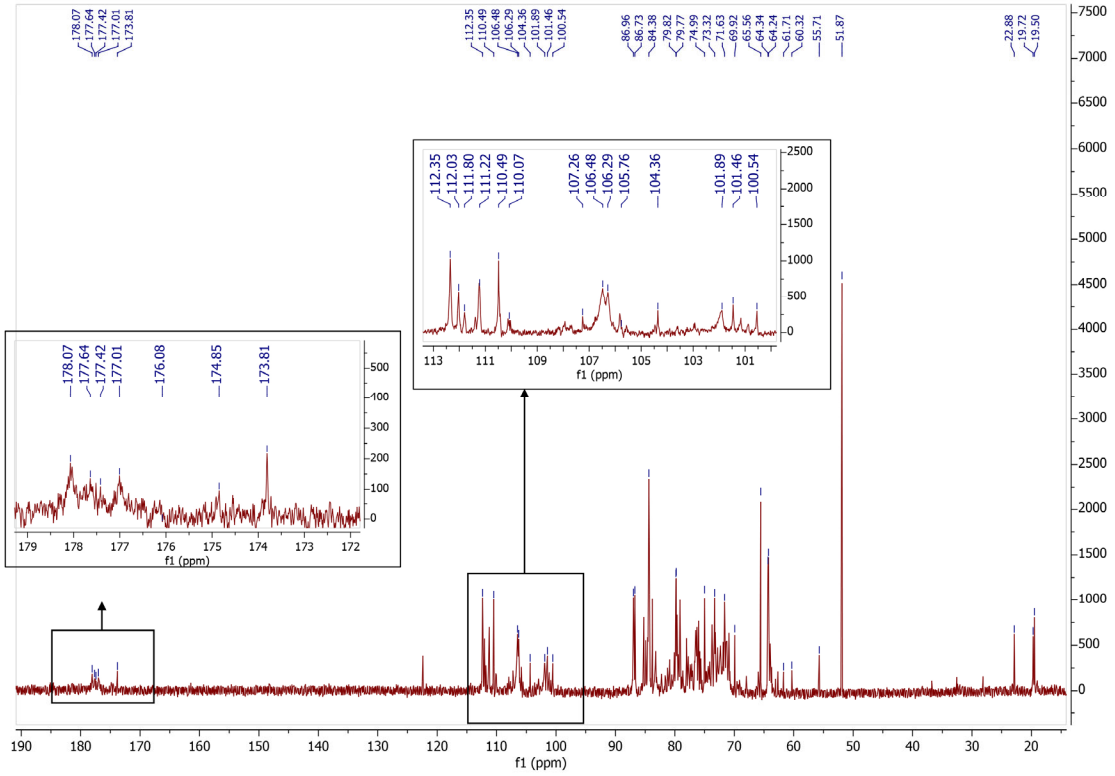


Fig. S2. GC chromatograms and MS spectra of glycosidic linkage types of AL-N (A), AL-I-I (B) and AL-I-II (C).

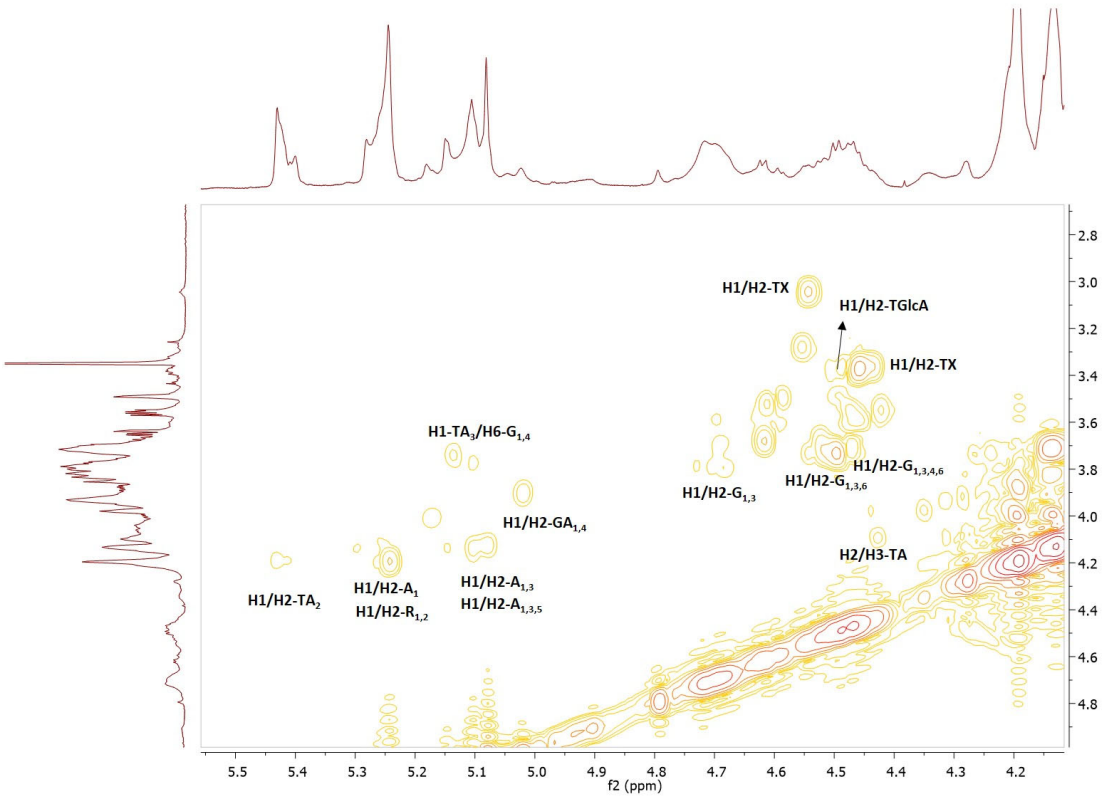
A


5.43
5.24
5.11
5.08
4.71
4.47
4.19
3.98
3.93
3.82
3.79
3.72
3.63
3.56
3.49
3.35
3.05
2.09
2.08
2.07
2.07
1.33
1.25
1.17
f1 (ppm)

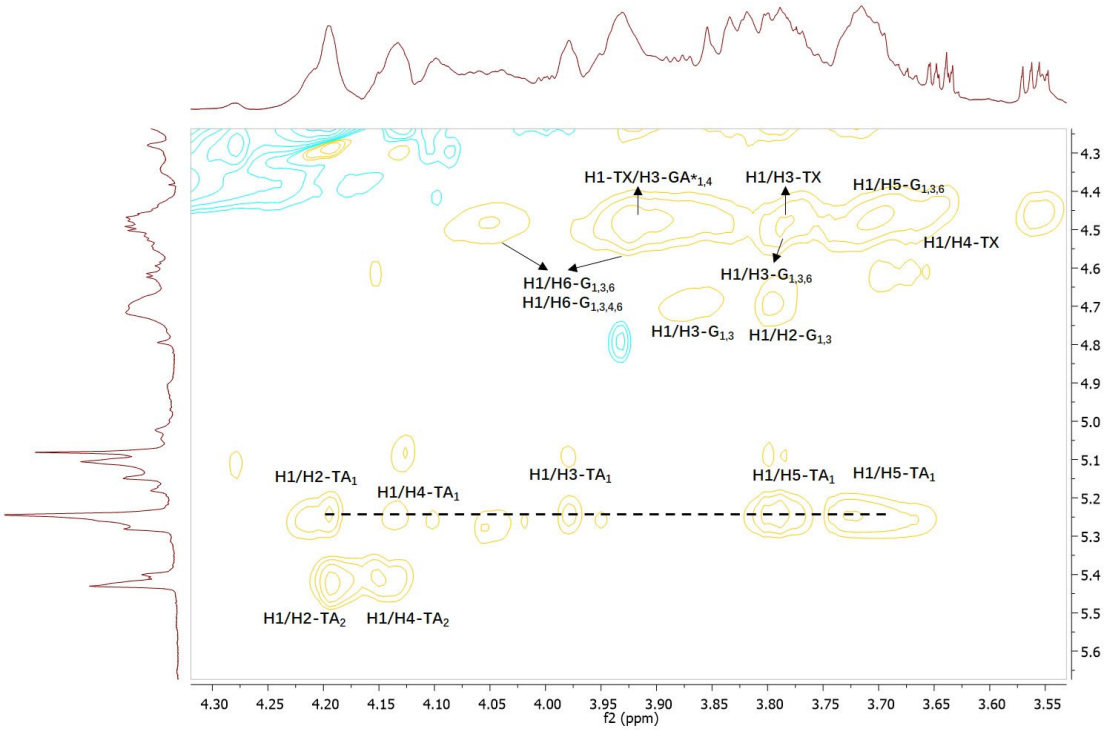
B


178.07
177.64
177.42
177.01
173.81
112.35
110.49
106.48
106.29
104.36
101.89
101.46
100.54
86.96
86.73
84.38
79.82
79.77
74.99
73.32
71.63
69.92
65.56
64.34
64.24
61.71
60.32
55.71
51.87
22.88
19.72
19.50
f1 (ppm)

C


H1/H2-TX
H1/H2-TGlcA
H1/H2-TX
H1-TA3/H6-G1,4
H1/H2-G1,3,4,6
H1/H2-G1,3,6
H1/H2-G1,3
H1/H2-GA1,4
H2/H3-TA
H1/H2-TA2
H1/H2-A1
H1/H2-R1,2
H1/H2-A1,3
H1/H2-A1,3,5
f2 (ppm)

D


H1-TX/H3-GA*1,4
H1/H3-TX
H1/H5-G1,3,6
H1/H4-TX
H1/H3-G1,3,6
H1/H6-G1,3,6
H1/H6-G1,3,4,6
H1/H3-G1,3
H1/H2-G1,3
H1/H2-TA1
H1/H4-TA1
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H1/H5-TA1
H1/H2-TA2
H1/H4-TA2
f2 (ppm)

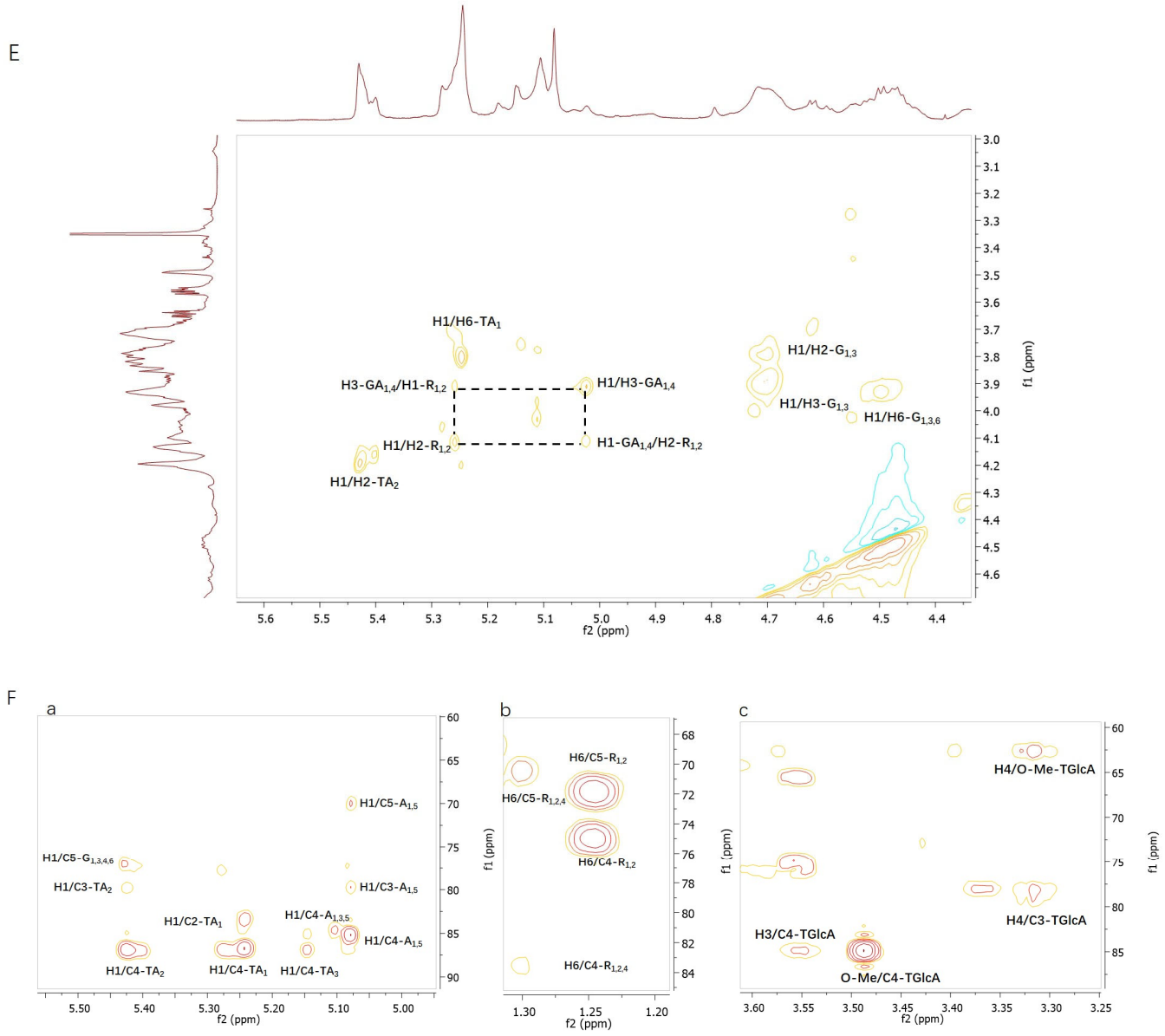
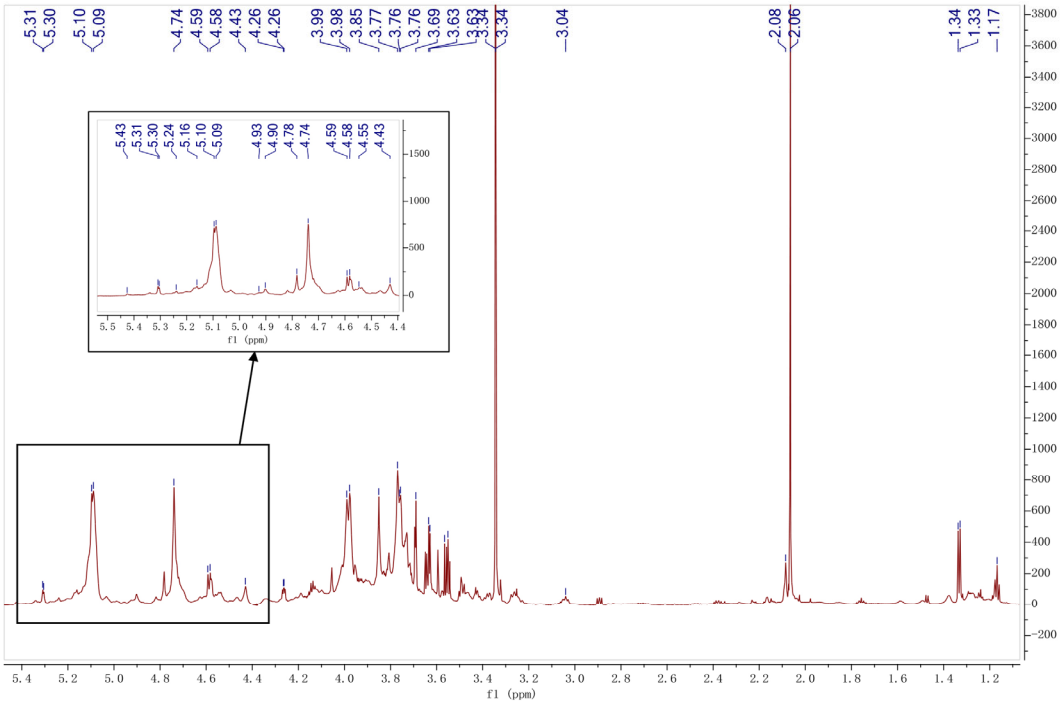


Fig. S3. ^{1}H-NMR (A), ^{13}C-NMR (B), COSY (C), ROESY (D), NOESY (E) and partial HMBC (F) spectra of AL-I-I.

