Identification of novel pharmaceutical compounds from traditional Norwegian medicinal plants with focus on immunomodulation and the gut microbiome

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All disease begins in the gut

- *Hippocrates*

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Oslo, May 2023

Emilie Steinbakk Ulriksen

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Abbreviations

Abbreviation	Substituent name
A. archangelica	*Angeliga archangelica* subsp. *archangelica*
A. litoralis	*Angeliga archangelica* subsp. *litoralis*
ACE2	Angiotensin-converting enzyme 2
AG-II	Arabinogalactan type II
AhR	Aryl hydrocarbon receptor
ASC	Adult somatic/primary tissue stem cell
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BMP	Bone morphogenic protein
CFSE	5- (and 6)-carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptors
ConA	Concanavalin A
DAMPs	Danger associated molecular patterns
Dectin	DC-associated C-type lectin
DMSO-d_6	Deuterated dimethyl sulfoxide
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray ionization-mass spectrometry
FZD	Wnt receptor of the frizzled
Gal	Galactose
GalA	Galacturonic acid
GI	Gastrointestinal
Glc	Glucose
GM-CSF	Granulocyte macrophage colony-stimulating factor
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CRP	C-reactive protein
HG	Homogalacturonan
HPLC-DAD	High performance liquid chromatography – diode array detector
IBD	Inflammatory bowel disease

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
ISAPP	International Scientific Association of Probiotics and Prebiotics
M-cells	Microfold cells
Man	Mannose
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
Mincle	Macrophage-inducible C-type lectin
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NF-kB	Nuclear factor kappa B
NK cells	Natural killer cells
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PAMPs	Danger associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PS	Polysaccharide
PSC	Pluripotent stem cells
iPSC	Induced pluripotent stem cells
Reg4	Regenerating islet-derived family member 4
RG-I	Rhamnogalacturonan type I
RG-IÏ	Rhamnogalacturonan type II
Rha	Rhamnose
ROS	Reactive oxygen species
SEAP	Secreted embryonic alkaline phosphatase
SPE	Solid-phase extraction
TCR	T cell receptor
T_h	Helper T cells

T_{reg}	Regulatory T cells
TEER	Transepithelial electrical resistance
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
Wnt	Acronym for wingless/integrated
Xyl	Xylose

List of papers

PAPER I

The discovery of novel immunomodulatory medicinal plants by combination of historical text reviews and immunological screening assays

Ulriksen, Emilie Steinbakk; Butt, Hussain Shakeel; Ohrvik, Ane; Blakeney, Rebecca Angelica; Kool, Anneleen; Wangensteen, Helle; Inngjerdingen, Marit & Tvete Inngjerdingen, Kari Journal of Ethnopharmacology. 2022; 296:115402. doi: 10.1016/j.jep.