A


5.31
5.30
5.10
5.09
4.74
4.59
4.58
4.43
4.26
4.26
3.99
3.98
3.85
3.77
3.76
3.76
3.69
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3.04
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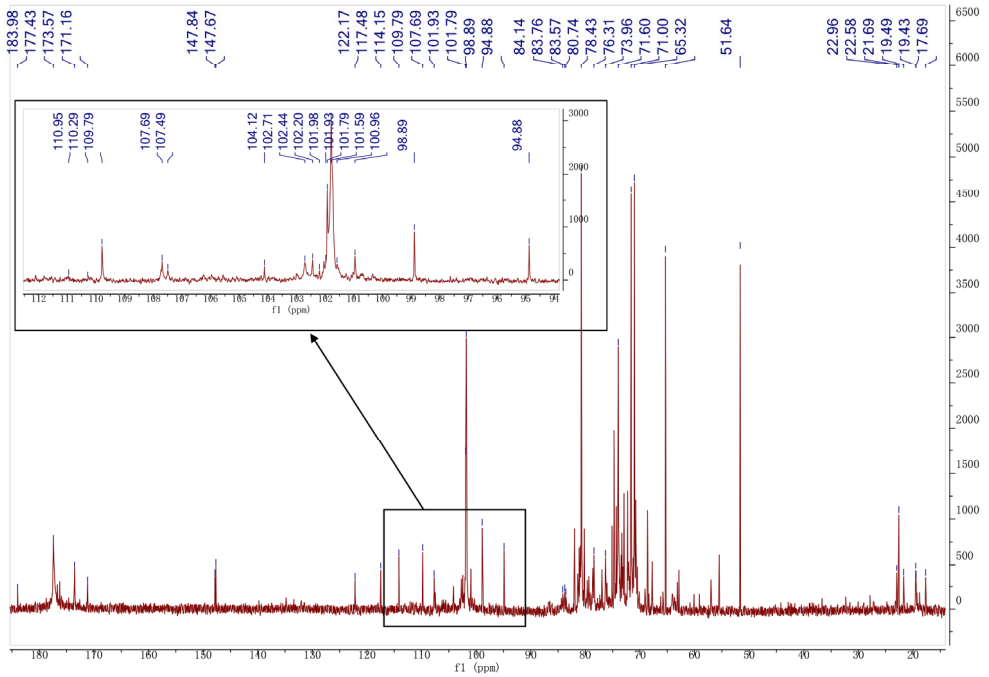
2.06
1.34
1.33
1.17
f1 (ppm)

B


183.98
177.43
173.57
171.16
147.84
147.67
122.17
117.48
114.15
109.79
107.69
101.93
101.79
98.89
94.88
84.14
83.76
83.57
80.74
78.43
76.31
73.96
71.60
71.00
65.32
51.64
22.96
22.58
21.69
19.49
19.43
17.69
f1 (ppm)

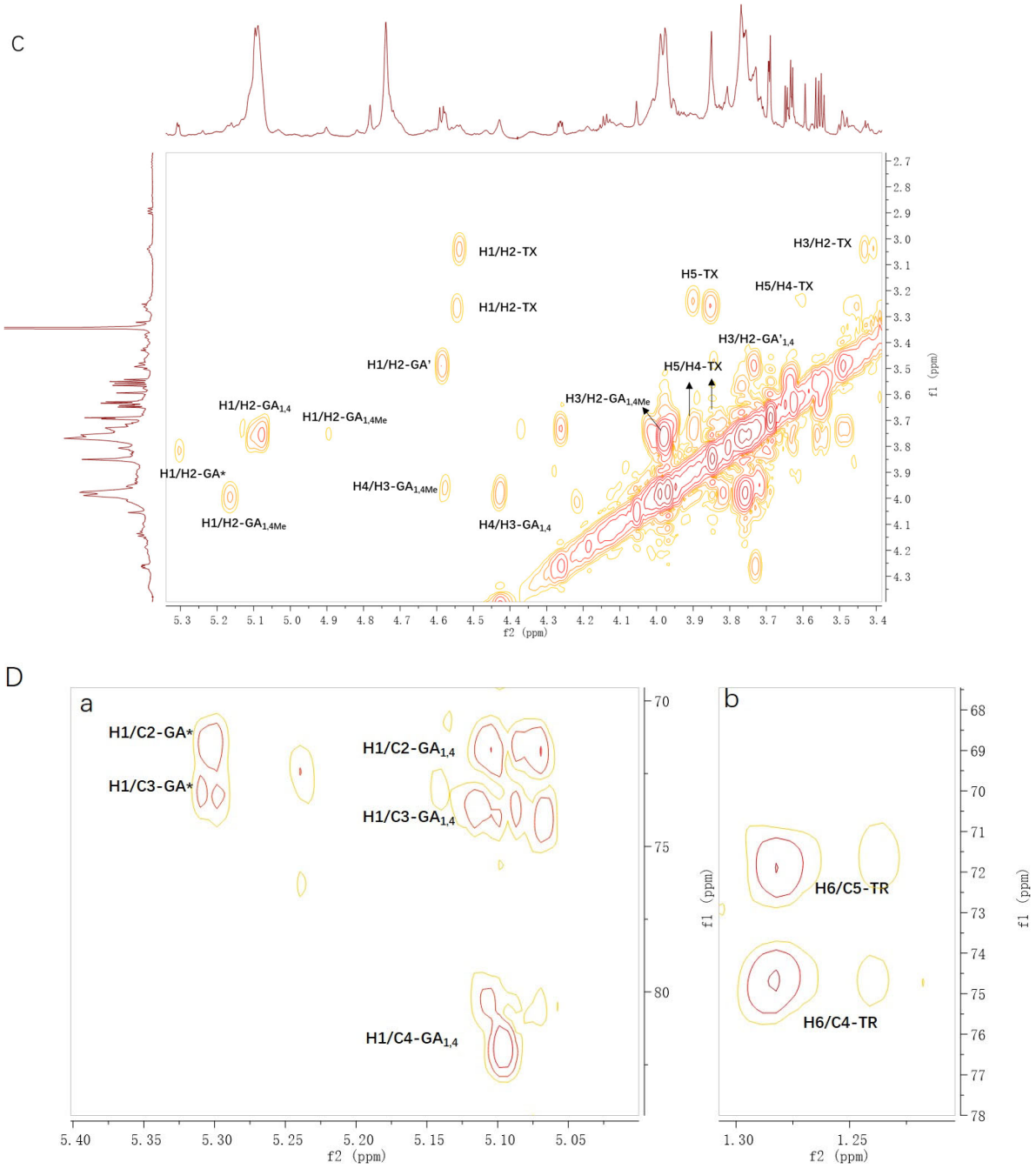


Fig. S4. ^{1}H-NMR (A), ^{13}C-NMR (B), COSY (C) and partial HMBC (D) spectra of AL-I-II

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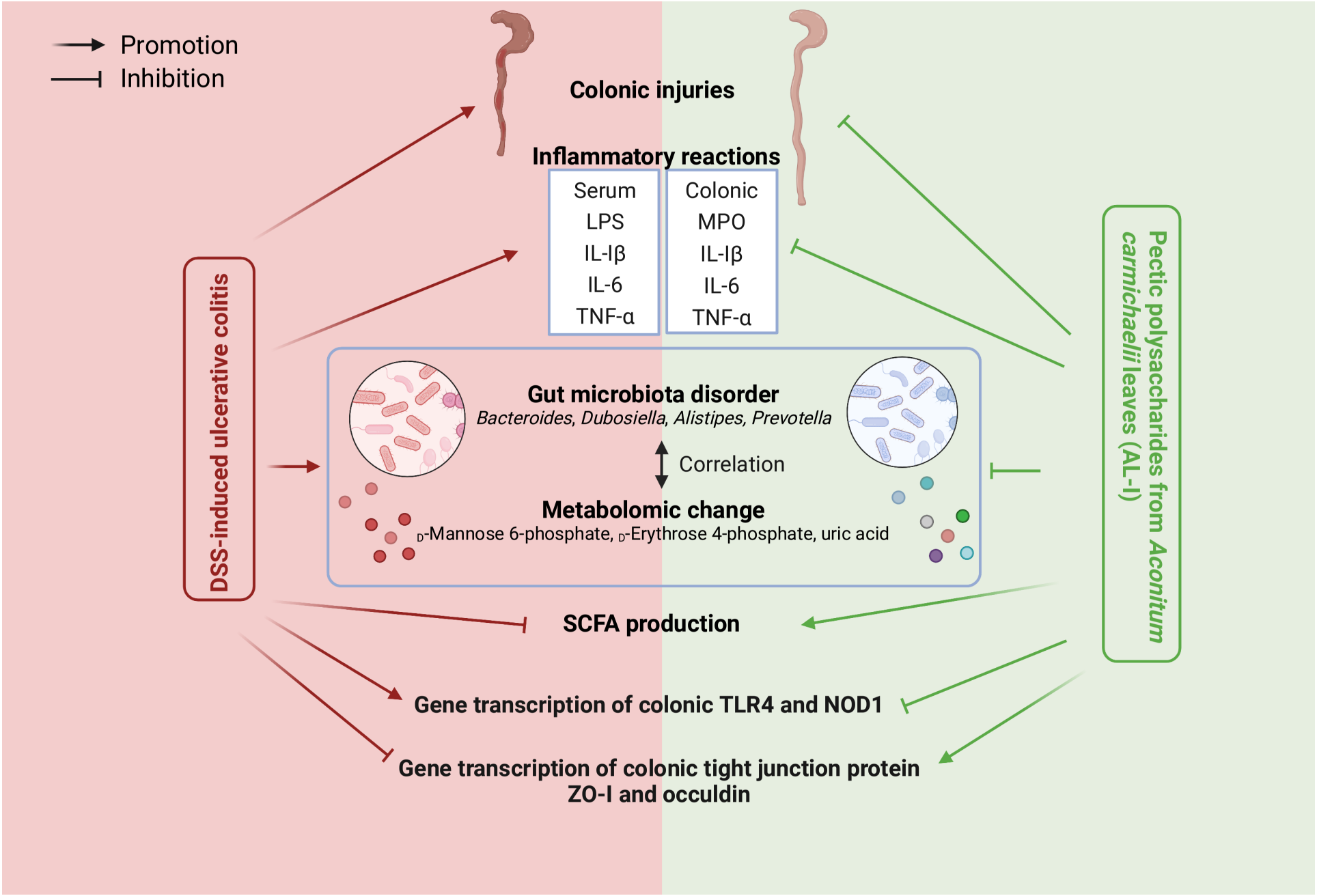
Promotion
Inhibition
Colonic injuries
Inflammatory reactions
Serum
LPS
IL-Iβ
IL-6
TNF-α
Colonic
MPO
IL-Iβ
IL-6
TNF-α
DSS-induced ulcerative colitis
Gut microbiota disorder
Bacteroides, Dubosiella, Alistipes, Prevotella
Correlation
Metabolomic change
D-Mannose 6-phosphate, D-Erythrose 4-phosphate, uric acid
Pectic polysaccharides from Aconitum carmichaelii leaves (AL-I)
SCFA production
Gene transcription of colonic TLR4 and NOD1
Gene transcription of colonic tight junction protein ZO-I and occuldin

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Pectic polysaccharides from *Aconitum carmichaelii* leaves protects against DSS-induced ulcerative colitis in mice through modulations of metabolism and microbiota composition

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Keywords:
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Pectic polysaccharide
Ulcerative colitis
Metabolomics
Gut microbiota

ABSTRACT

The industrial processing of *Aconitum carmichaelii* roots for use in Traditional Chinese Medicine generates a high amount of waste material, especially leaves. An acidic polysaccharide fraction isolated from these unutilized leaves, AL-I, was in our previous work shown to contain pectic polysaccharides. This study aimed to investigate the protective effect of AL-I on ulcerative colitis for the possible application of *A. carmichaelii* leaves in the treatment of intestinal inflammatory diseases. AL-I was found to alleviate symptoms and colonic pathological injury in colitis mice, and ameliorate the levels of inflammatory indices in serum and colon. The production of short- and branched-chain fatty acids was also restored by AL-I. The observed protective effect could be due to the inhibition of NOD1 and TLR4 activation, the promotion of gene transcription of tight-junction proteins, and the modulation of gut microbiota composition like *Bacteroides*, *Dubosiella*, *Alistipes* and *Prevotella*,. A regulation of serum metabolomic profiles being relevant to the bacterial change, such as D-mannose 6-phosphate, D-erythrose 4-phosphate and uric acid, was also observed.

1. Introduction

During the industrial production of Traditional Chinese medicine (TCM), a huge amount of unutilized plant parts are generated without further usage, such as stems and leaves for traditional medicines based on roots. These plant parts could be suitable plant resources for the production of phytochemicals [1]. The lateral ("Fuzi") and mother roots ("Chuanwu") of *Aconitum carmichaelii* Debeaux (Ranunculaceae) are commonly used in TCM as analgesic and anesthetic agents, and to heal shock resulting from acute myocardial infarction and coronary heart disease [2,3]. After the roots of *A. carmichaelii* are harvested, the aerial parts are normally discarded, resulting in a vast amount of waste of this medicinal plant. To date, the aerial parts of *A. carmichaelii* have shown similar analgesic and anti-inflammatory activities as for the roots [4]. An acidic polysaccharide fraction composed of pectic polysaccharides isolated from *A. carmichaelii* leaves and structurally characterized in a previous study, was shown to possess immunomodulatory and intestinal anti-inflammatory activities in vitro [5].

Ulcerative colitis is a chronic intestinal inflammatory bowel disease, and the number of cases worldwide has increased during the last decades. Inflammation of colonic epithelial cells and defects on mucous and epithelial barriers are factors strongly implicated in the pathogenesis of ulcerative colitis, in addition to microbiota dysbiosis [6]. The stability of gut microbiota is essential for human health and its interaction with the host determines the integrity of the intestinal mucosal barrier. In ulcerative colitis, the microbiota is characterized by reduced biodiversity, abnormal composition, and altered spatial distribution [7]. Further, certain classes of metabolites, deriving from bacterial metabolism of dietary substances or other molecules, have been implicated in the pathogenesis of intestinal bowel diseases [8].

There are mainly three types of classic drugs used for treating intestinal bowel disease at present, including aminosalicylic acid derivatives, glucocorticoids, and immunosuppressants. However, due to the possible side effects after long-term of treatment, new treatment options are needed. Natural plant polysaccharides, as indigestible polymers, have low toxicity and have been shown to affect the intestinal immune system and restore barrier function in ulcerative colitis [9,10]. Pectins, for instance, have attracted growing attention in treatment or

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prevention of ulcerative colitis through regulation of intestinal microbiota composition, strengthening of intestinal barrier function, enhancement of antioxidant activities, promotion of short-fatty acids (SCFAs) production and reduction of pro-inflammatory mediators [9,11,12]. Therefore, based on the promising intestinal anti-inflammatory effects observed in vitro, we hypothesized that the pectic polysaccharides of *A. carmichaelii* leaves could alleviate dextran sulfate sodium (DSS)-induced ulcerative colitis in an in vivo mouse model.

This study aimed to broaden the utilization of *A. carmichaelii* leaves from the aspect of polysaccharides and to investigate the protective effects on DSS-induced ulcerative colitis. Metabolomic and microbiota analysis were performed to study the underlying mechanisms of the protective effect.

2. Materials and methods

2.1. Materials

The whole plant of *A. carmichaelii* Debeaux was collected from Jiangyou City, Sichuan Province, P.R. China in June 2019 (31°50'24.0"N/ 104°47'24.0"E, 517 m. a.s.l.). The fresh leaves were separated and processed as earlier reported [5].

2.2. Isolation, chemical composition and average-molecular weight of an acidic polysaccharide from A. carmichaelii leaves

A major acidic polysaccharide fraction, AL-I, was isolated from the water extract of dried leaves of *A. carmichaelii* by anion exchange chromatography, as earlier described [5]. The monosaccharide composition of AL-I was determined as trimethylsilylated (TMS) derivatives, after methanolysis by GC (Trace™ 1300 GC, Thermo Scientific™, Milan, Italy), as described by Chambers and Clamp [13] with modifications [14]. The total amounts of phenolic compounds and proteins of AL-I were determined by Folin-Ciocalteu [15] and Bio-Rad protein assay [16], respectively. The average-weight molecular weight (*Mw*) was determined by size exclusion chromatography and calculated by known dextran standards as previously described [5].

2.3. Protective effects of AL-I on ulcerative colitis mice

2.3.1. Animal care and experimental design

Thirty-six male SPF C57BL/6 mice (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing China) and maintained for adapting to the new environment (24 ± 1 ℃) for one week. All experiments were performed under the supervision of the Ethics Committee for Animal Experiment at Sichuan Agricultural University (Confirmation number: DYXY141642008).

As shown in Fig. 1, mice were randomly divided into 6 groups, with 6 mice in each group. The mice were pre-administrated with the commercial drug Mesalazine Enteric Coated Tablets (containing mainly anti-inflammatory 5-aminosalicylic acid, Sunflower Pharmaceutical Industry Co. Ltd, China), as the positive control, or different dosages of AL-I for 14 days (D1 to D14, dissolved in germ-free distilled water) by gastric gavage. One week after administration, 2.5 % (w/v) of dextran sodium sulfate (DSS) (dissolved in germ-free distilled water, with *Mw* 36,000–50,000 Da, MP Biomedicals, Ontario, CA, United States) was used to induce acute ulcerative colitis [17], given through drinking water for 7 days (from D8 to D14). The mice with no treatment of DSS during the whole experimental period was set as negative control (control group), while those treated only with DSS was set as colitis control (model group). Body weight, stool characteristics and the presence of occult blood in the feces were recorded daily after DSS was given (D8 to D14), by which the disease activity index (DAI) was calculated according to an evaluation system (Table S1) [18]. 24 h after the last administration, mice were euthanized with carbon dioxide followed by cervical dislocation, and blood, cecal content and colonic tissues were collected. Colon length was measured immediately before being collected, as shown in the following sections.

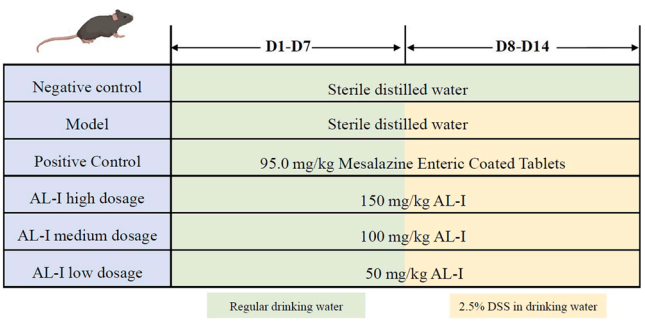

Fig. 1. Schematic overview of experimental design. Mesalazine and AL-I were given by gastric gavage.

2.3.2. Determination of myeloperoxidase (MPO) level and secretions and gene expressions of pro-inflammatory cytokines

Colon tissues were ground under liquid nitrogen (N_2). 40 mg of tissues were homogenized in 2 mL Phosphate Buffered Saline (PBS, 1 ×), centrifuged at 1500g for 15 min, and the supernatants were used for the analysis of secretions of MPO, IL-1β, IL-6, and TNF-α by ELISA kits according to manufacturer's instructions. Serum cytokine IL-1β, IL-6 and TNF-α, as well as the levels of LPS, were also quantified by ELISA kits.

Around 10 mg of colon tissues were used for the analysis of gene expressions of NOD and TLR4 receptors and tight junction proteins by qRT-PCR. Briefly, total RNA of colonic tissues was isolated using Trizol Reagent (Biomed, RA101-12, China), and reverse transcribed into cDNA using M-MLV 4 First-Strand cDNA Synthesis Kit (Biomed, RA101-12, China). All real-time PCR analysis were performed by SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Mei5Bio, China), and the gene expressions were quantified as relative regulation fold compared with *β-actin* (normalizing reference). Primers are shown in Table S2.

2.3.3. Histological evaluation

After fixation with 4 % paraformaldehyde solution for more than 24 h, paraffin slices (4 μm) of colon tissues were cut and stained with hematoxylin and eosin (H&E). The colon slices were further analyzed in a blinded fashion, and the histological assessment index (HAI) was scored according to the evaluation system listed in Table S3 [18]. Brightfield images at 200 × magnification were captured on Nikon Eclipse Ti microscope (Melville, NY, USA).

2.3.4. Analysis of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) in the cecal content

The content of SCFAs, including acetic, butyric, and propionic acids, and BCFAs such as isovaleric and isobutyric acids of cecum content were analyzed by GC based on methods described previously [19] with minor modifications. See details for sample preparation and GC in supplementary methods. Crotonic acid (internal standard) and standards were used as shown in Table S4.

2.3.5. Serum untargeted metabolomics analysis

Fresh serum samples (100 μL) were analyzed by Novogene Co., Ltd. (Beijing, China) for a host metabolomic study. Briefly, processed samples were injected into an LC-MS/MS system with a Vanquish UHPLC system (ThermoFisher, Germany) and were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher). All metabolites were identified with mzCloud (https://www.mzcloud.org/), mzVault and MassList, and annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/pathway.html), HMDB (https://hmdb.ca/metabolites) and LipidMaps (http://www.lipidmaps.org/) databases. Principal Components Analysis (PCA) and

Partial Least Squares Discriminant Analysis (PLS-DA) were performed by metaX. The functions of these metabolites and metabolic pathways were screened in the KEGG database. Details can be found in supplementary methods.

2.3.6. Gut microbiota analysis

Fresh feces of mice were processed and analyzed by Beijing Novogene Science and Technology Co., Ltd, as predicted in supplementary material. Briefly, the 16 S rRNA genes of the V4 regions were amplified from feces genome, and were further sequenced and analyzed. Sequences with ≥ 97 % similarity were assigned to the same operational taxonomic units (OTUs). Alpha and beta diversity analysis were used to evaluate microbiota diversity and differences. Cluster analysis was performed by PCA, principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) by R software. Different and enriched bacterial species among groups were analyzed by MetaStat and

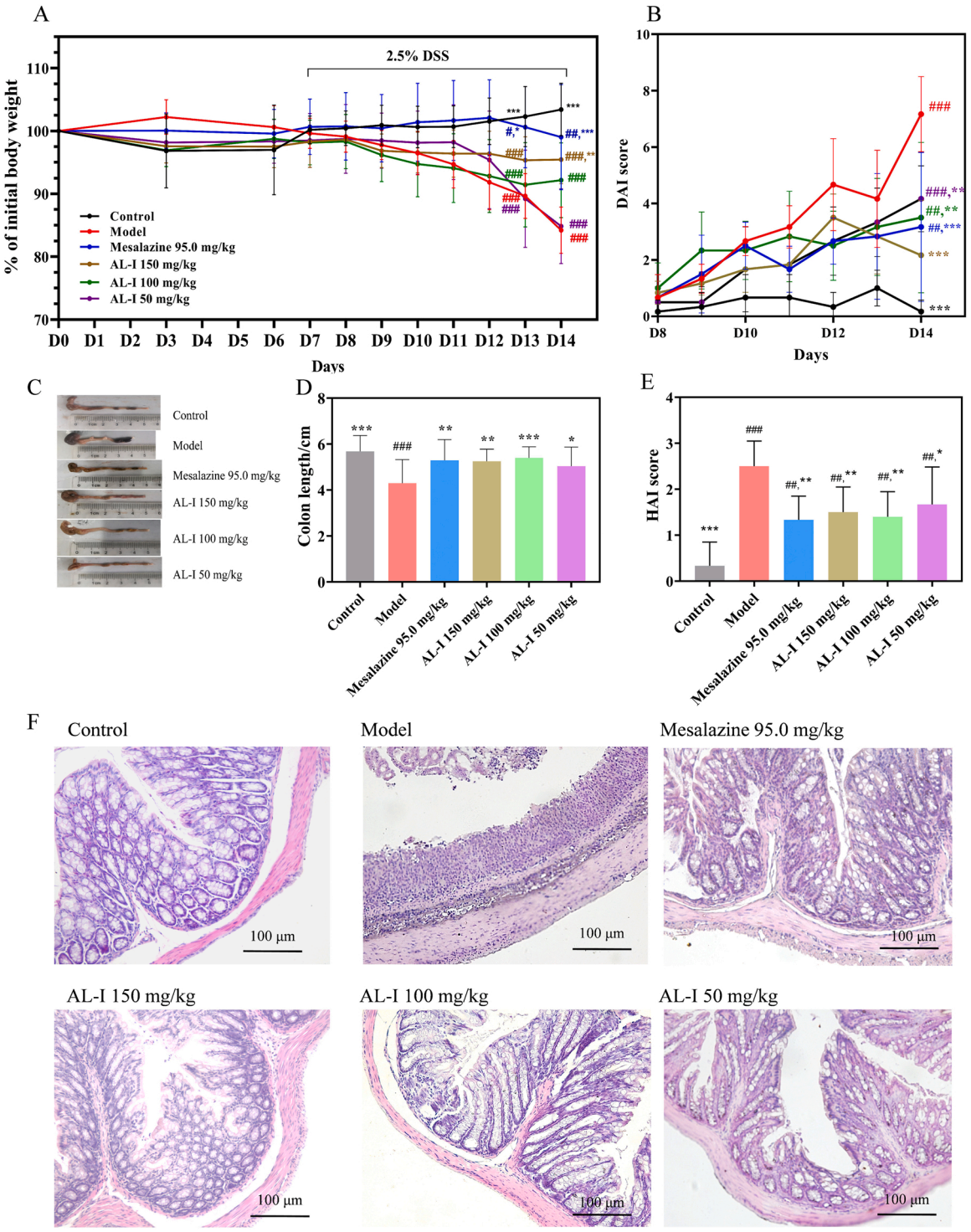

Fig. 2. Effects of AL-I on body weight (A, D1 to D14), DAI scores (B, D8 to D14), colon tissues (C, D14) and lengths (D, D14), HAI scores (E, D14) and histological changes (F, D14) of DSS-induced ulcerative colitis mice. # $p < 0.05$ versus control group; ## $p < 0.01$ versus control group; ### $p < 0.001$ versus control group; * $p < 0.05$ versus model group; * * $p < 0.01$ versus model group; * ** $p < 0.001$ versus model group; n = 6.

LDA effect size (LEfSe) with statistical analysis of t-test.

2.4. Statistical analysis

Besides the statistical analysis used in metabolomic and microbiota experiments, the rest of data were expressed as the mean± S.D. and analyzed using one-way ANOVA analysis of variance and LSD test (IBM SPSS Statistics version 24, IBM Corp., Armonk, New York, USA). #, $p < 0.05$ versus control group; ##, $p < 0.01$ versus control group; ### $p < 0.001$ versus control group; * , $p < 0.05$ versus model group; * *, $p < 0.01$ versus model group; * ** , $p < 0.001$ versus model group; ns, no significant difference was observed.

3. Results and discussion

3.1. Isolation, chemical composition and molecular weight determination of AL-I

A crude polysaccharide from *A. carmichaelii* leaves, ALP, was isolated after water extraction and ethanol-precipitation, and an acidic fraction AL-I was further obtained after anion exchange chromatography [5]. A protein content of 1.0 % was determined in AL-I, while no phenolic compounds were found. It was further shown that AL-I was composed of mainly galacturonic acid (GalA, 50.3 mol%), followed by arabinose (Ara, 15.1 mol%), galactose (Gal, 14.3 mol%), xylose (Xyl, 6.9 mol%), rhamnose (Rha, 6.3 mol%), and minor amounts of glucose (Glc, 2.9 mol %), glucuronic acid (GlcA, 1.9 mol%), fucose (Fuc, 1.2 mol%) and mannose (Man, 1.0 mol%) (Fig. S1). AL-I could consist of typical pectic polysaccharide units due to the high amount of GalA and the presence of Rha which are the main monomers in type I rhamnogalacturonan (RG-I) domains [20]. This is consistent with previous results showing that AL-I was composed of pectic polysaccharides where Gal and Ara could be due to the pectic structural elements arabinogalactans, galactans and/or arabinans [5]. Additionally, AL-I was shown to be a fraction with wide range *Mw*, from 26 kDa to 270 kDa, after determination on size exclusion chromatography packed with Superose 6.

3.2. AL-I protected ulcerative colitis mice from severe colitis symptoms

Compared with mice in the control group, a significant reduction of bodyweight (Fig. 2A), increase in DAI index (Fig. 2B), and shortening of colonic lengths in the model group (Fig. 2C–D) indicated that the

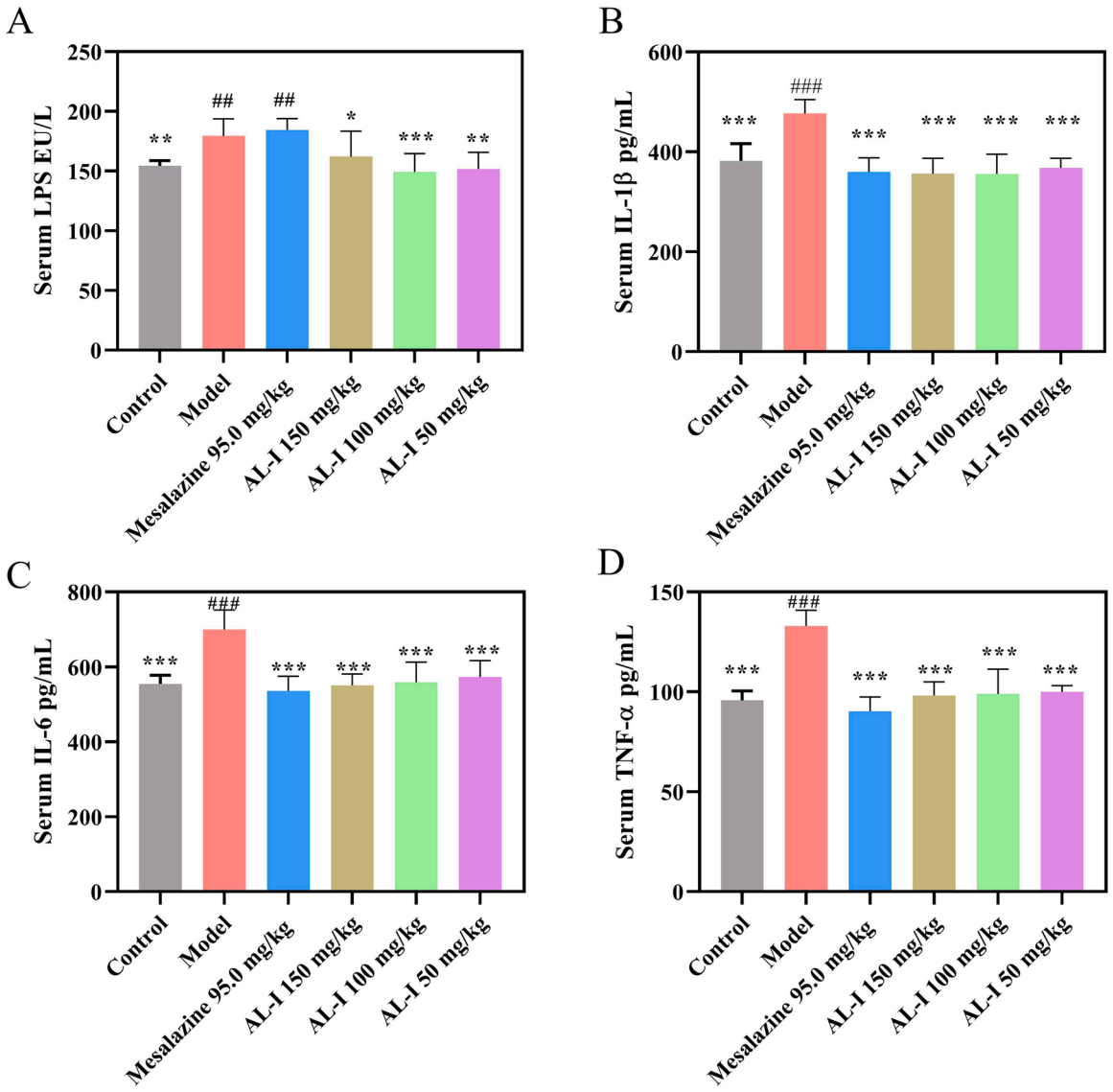

Fig. 3. Effects of AL-I on serum LPS (A), IL-1β (B), IL-6 (C) and TNF-α (D) of DSS-induced ulcerative colitis mice. # $p < 0.05$ versus control group; ## $p < 0.01$ versus control group; ### $p < 0.001$ versus control group; * $p < 0.05$ versus model group; * * $p < 0.01$ versus model group; * ** $p < 0.001$ versus model group; n = 6.

ulcerative colitis model in mice was successfully established, as described in a previous study [17,21]. The obvious damage on colonic surface epithelium, crypt and mucosal structure, and increased infiltration of inflammatory cells also proved colitis severity caused by DSS (Fig. 2F). After oral administration of 50, 100 or 150 mg/kg AL-I, these symptoms of colitis mice were ameliorated. This was especially observed on D14 when both the body weight and DAI index were statistical significantly reversed by AL-I treatment ($p < 0.01$) compared to the model mice, as presented in Figs. 2A and 2B. Similar restorative effects also occurred in mice treated with the positive control Mesalazine (Fig. 2C–F). The HAI scores according to colonic histological observations also gave a clear indication for these restorations by AL-I and Mesalazine (Fig. 2E). No change was observed in body weight (Fig. 2A) of mice before DSS intervention.

The levels of serum pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were notably elevated in colitis mice, but the levels in mice supplemented with all doses of AL-I were considerably lower ($p < 0.001$), being close to normal levels (no significant difference with the control group) (Fig. 3B–D). A rising secretion of serum LPS is one of the indicators of systematic inflammation of colitis mice ($p < 0.001$), but this was relieved in all AL-I groups ($p < 0.001$) (Fig. 3A). Therefore, it was demonstrated that AL-I intervention at different dosages were able to protect against colitis-associated symptoms and attenuate inflammation in DSS-induced ulcerative colitis mice.

3.3. AL-I ameliorated colonic inflammation and repaired gut barrier function in DSS-induced ulcerative colitis mice

As a local mediator of tissue damage and the resulting inflammation in various inflammatory diseases and ulcerative colitis [21,22], the amount of MPO in colon tissues was significantly enhanced in the model group, which revealed a severe inflammatory reaction in the colon of ulcerative colitis mice [22]. It was however dramatically reversed by all doses of AL-I ($p < 0.001$) (Fig. 4A). Similar inhibition on the secretions of IL-1β, IL-6 and TNF-α (Fig. 4A) were also observed in all AL-I-treated mice. The down-regulations of these inflammatory mediators could be due to the inhibition of AL-I on activation of pattern-recognition receptors (PRRs), such as nucleotide-binding oligomerization domain 1 (NOD1) and Toll-like receptor 4 (TLR4) on the surface of intestinal epithelial cells, evidenced by the reduced colonic mRNA expressions of these receptors compared to model group (Fig. 4B). These receptors could be the possible active sites of AL-I for its anti-inflammatory effects, as shown by other pectic polysaccharides [11,12,20].

Upon epithelial injury, a damaged intestinal mucosal integrity by DSS is one of the causes of intestinal inflammation [17]. An observed increase of serum LPS suggested a possible invasion of endotoxin after intestinal mucosa injury (Fig. 3A), as well as down-regulation of colonic tight junction (TJ) proteins such as ZO-1 and occludin (Fig. 4B) that are crucial to protect the intestinal epithelial barrier. However, gene expressions of both ZO-1 and occludin were improved after14-day treatment of AL-I (Fig. 4B), being equivalent to those reported for other natural polysaccharides [10,23–25]. These results suggest that AL-I administration could protect mice from reduced intestinal barrier functions by promoting the expressions of TJ proteins.

3.4. AL-I promoted production of SCFAs and BCFAs in DSS-induced ulcerative colitis mice

Total SCFAs, acetic acid, and isovaleric acid have been reported as the main fermentation products of water-soluble polysaccharides, and exhibit immunomodulatory effects and are involved in intestinal protection [20,26,27]. The BCFAs isovaleric acid and isobutyric acid are metabolic end products of undigested protein when fermentable fiber supply decrease, but are generally omitted as they are present in lower quantities than SCFAs [28]. After DSS induction, the contents of total SCFAs, acetic acid, and isovaleric acid visibly decreased ($p < 0.05$) compared to the control group (Fig. 5), whereas in mice administered with AL-I the contents were significantly restored in a dosage-dependent manner, with acetic acid being the predominant one promoted by AL-I. These changes could be another explanation for the protective activities of AL-I on colitis mice, as SCFAs can play a key role in the protective activities of colitis mice through anti-inflammatory effects, regulation of the differentiation of intestinal epithelial cells and maintenance of intestinal barrier [20,27]. Additionally, BCFAs may serve as alternative fuels in colonocytes [29], which could potentially be an indirect way for the activity of AL-I. Comparatively, the contents of these SCFAs were not affected by the chemical therapeutic medication Mesalazine. The production of SCFAs has been reported to be dramatically reduced in colitis mice, probably due to less bacterial diversity and reduction of abundance of SCFAs-producing bacteria [20,27]. Thus, an underlying balance of AL-I on DSS-induced dysbacteriosis might also contribute to its modulatory effects on SCFAs, as well as other positive reactions on colitis.

3.5. AL-I modulated host metabolomics in DSS-induced ulcerative colitis mice

For a better understanding of the active mechanism of AL-I, an untargeted metabolomics analysis was performed to discover the potential metabolites and pathways that are affected by AL-I (150 mg/kg)

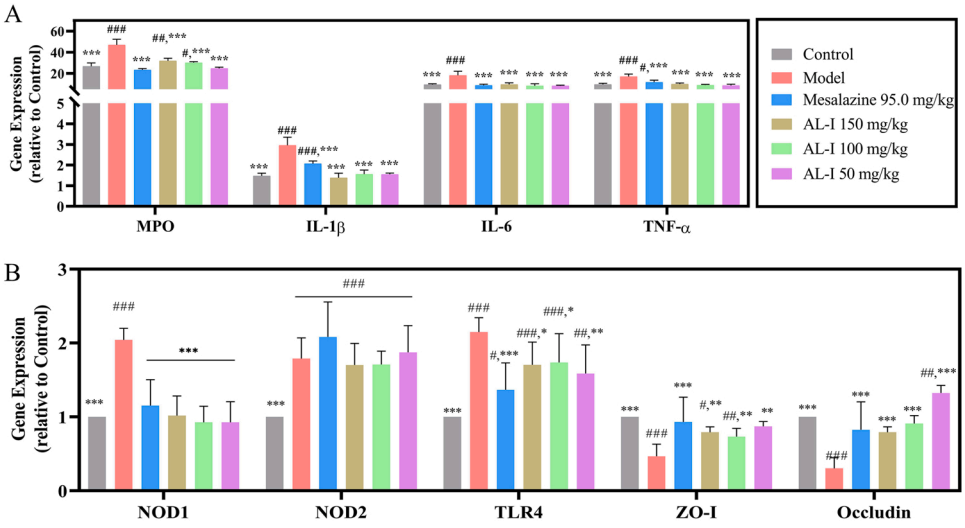

Fig. 4. Effects of AL-I on colonic inflammation and barrier functions of DSS-induced ulcerative colitis mice. Colonic MPO levels and secretions of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were determined using ELISA kits (A); Gene expressions of inflammatory signal receptors and tight junction proteins were quantified using qRT-PCR (B). # $p < 0.05$ versus control group; ## $p < 0.01$ versus control group; ### $p < 0.001$ versus control group; * $p < 0.05$ versus model group; * * $p < 0.01$ versus model group; * ** $p < 0.001$ versus model group; ns, no significant difference; n = 6.

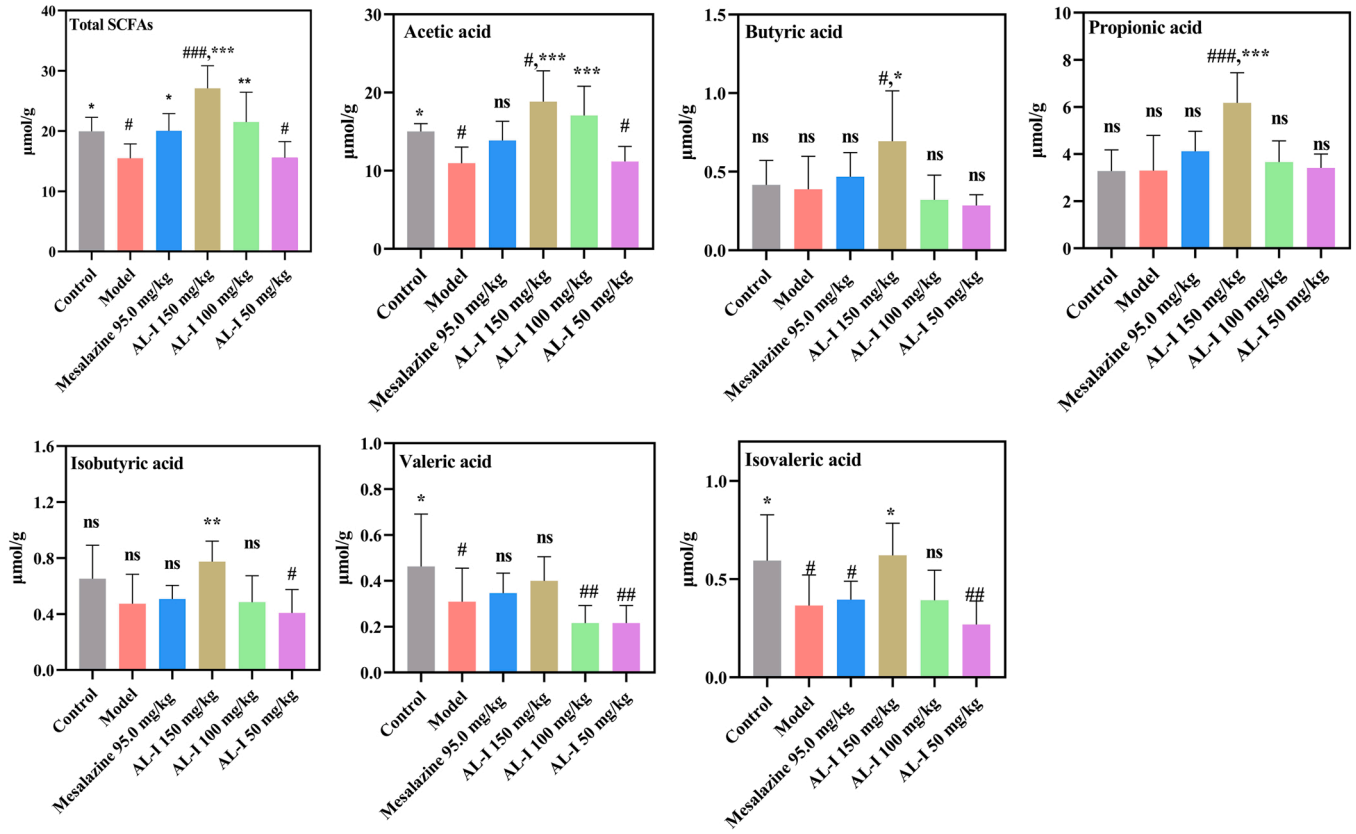

Fig. 5. Effects of AL-I on concentrations of SCFAs and BCFAs in cecum content. # $p < 0.05$ versus control group; ## $p < 0.01$ versus control group; ### $p < 0.001$ versus control group; * $p < 0.05$ versus model group; * * $p < 0.01$ versus model group; * ** $p < 0.001$ versus model group; ns, no significant difference; n = 6.

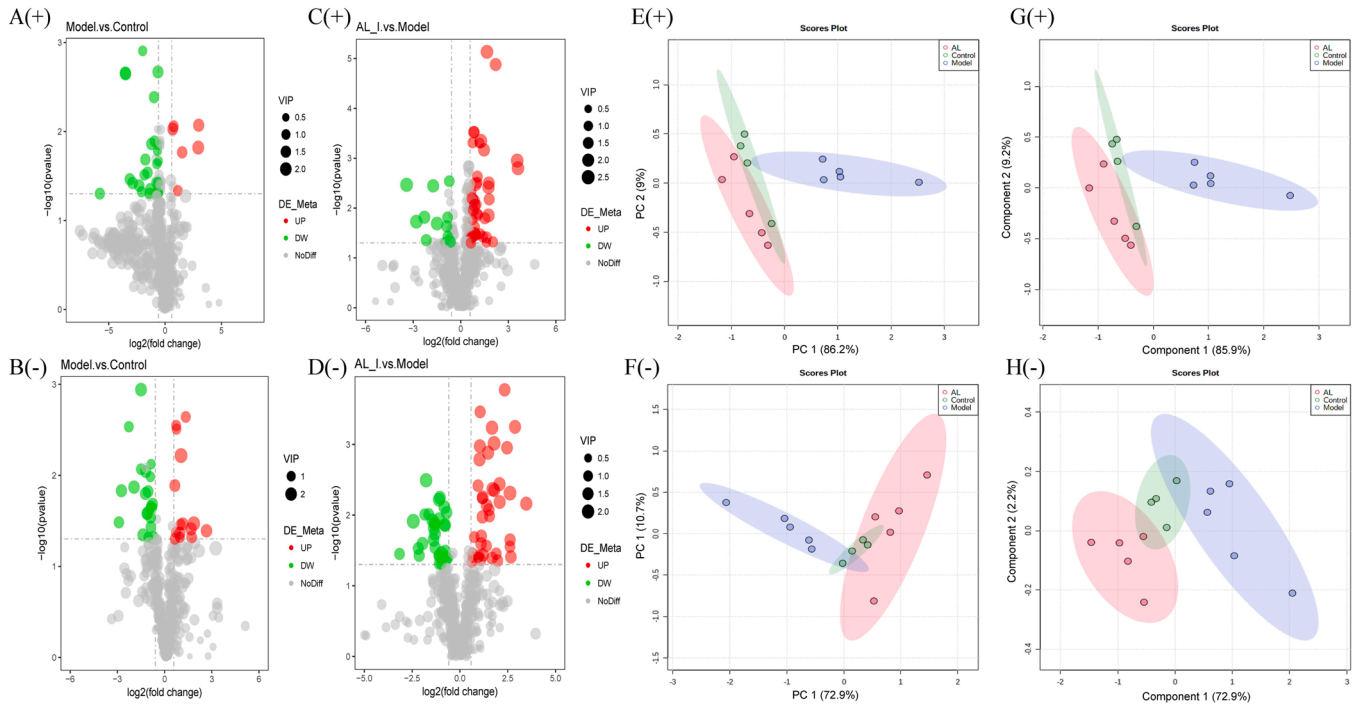

Fig. 6. Effects of AL-I on serum metabolomic profile of DSS-induced ulcerative colitis mice. Volcano plots in positive mode (+, A, C) and negative mode (-, B, D) altered within groups. PCA and PLS-DA analysis of modulated differential metabolites in positive (+, E, G) and negative mode (-, F, H), respectively. AL/AL_I represents the AL-I 150 mg/kg group, Model represents model mice treated with DSS only, and Control represents control mice without treatment.

on colitis mice. Generally, 656 metabolites in positive ionization mode (+) and 469 in negative mode (-) were identified and quantified based on mzCloud, mzVault, and MassList database (Table S5). Considerable differences in total (Fig. S2) and differential metabolites among groups (Fig. S3) were observed after PCA and PLS-DA analysis. Metabolites with significant up- or down-regulation within groups were further chosen (Table S5, Fig. 6A–D) after PCA (Fig. 6E, F) and PLS-DA (Fig. 6G, H) analysis. Herein, 8 (+) and 19 (-) metabolites were altered by AL-I compared to the model and control groups (Fig. 7). Every differential metabolite was verified for accuracy by receiver operating characteristic (ROC, not shown).

Relevant metabolites and the included metabolic pathways are listed in Table S6 after screening in KEGG database. In positive mode, the nicotinate and nicotinamide metabolism ($p = 0.001$) and porphyrin and chlorophyll metabolism ($p = 0.005$) pathways were suppressed by DSS (Table S6, Fig. 8A), represented by the alterations of nicotinamide and biliverdin (Fig. 8G) respectively. However, AL-I failed to restore the levels of these metabolites and their included pathways, except modulation on the glycerophospholipid metabolism compared to both model and control groups (Fig. 8C, E). In negative mode, 5 pathways were tentatively found to be affected by DSS compared to normal mice (Fig. 8B) and were further significantly restored by AL-I compared with the model group, including the pentose phosphate pathway ($p = 0.036$, enriched with $_D$-erythrose 4-phosphate and $_D$-sedoheptulose 7-phosphate), fructose and mannose metabolism ($p = 0.019$, enriched with $_D$-mannose 6-phosphate and dihydroxyacetone phosphate), amino sugar and nucleotide sugar metabolism ($p = 0.018$, enriched with $_D$-mannose 6-phosphate), purine metabolism ($p = 0.001$, enriched with uric acid, xanthosine, and 2,6-dihydroxypurine) and the biosynthesis of unsaturated fatty acids pathway ($p = 0.011$, enriched with linoleic acid, arachidonic acid, 8Z,11Z,14Z-eicosatrienoic acid, docosahexaenoic acid and eicosapentaenoic acid), as shown in Fig. 8D, H and Table S6. In addition, the linoleic acid metabolism and arachidonic acid metabolism (Fig. 8D–F) pathways were inhibited by AL-I represented mainly by linoleic acid (Table S6 and Fig. 8H).

The pentose phosphate pathway is a fundamental component in cellular metabolism, which is crucial for maintaining carbon homeostasis, nucleotide, and amino acid biosynthesis, as well as defeating oxidative stress [30]. The levels of two constituents in the non-oxidative branch of this pathway, $_D$-erythrose 4-phosphate, and $_D$-sedoheptulose 7-phosphate were promoted after AL-I treatment (Fig. 8H), which suggested a possible modulatory effect of AL-I on the transaldolase-catalysed reaction. The process of mannose 6-phosphate generation, a metabolite included in the fructose and mannose metabolism and amino sugar and nucleotide sugar metabolism, has been proposed to inhibit IL-1β production in macrophages via suppressing glycolysis by reducing glucose 6-phosphate [31]. It is also responsible for the inhibition of TLR4 expression and suppression of the inducible nitric oxide synthase (iNOS) expression and NO and cytokine production, by competitively binding with insulin-like growth factor II/mannose-6-phosphate (IGF-II/M6P) receptor [32]. Therefore, the significantly increased mannose-6-phosphate might be one of the contributors to the anti-inflammatory effect of AL-I. Lipids derived from polyunsaturated fatty acids, such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, are not only functioning as anti-inflammatory activators but also act as immunomodulators and signaling molecules (in the family of prostanoids) to drive some of the signs of inflammation. They can be activated and released from cell membrane phospholipid stores when stimuli such as a microorganism or damage appear [33]. The levels of these metabolites increased in colitis mice both in current and previous studies [34,35], and AL-I suppressed their levels (Fig. 8H), as well as their associated metabolic pathways

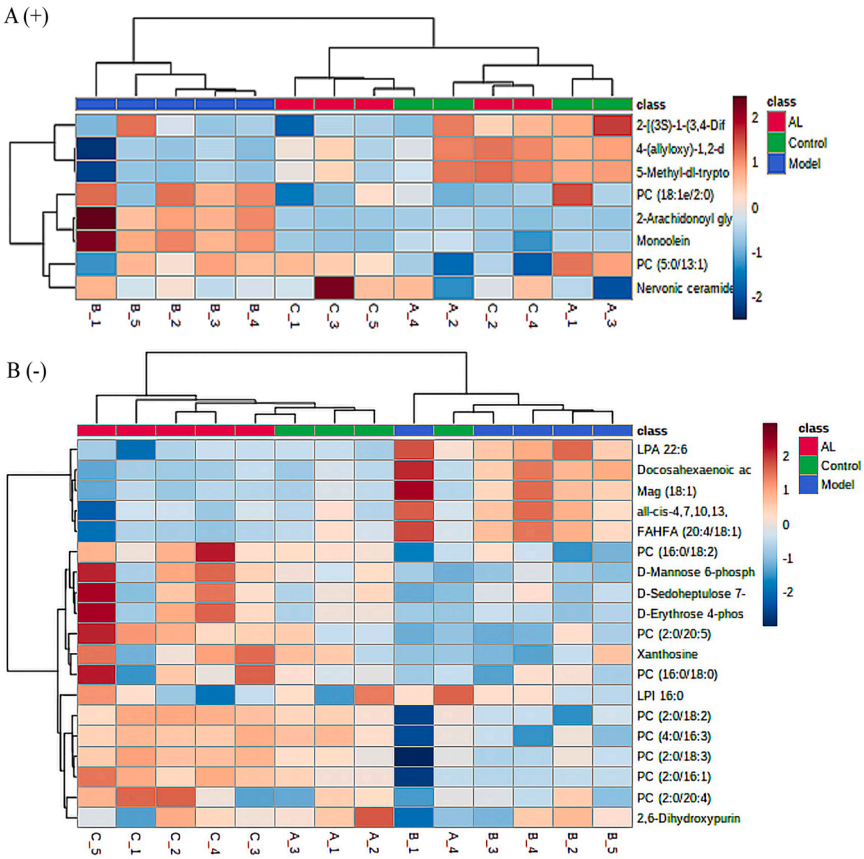

Fig. 7. Clustered heatmaps of modulated differential metabolites within three groups in positive mode (+, A) and negative mode (-, B). AL represents the AL-I 150 mg/kg group, Model represents model mice treated with DSS only, and Control represents control mice without treatment.

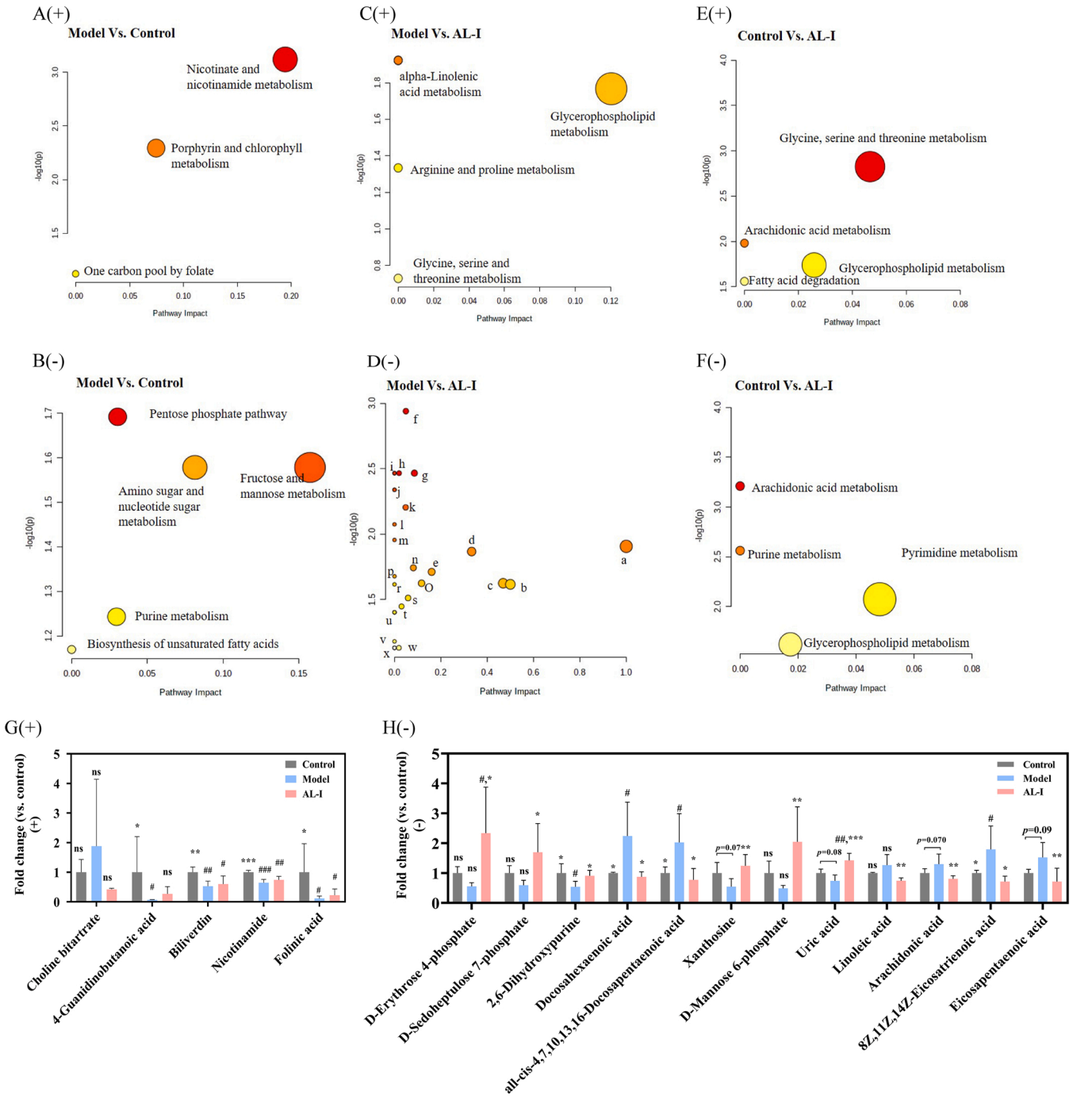

Fig. 8. Effects of AL-I on metabolic pathways and metabolites of DSS-induced colitis mice. Pathways were concluded based on metabolites in both positive (+) and negative (-) modes, and analyzed between model vs control (A, B), model vs AL-I (C, D) and control vs AL-I (E, F). Detailed metabolic pathways of (D) are: a, Linoleic acid metabolism; b, D-glutamine and D-glutamate metabolism; c, Alanine, aspartate and glutamate metabolism; d, Arachidonic acid metabolism; e, Fructose and mannose metabolism; f, Purine metabolism; g, Arginine and proline metabolism; h, Glutathione metabolism; i, Nitrogen metabolism; j, Aminoacyl-tRNA biosynthesis; k, Pyrimidine metabolism; l, Pentose and glucuronate interconversions; m, Biosynthesis of unsaturated fatty acids; n, Amino sugar and nucleotide sugar metabolism; o, Arginine biosynthesis; p, Steroid hormone biosynthesis; q, Butanoate metabolism; r, Citrate cycle (TCA cycle); s, Pentose phosphate pathway; t, Pantothenate and CoA biosynthesis. Specific metabolites in positive (G) and negative mode (H) are included in the metabolic pathways above. AL-I represents the AL-I 150 mg/kg group, Model represents model mice treated with DSS only, and Control represents control mice without treatment.

(Fig. 8D, Table S6). Linoleic acid is also a biomarker for the development of colitis into colorectal cancer [35], which was successfully reduced by AL-I in the current study. Collectively, these results suggested that the protection of AL-I from a disordered broad-scale metabolism process is a potential mechanism of its protective role in colitis.

Decreased levels of uric acid, 2,6-dihydroxypurine, and xanthosine were observed in the colitis mice (Fig. 8H). However, it is still controversial whether they are related to colitis development. Serum uric acid, for instance, was previously indicated to be associated with ulcerative colitis patients having higher levels compared to healthy people [36]. However, the disease activity index of ulcerative colitis was not related with the uric acid level [37]. Moreover, 2,6-dihydroxypurine and xanthosine have also been previously reported to decrease in DSS-induced colitis mice [38]. It is consequently still a challenge to

distinguish whether these metabolites would induce or alleviate inflammatory processes in the current study, as the inflammatory threshold levels in ulcerative colitis mice remain unclear. Meanwhile, since gut dysbacteriosis was noticed not only participating in the pathogenesis of intestinal bowel diseases, but also affecting the metabolic pathways of urate [36], the change in microbiota composition might be a possible reason for the uric acid increase. A statistical modulation of serum uric acid compared to normal mice was only observed in AL-I-treated mice rather than in colitis model mice without any treatment (Fig. 8H), which also indicates that uric acid may have a greater relationship with microbiota modulation, rather than the metabolic pathways or inflammatory status, which will be discussed after the analysis of bacterial composition.

3.6. AL-I modulated the gut dysbiosis on DSS-induced ulcerative colitis mice

3.6.1. Alpha and beta diversity analysis

Gut microbiota dysbiosis has been considered as one of the factors in ulcerative colitis development [7]. After DSS induction for 7 days, the indices of the alpha diversity of ulcerative colitis mice (model group) were significantly reduced compared to normal mice (control group) (Table S7), which indicated a decrease of microbiota diversity caused by DSS. However, alpha diversity indices of the mice treated with AL-I were significantly lower than the control group ($p < 0.05$) and no difference with colitis mice was observed. It was consequently shown that 14 days of AL-I treatment had not reversed the reduced richness and diversity of microbial communities caused by DSS. The rarefaction curve (Fig. S4A) and rank abundance curve (Fig. S4B) also suggested a lower abundance of microbiota diversity in model and AL-I groups compared to the

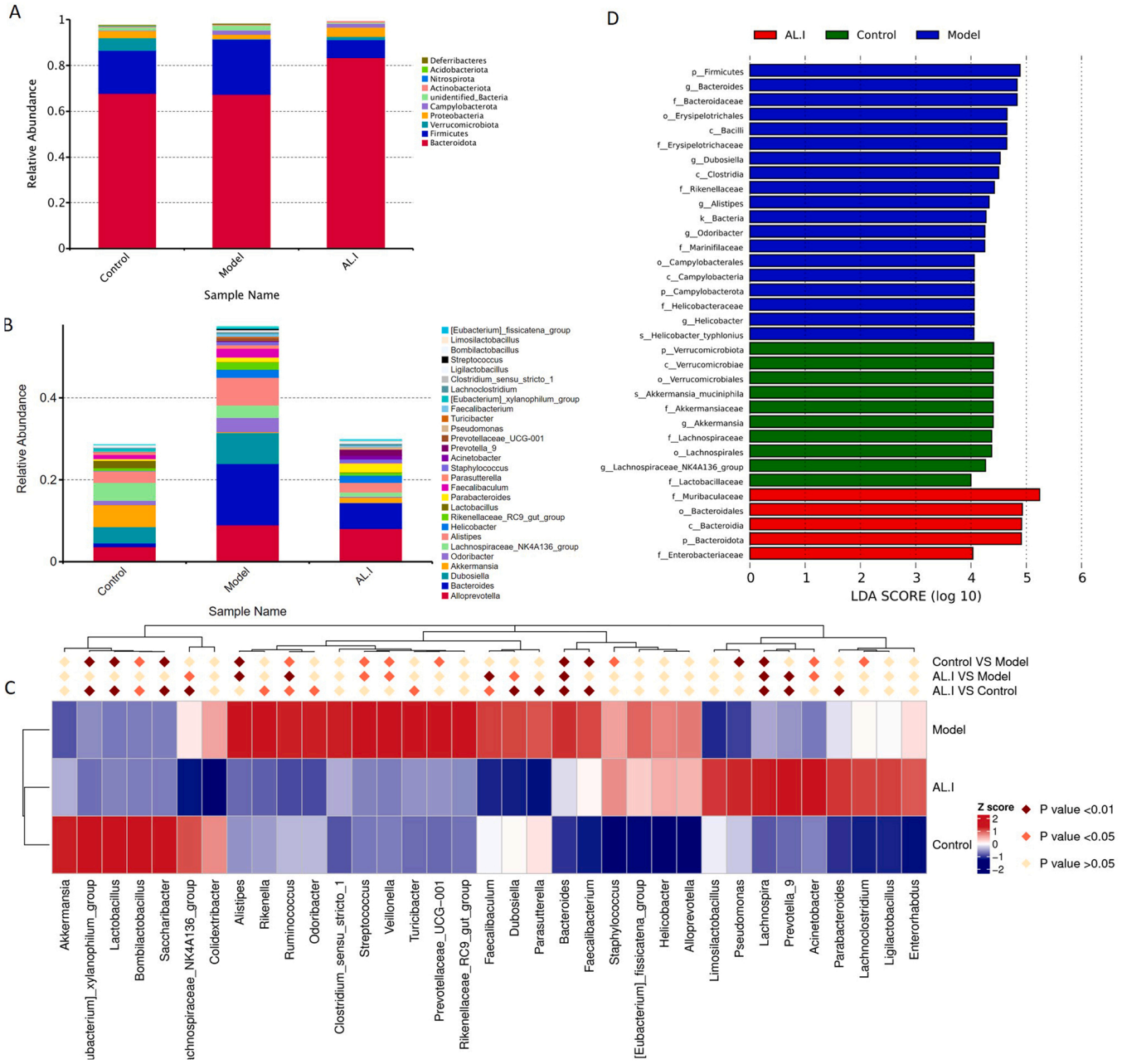

Fig. 9. Microbiota compositions at phylum (A) and genus levels (B) and MetaStat (C, genus) and LEfSe (D) analysis of bacterial changes of DSS-induced ulcerative colitis. AL.I represents the AL-I 150 mg/kg group, Model represents model mice treated with DSS only, and Control represents control mice without treatment.

control group. In addition, beta diversity is utilized to compare the microbiota composition among groups based on the OTUs abundance and annotation. Clear separations were observed between the control and model groups in PCoA (Fig. S4C) and PCA analysis (Fig. S4D), and NMDS analysis (Fig. S4E), which also demonstrated a huge modulation in species composition after DSS induction. Clear shifts between the model group and AL-I treated group was also observed in PCoA and NMDS analysis (Fig. S4D,E), indicating that a significant change in gut microbiota composition occurred after administration of AL-I.

3.6.2. Modulation of microbiota composition

The statistical difference in microbiota composition between groups was determined by Anosim and MRPP analysis (Table S7). The relative abundance of phylum, class, order, family, genus and species levels are shown in Fig. 9A, Fig. S5A–C Fig. 9B and Fig. S5D, respectively. The significantly different bacteria among groups on phylum and genus levels were further investigated by MetaStat (Fig. 9C, Fig. S6A) and LEfSe (Fig. 9D) analysis in order to determine the different bacterial species present among three groups.

Bacteroidota, *Firmicutes*, *Verrucomicrobiota*, *Proteobacteria* and *Camphylobacterota* are the dominant phyla in the samples (Fig. 9A), and modulation of the microbiota composition was observed both in the model group and AL-I treated group. The relative abundance of *Bacteroidota* and *Firmicutes* in the model group was not affected by DSS treatment (Fig. 9C, $p > 0.05$). However, as shown in Fig. S6A and Fig. 9A, the abundance of *Acidobacteriota* and *Nitrospirota* were significantly decreased in the model group ($p < 0.05$). A different pattern of microbiota composition was found in AL-I-treated colitis mice, with a more relative abundance of *Bacteroidota* ($p < 0.05$), and less *Firmicutes*, *Verrucomicrobiota*, *Acidobacteriota* and *Deferribacteres* ($p > 0.05$) (Fig. 9A and Fig. S6A). The imbalance of different phyla in ulcerative colitis is variable across studies, however, a decrease in the abundance of *Bacteroides* spp has also been observed in ulcerative patients [7,39] and in experimental mice [40,41]. From the aspect of pectin degradation, the *Bacteroidetes* are generally regarded as the more dominant plant polysaccharide degraders in the human gut, and the relative contribution of *Bacteroidetes* versus *Firmicutes* for the in-situ pectin degradation is yet unclear [26].

For a better understanding of the modulatory effect of AL-I on colitis microbiota, changes at the dominant genus level were further analyzed. As shown in Fig. 9B and **C**, 16 bacterial genera were significantly regulated in the model group compared to the control group. Some of them that had been promoted by DSS were reduced significantly by AL-I administration, including the *Bacteroides*, *Alistipes*, *Streptococcus* and *Ruminococcus* genera, and some that were inhibited by DSS were promoted by AL-I such as *Acinetobacter*, *Prevotella 9* and *Lachnospira* (Fig. 9C). *Bacteroides*, *Dubosiella* and *Alistipes* were enriched in the model group, as shown in the LEFSe analysis (Fig. 9D). *Muribaculaceae*, belonging to the *Bacteroidetes* phylum, was mostly enriched in AL-I treated group (Fig. 9D, Fig. S5C), which might explain the higher relative abundance of *Bacteroidetes* phylum than those of the model group. Noticeably, *Bacteroides* and *Alistipes* genera were dramatically reduced by AL-I compared to the model group (Fig. 9C), and all of them belong to the *Bacteroidetes* phylum.

The abovementioned bacterial species altered by AL-I might contribute to the protective effects on colitis. One of the top 10 bacterial genus *Bacteroides* is known to play a central role in the degradation of a wide range of plant polymers in the gut intestine and to possess a wide range of carbohydrate active enzymes in their genomes. However, they can also break down the host-derived mucin glycans, the major constituent in the intestinal mucosal layer [42], and have been suggested to be correlated with colitis [7,39,43]. *Dubosiella* has seldom been studied and its biological properties is still unclear. It has been reported with a positively correlation with butyric acid production and might be a potentially beneficial bacterial agent against colitis because of its lower richness in colitis mice than in normal ones [44]. However, a contrary result was recently observed with higher relative abundance in DSS-induced colitis than the normal ones [45]. Therefore, the function of *Dubosiella* on colitis and other intestinal inflammation should be further investigated by more in vivo studies. Similarly, the commonly considered beneficial bacteria *Ruminococcus* has also been observed to be increased in some colitis patients [7,39]. *Alistipes* is recently discovered as a new genus of bacteria in the *Bacteroidetes* phylum, and it is still controversy around its protective and pathogenic effects [46]. For example, *A. finegoldii* was reported showing a protective effect against colitis as it was decreased in colitis mice [43], but correlations between *Alistipes* genera and colitis and colorectal cancer were also noticed [47]. In the current study, the relative abundance of *Alistipes* genera was strikingly enhanced in the model group, but was further reduced by AL-I, which is in line with a previously published study on a mannoglucan isolated from Chinese yam [48]. The abundance of *A. finegoldii* was also found to be increased after treatment with AL-I (Fig. S6B), which, to some extent, also indicated an underlying restoration of AL-I on gut microbiota to protect against colitis. These enriched bacterial genera (*Bacteroides*, *Dubosiella,* and *Alistipes*) were significantly reduced by AL-I, which could be a potential way for pectin to protect against colitis. As for the genus of *Lachnospira*, *Prevotella*, *Parabaceroides* and *Faecalibacterium* that increased by AL-I relative to either model or control group, they have been reported with fermentative ability on pectins and their relative abundance would be increased during the fermentation [49–51]. However, the relative abundance of beneficial bacteria, such as *Akkermansia* and *Lactobacillus*, in the AL-I treated group was still statistically lower than the control group (Fig. 9C), but a mild improvement in *Akkermansia* genera was observed in Fig. 9B, C. All results demonstrated that the pectic polysaccharide AL-I could significantly reverse the microbiota changes induced by DSS treatment, and suggested that these variations on the abundance of bacteria could be involved in its protective effects against ulcerative colitis.

3.7. Correlation between metabolomic and microbiota composition

The correlation between the different metabolites in serum and different microbiota genera was determined using correlation heatmap analysis (Fig. 10). Six metabolites that increased after AL-I treatment compared to the model mice, including L-glutamic acid, D-mannose 6-phosphate, uric acid, xanthosine, orotic acid, and D-erythrose 4-phosphate (negative mode, Table S6), were classified with certain correlations with bacterial genera. Four of them were significantly negatively correlated with *Alistipes* genera. However, the *Prevotella* and *Bacteroides*

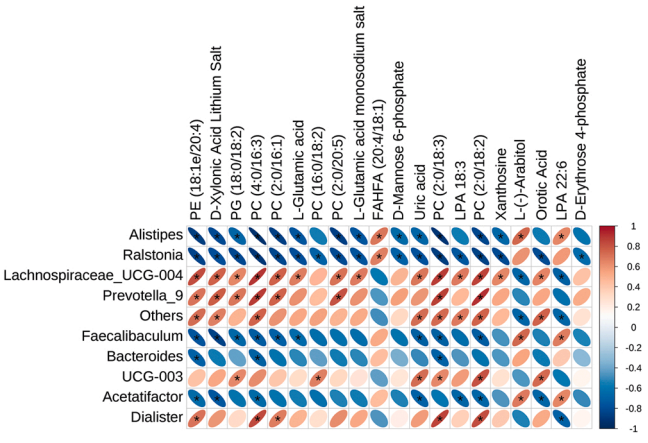

Fig. 10. Heatmap showing the correlation between the differentiated microbiota genera and identified metabolites. Metabolites included were the ones with significant differences between AL-I and the model group in negative ionization mode. * , represents that the correlation within specific metabolite and bacterial species was statistically significant ($p < 0.05$).

were correlated with other metabolites, such as PE (18:1e/20:4), PC (4:0/16:3) and PC (2:0/18:3). At the same time, it was noticed that the level of uric acid presented significant correlations with six bacterial genera, which further verified the forementioned speculation that the potent alteration of uric acid was related more to the microbiota composition modulated by AL-I. However, other bacteria that was promoted by AL-I had no significant correlation observed with these metabolites, which could be due to the relatively short intervention time in the current study. Long-term oral administration, as well as microbiota transplantation is needed in further research in order to investigate a clearer relation between gut microbiota and host metabolism. Moreover, the imbalance of intestinal inflammatory effector cells and regulatory cells contributes to strong activation of the immune system with increased inflammatory effector cells. This may also lead to prolonged or chronic inflammation, which is detrimental and closely associated with the development of ulcerative colitis [52,53]. For instance, the change in bacterial composition, as well as the resulting modulation on SCFAs, might be involved in the differentiation and expansion of T regulatory or Th17 cells and thereby introducing inflammatory and bacterial infection to the intestinal mucosa [52]. This would be of interest to further investigate on colitis mice, normal or germ-free mice, in order to identify the exact modulatory effects of AL-I on intestinal immune homeostasis.

4. Conclusions

In this study, different dosages of a pectic type polysaccharide fraction (AL-I) isolated from *A. carmichaelii* leaves were orally administrated in DSS-induced ulcerative colitis mice and significantly ameliorated colitis symptoms, attenuated serum and colonic inflammatory indices and injury on colonic histological structure. AL-I showed a potent protective effect through inhibiting the activation of NOD1 and TLR4 receptors, promoting expressions of TJ proteins and restoring the production of SCFAs and BCFAs. In addition, metabolomic biomarkers such as $_{L}$-glutamic acid, $_{D}$-mannose 6-phosphate, uric acid, xanthosine, orotic acid, and $_{D}$-erythrose 4-phosphate were modulated by high dosage of AL-I, and the dysbiosis of gut microbiota represented by *Bacteroides*, *Alistipes*, *Streptococcus* and *Ruminococcus*, *Acinetobacter*, *Prevotella 9* and *Lachnospira* genera were restored. These findings suggested that AL-I exert promising protection on DSS-induced ulcerative mice, and that the leaves of *A. carmichaelii* have a potential to be utilized as a medicinal plant resource for the treatment of inflammatory intestinal diseases.

CRediT authorship contribution statement

Yu-Ping Fu: Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Cen-Yu Li:** Data curation, Investigation, Methodology, Visualization. **Xi Peng:** Data curation, Software. **Helle Wangensteen:** Project administration, Supervision, Writing – review & editing. **Kari Tvete Inngjerdingen:** Methodology, Project administration, Supervision, Writing – review & editing. **Yuan-Feng Zou:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113767.

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Supplementary data

Pectic polysaccharides from *Aconitum carmichaelii* leaves protects against DSS-induced ulcerative colitis in mice through modulations of metabolism and microbiota composition

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Contents of supplementary materials

Supplementary methods

1. Serum metabolomics analysis

1.1 Sample processing

Fresh serum samples (100 μL) were resuspended with prechilled 80% methanol and 0.1% formic acid (FA) by well vortex. Then the samples were incubated on ice for 5 min and centrifuged at 15,000 g, 4°C for 20 min. Supernatant (400 μL) was diluted with LC-MS grade water to a final concentration of 53% methanol. The samples were subsequently centrifuged at 15000 g, 4°C for 20 min, and the supernatant was injected into the LC-MS/MS system.

1.2 UHPLC-MS/MS Analysis

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q ExactiveTMHF-X mass spectrometer (Thermo Fisher, Germany) in Novogene Co., Ltd. (Beijing, China), with Hypesil Gold column (100×2.1 mm, 1.9μm) using a 17-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 17 min. Q Exactive^{TM} HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 40 arb and aux gasflow rate of 10 arb.

1.3 Data processing and metabolite identification

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. Then, peaks were matched with the mzCloud (https://www.mzcloud.org/), mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release

6.6). When data were not normally distributed, normal transformations were attempted using of area normalization method.

1.4 Data Analysis

After annotation using databases, the principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed by metaX (a flexible and comprehensive software for processing metabolomics data). Univariate analysis (t-test) was applied to calculate the statistical significance (P-value). The metabolites with VIP > 1 and P-value< 0.05 and fold change≥2 or FC≤0.5 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest which based on log2 (FoldChange) and -log10(p-value) of metabolites by ggplot2 in R language. For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were ploted by Pheatmap package in R language. The correlation between differential metabolites were analyzed by cor () in R (method=pearson). Statistically significance of correlation between different metabolites were calculated by cor.mtest() in R. P-value < 0.05 was considered as statistically significant and correlation plots were plotted by corrplot package in R. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was performed. When ratio was satisfied by x/n > y/N, metabolic pathway was considered as enrichment. When P-value of metabolic pathway < 0.05, metabolic pathway was considered as statistically significant enrichment.

2. Methods of SCFA and BCFA analysis in the cecal content.

Fifty milligram of cecal content was weighted and extracted with 1.5 mL of deionized water, mixed well and kept in room temperature for 30 min. After centrifugation at 5000 rpm for 10 min, 100 μL of supernatant from sample solutions, as well as mixed-standards solutions (Table S4), were taken, then 20 μL of 25% (w/v) metaphosphoric acid and 1.52 μL of 210 mmol/L crotonic acid were added and incubated at 4°C for 30 min after mixing. Methanol was added with the same volume of clear supernatant (1:1 dilution) after centrifuging at 12,000 rpm/min for 10 min, and was further centrifuged at 10,000 rpm for 20 min. 1.0 μL of the supernatant filtered with a 0.22 μm membrane was injected to Varian CP-3800 GC system (Palo Alto, CA, USA) with flame ionization detector (FID), attached to a HP-FFAP column (30 m×0.535 mm×1 μm). Column temperature program was set as increasing temperature by 5°C/min from 100°C

to 150°C, and keeping for 5 min at 150°C. The carrier gas was nitrogen, at a flow rate of 2 mL/min. The inlet temperature was 270°C, and the detector temperature was 280°C.

3. Methods of gut microbiota analysis.

The genome DNA of feces was extracted using cetyltrimethylammoniumbromide (CTAB)-based extraction method and diluted to 1 ng/μL using sterile water. 16S rRNA genes of the V4 regions were amplified using specific primer (F: GTGCCAGCMGCCGCGGTAA; R: GGACTACHVGGGTWTCTAAT) with barcode, and the PCR products were purified using Qiagen Gel Extraction lit (Qiagen, Germany). TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to generate the sequencing libraries, which were assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. This library was sequenced on a NovaSeq platform and 250 bp paired-end reads were generated. These reads were merged, filtered, and compared with the Silva database (https://www.arb-silva.de/). Sequence analysis were performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/), and those with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). The representative sequence for each OTU was screened for further annotation on the Silva database (http://www.arb-silva.de/).

Alpha diversity is applied in analyzing the complexity of species diversity for a sample through 6 indices, including the observed species, Chao1, Shannon, Simpson, ACE and PD whole tree. All these indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity analysis was used to evaluate differences of samples in species complexity. Cluster analysis was performed by principal component analysis (PCA), principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) by R software. Different and enriched bacterial species among groups were analyzed by MetaStat and LDA effect size (LEfSe) with statistical analysis of t-test.

Supplementary Tables

Table S1. The DAI score evaluation system

Score	Diarrheal stool score	Bloody stool score*	Weight loss (%)
0	Normal	No rectal bleeding or bloody stool	<1%
1	Stool is soft/adheres to cage	A slight positive hemoccult in the stool	≥1-<5%
2	Moderate diarrhea/unformed stool	Hematocele in stool	≥5-<10%
3	Diarrhea/watery stool	Fresh rectal bleeding	≥10-<15%
4	/	/	≥15%

Note: *, detected using commercial occult blood test kit (BA2020B, Baso Biotechnology Co.)

Table S2. Primer sequences for qRT-PCR

Gene	Primer sequence	NCBI no.	bp
TLR4	F: CACTTTATTCAGAGCCGTTG R: AGGCGATACAATTCCAC	NM_021297.3	146
NOD1	F: TCAGCGTCAACCAGATCACC R: CACATACCTGGCTCCGAT	NM_001171007.1	125
NOD2	F: TTCCTTCTACAGCACGTCA R: AAAGATTCTCCGACCCAT	NM_145857.2	148
ZO-1	F: TCCAGTCAGCCCGCAAA R: GGCCAATCGAAGACCAT	NM_001163574.1	78
β-actin	F: GAGACCTTCAACACCCC R: AGAGCATAGCCCTCGTA	NM_007393.5	140
Occludin	F: TCTGGATCTATGTACGGCTCA R: ATATTGATCCACGTAGAGACCA	NM_001360536.1	87

Table S3. Histological assessment index (HAI) system of the pathological severity on colon tissues.

Scores	Degree of inflammatory cell infiltration	Range of inflammatory cell infiltration	Damage in intestinal crypt architecture	Loss of goblet cells
0	None	None	None	None
1	Mild /Focal	Mucosa	One third of crypt	Mild/Focal
2	Moderate /Multifocal	Mucosa and submucosa	Two third of crypt	Moderate/Multifocal
3	Severe /Diffuse	All mucosal and muscle layers	Entire crypt damage	Severe/Diffuse
4	/	Transmural infiltration	Entire crypts and epithelium damage	/

Table S4. Standard solutions with final concentrations used in SCFA and BCFA determination by GC

	Acetic acid /mmol/L	Butyric acid /mmol/L	Propionic acid /mmol/L	Isovaleric acid /mmol/L	Isobutyric acid /mmol/L	Crotonic acid /mmol/L
1	2.368	0.781	0.313	0.313	0.304	0.304
2	4.736	1.563	0.626	0.626	0.607	0.607
3	7.103	2.344	0.939	0.939	0.911	0.911
4	9.471	3.125	1.252	1.252	1.215	1.215
5	11.839	3.905	1.565	1.565	1.5185	1.585

Table S5. Differential metabolites screening within groups

	Model vs Control		AL-I vs Control		AL-I vs Model	
	(+)	(-)	(+)	(-)	(+)	(-)
Num. of total metabolites	656	469	656	469	656	469
Num. of total significantly changed metabolites	34	37	66	49	56	71
Num. of up-regulated metabolites	6	15	28	21	43	39
Num. of down-regulated metabolites	28	22	38	28	13	32

145 **Table S6** Metabolomic KEGG pathways and their differential enriched metabolites

	KEGG pathways	Total Com.	Hits Com.		Trends	P value	-log10 (p)	Impact
Model Vs. Con (neg)	Pentose phosphate pathway	22	2	D-Erythrose 4-phosphate	↓	0.020	1.692	0.030
				D-Sedoheptulose 7-phosphate	↓			
	Fructose and mannose metabolism	18	1	D-Mannose 6-phosphate	↓	0.026	1.578	0.157
	Amino sugar and nucleotide sugar metabolism	37	1	D-Mannose 6-phosphate	↓	0.026	1.578	0.081
	Purine metabolism	66	3	Adenosine diphosphate ribose	↓	0.057	1.243	0.030
				2,6-Dihydroxypurine	↓			
				Xanthosine	↓			
	Biosynthesis of unsaturated fatty acids	36	1	Docosahexaenoic acid	↑	0.068	1.169	0
Model VS AL (neg)	Purine metabolism	66	3	Uric acid	↓	0.001	2.940	0.049
				Xanthosine	↓			
				2,6-Dihydroxypurine	↓			
	Pyrimidine metabolism	39	1	Orotic Acid	↓	0.006	2.205	0.048
	Biosynthesis of unsaturated fatty acids	36	5	Linoleic acid	↑	0.011	1.956	0
				Arachidonic acid	↑			
				8Z,11Z,14Z-Eicosatrienoic acid	↑			
				Docosahexaenoic acid	↑			
				Eicosapentaenoic acid	↑			
	Linoleic acid metabolism	5	1	Linoleic acid	↑	0.012	1.907	1
	Arachidonic acid metabolism	36	2	Arachidonic acid	↑	0.014	1.867	0.333
				Prostaglandin D2	↓			
	Amino sugar and nucleotide	37	1	D-Mannose 6-phosphate	↓	0.018	1.743	0.081

	sugar metabolism							
	Fructose and mannose metabolism	18	2	D-Mannose 6-phosphate	↓	0.019	1.712	0.160
				Dihydroxyacetone phosphate	↓			
	Glycerophospholipid metabolism	36	1	Dihydroxyacetone phosphate	↓	0.074	1.131	0.019
	D-Glutamine and D-glutamate metabolism	6	2	L-Glutamic acid	↓	0.024	1.616	0.5
				alpha-Ketoglutaric acid	↑			
	Pentose phosphate pathway	22	2	D-Erythrose 4-phosphate	↓	0.036	1.448	0.030
				D-Sedoheptulose 7-phosphate	↓			
Al vs con (neg)	Arachidonic acid metabolism	36	1	Prostaglandin D2	↑	0.000	3.210	0
	Purine metabolism	66	1	Adenosine diphosphate ribose	↓	0.003	2.562	0
	Pyrimidine metabolism	39	1	Orotic Acid	↑	0.008	2.074	0.048
	Glycerophospholipid metabolism	36	1	1-Oleoyl-Sn-Glycero-3-Phosphocholine	↓	0.024	1.621	0.017
Model Vs. Con (pos)	Nicotinate and nicotinamide metabolism	15	1	Nicotinamide	↓	0.001	3.121	0.194
	Porphyrin and chlorophyll metabolism	30	1	Biliverdin	↓	0.005	2.294	0.075
	One carbon pool by folate	9	1	Folinic acid	↓	0.076	1.122	0
Model vs AL (pos)	alpha-Linolenic acid metabolism	13	1	PC(16:0/16:0)	↑	0.012	1.922	0
	Glycerophospholipid metabolism	36	2	PC(16:0/16:0)	↑	0.017	1.767	0.120
				Choline bitartrate	↑			
	Arginine and proline metabolism	38	1	4-Guanidinobutyric acid	↓	0.046	1.333	0
	Glycine, serine and threonine metabolism	34	1	Choline bitartrate	↑	0.187	0.729	0
AL Vs. Con (pos)	Glycine, serine and threonine metabolism	34	2	Betaine	↓	0.001	2.825	0.047
				Choline bitartrate	↓			

	Arachidonic acid metabolism	36	1	16(R)-HETE	↑	0.010	1.981	0
	Glycerophospholipid metabolism	36	1	Choline bitartrate	↓	0.018	1.739	0.026
	Fatty acid degradation	39	1	Palmitoylcarnitine	↓	0.027	1.559	0

Table S7. Alpha diversity indexes

	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage	PD whole tree
Control	763.60±38.07 ***	5.96±0.17 *	0.96±0.01 **	813.55±44.72 ***	818.24±43.82 ***	0.998±0.0005 *	62.01±5.56 ***
Model	487.20±137.06 ###	5.43±0.42 ##	0.94±0.01 ##	518.16±154.23 ###	521.35±153.93 ###	0.998±0.0005 #	33.69±12.93 ###
AL-I	387.75±18.08 ###	5.11±0.07 ##	0.93±0.02 ##	467.11±118.32 ###	435.41±44.82 ###	0.999±0.000 #	24.18±1.56 ###

Note: Observed species represents the number of the detected bacterial species (equals to OTUs). Shannon represents the taxonomy overview of samples; the higher value means the higher community diversity and the more uniform of the distribution of these species. Simpson represents the diversity and homogeneity of bacterial species. Chao1 and ACE represent approximately numbers of species in the community. Good's coverage represents the index of genome sequencing depth. PD whole tree represents the relationships within species of bacterial community. The statistical analysis was performed by IBM SPSS using one-way analysis of variance and LSD test. # $p<0.05$ versus control group; ## $p<0.01$ versus control group; ### $p<0.001$ versus control group; * $p<0.05$ versus model group; ** $p<0.01$ versus model group; *** $p<0.001$ versus model group; n=5.

Table S8. Beta diversity indexes

	Anosim		MRPP			
	R-value	p-value	A	Observed delta	Expected-delta	significance
Control-Model	0.924	0.006	0.1784	0.4896	0.596	0.011
AL-Model	0.624	0.008	0.1131	0.5084	0.5732	0.004
AL-I-Control	0.992	0.01	0.208	0.4502	0.5685	0.007

Anosim is used to compare the difference between samples within a group and between groups. R-value >0 means there is a larger significant difference between tested groups than within each group. MRPP is a similar method to the Anosim analysis for comparing and analyzing the microbiota structure between groups, but based on Bray-Curtis distance; a lower delta value means a less difference within each group; a higher expect delta value means a greater difference between different groups; A>0 represents the difference between groups is greater than the differences within groups; significance lower than 0.05 represents significant difference was determined; n=5.

Supplementary Figures

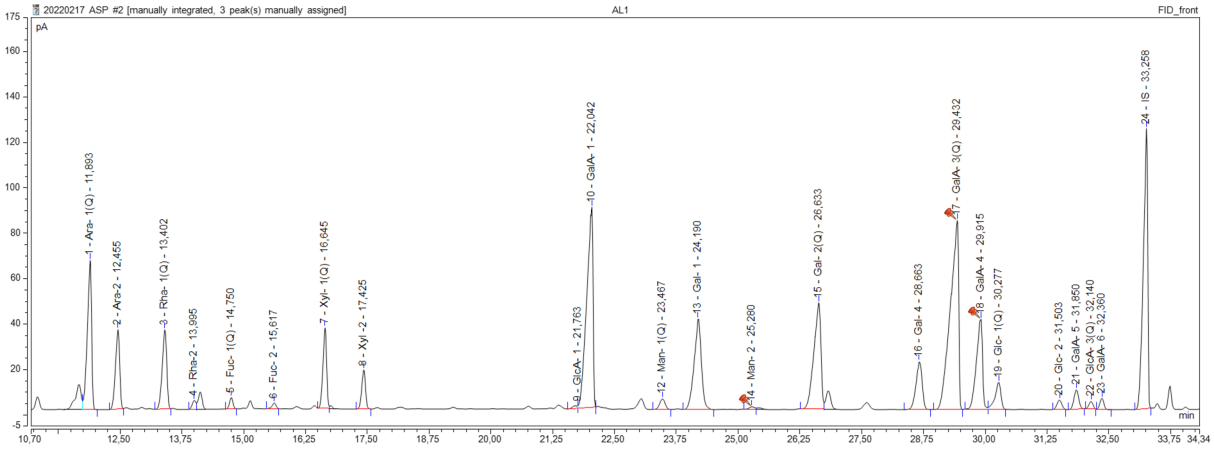


Fig. S1. GC chromatogram of monosaccharide composition of AL-I after methanolysis and TMS-derivation.

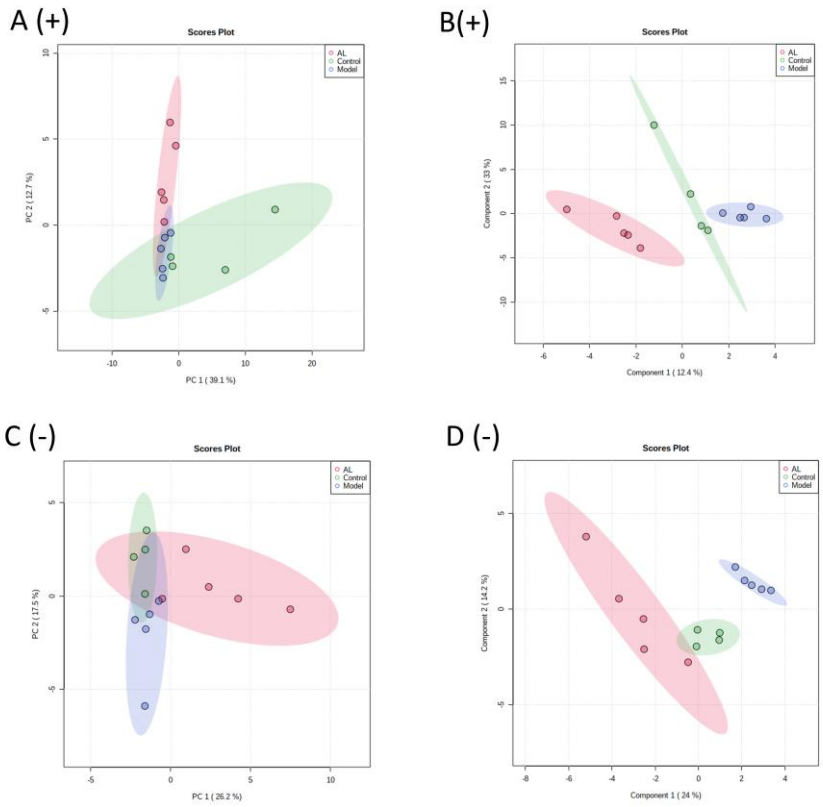


Fig. S2. PCA and PLS-DA analysis of total identified metabolites in positive (A, B) and negative mode (C, D).

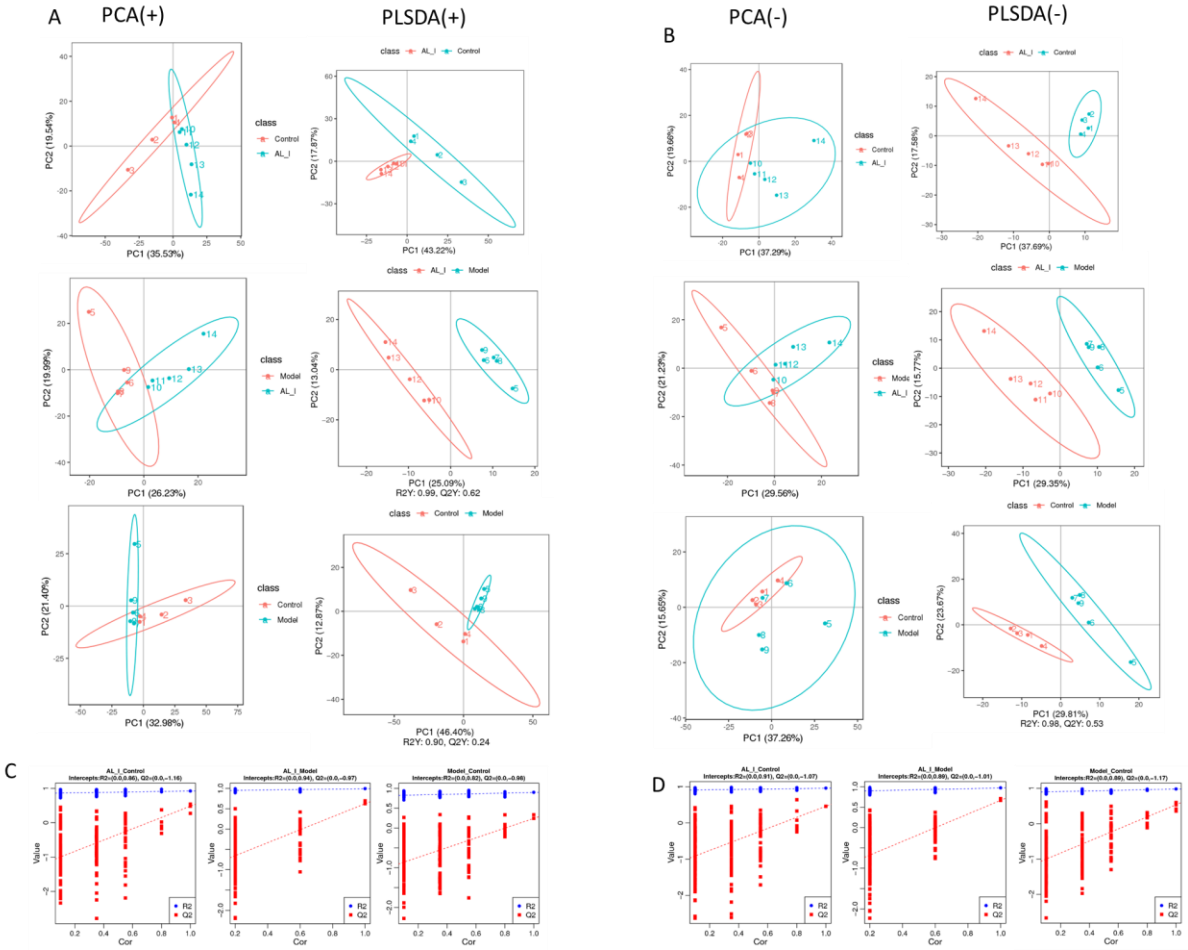
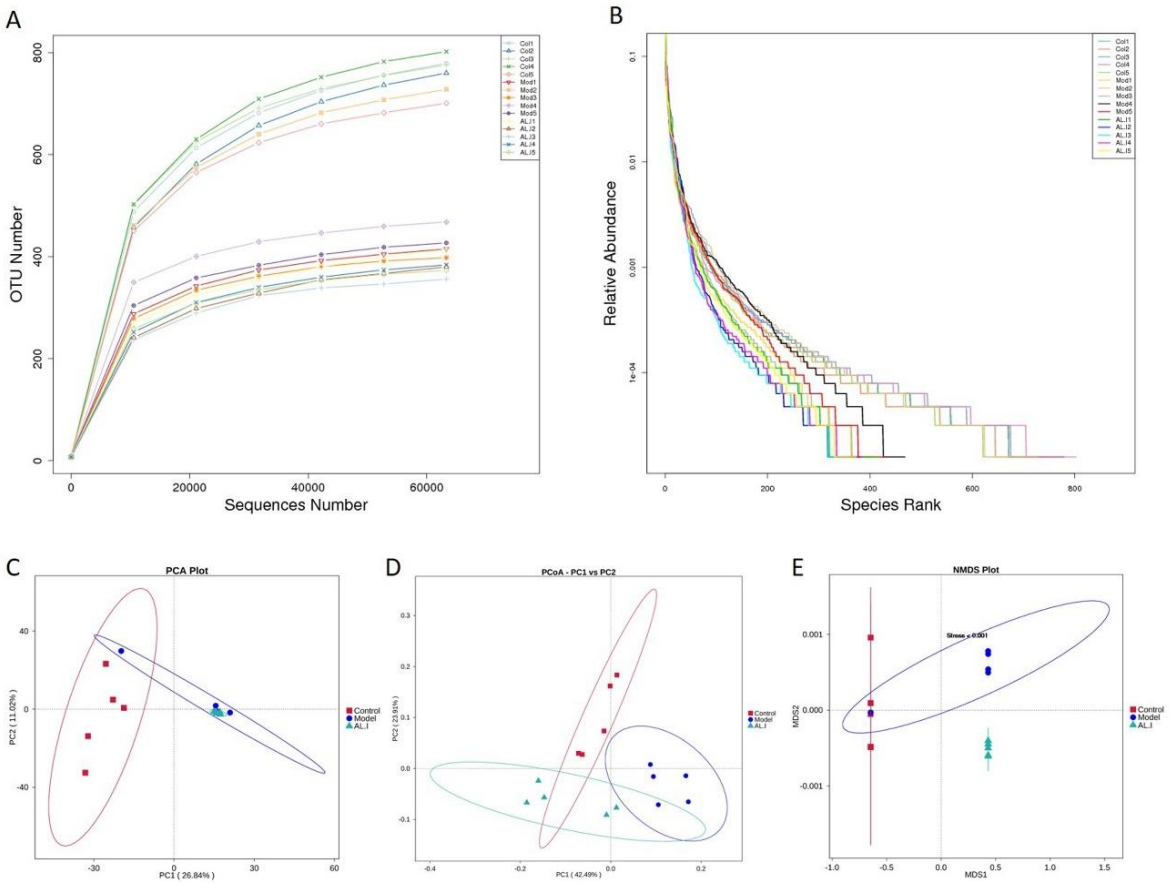


Fig. S3. PCA (A) and PLSDA (B) analysis and model evaluation (C and D) of different metabolites within groups. The trend of metabolites distribution can be observed. The analysis model of PLSDA were evaluated and identified as stable and valid model by the values of parameters R2 and Q2 (should be close to 1). When R2 is higher than Q2, and Y-axis intercept in Q2 regression line is less than 0, it indicates that the model is not "overfitting".



182

183 **Fig. S4.** The alpha and beta diversity analysis of microbiota modulated by AL-I on
184 DSS-induced ulcerative colitis. The alpha diversity analysis includes the rarefaction
185 curve (A) and rank abundance curve (B) performed based on the OTU level. The beta
186 diversity analysis includes PCA (C), PCoA (D) and NMDS (E) analysis of weighted
187 Unifrac distance.

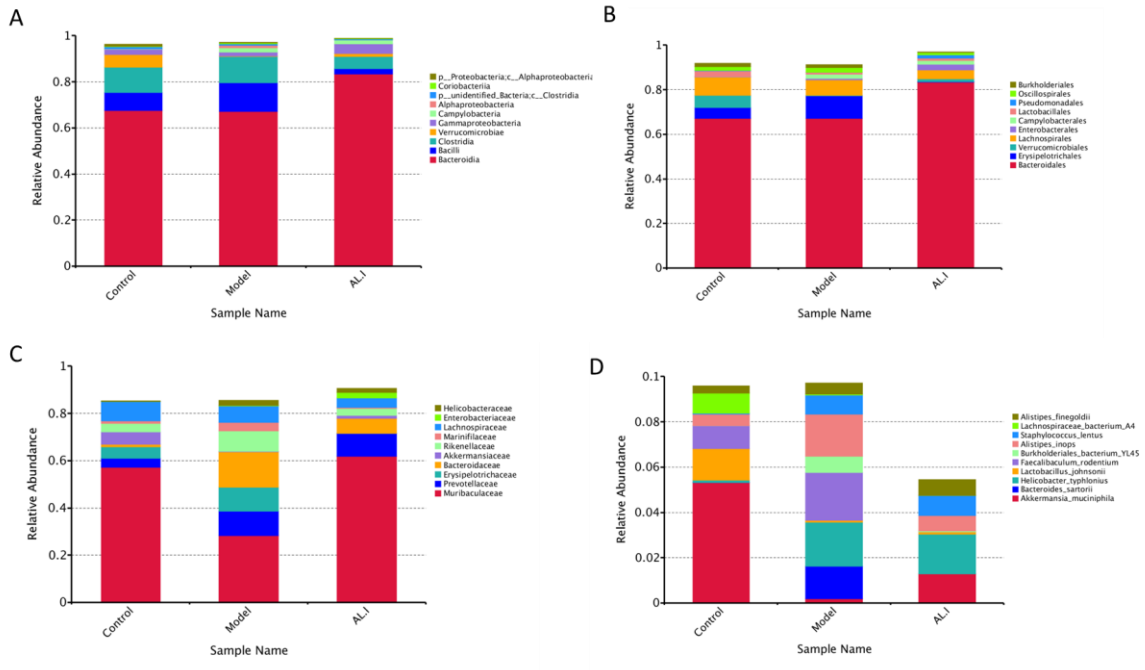


Fig. S5. The microbiota compositions at class (A), order (B), family (C) and species (D) levels regulated by AL-I on DSS-induced ulcerative colitis.

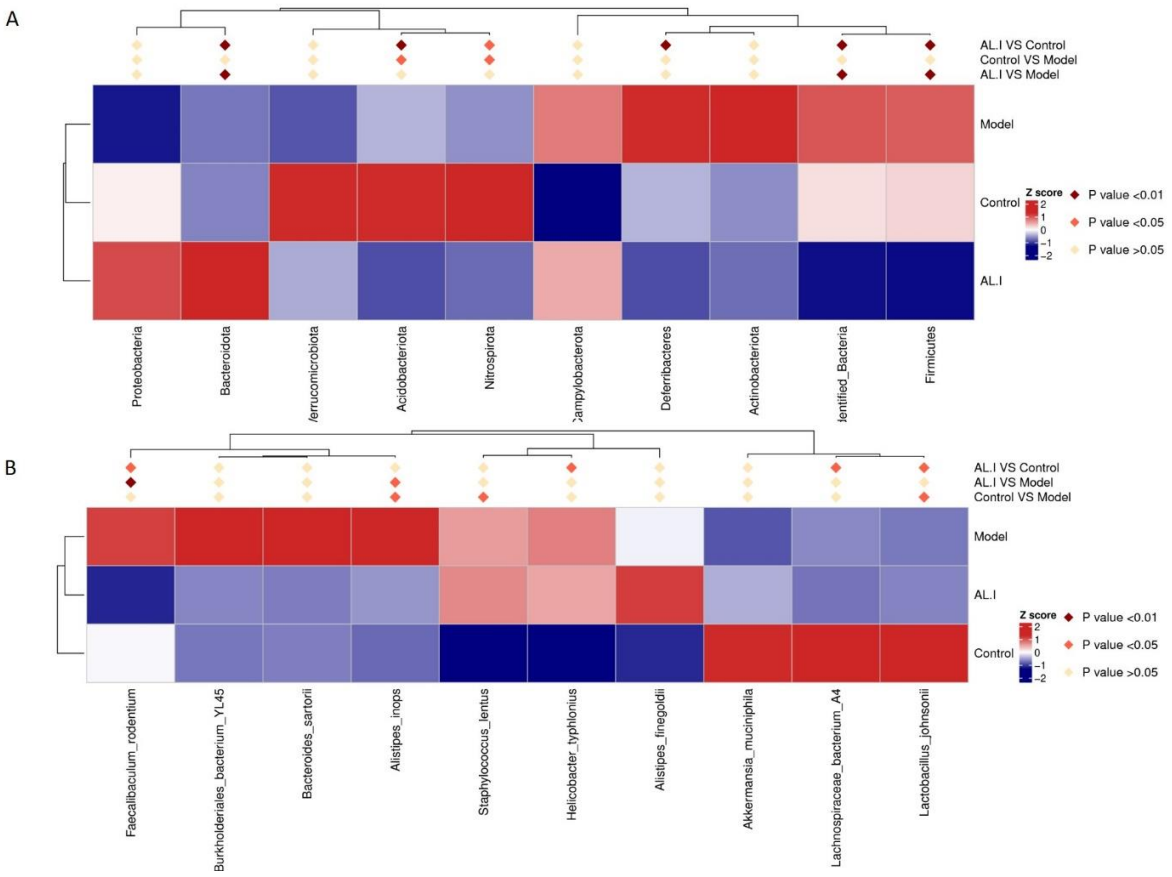


Fig. S6. The differential species performed by MetaStat at phylum (A) and species (B) levels.

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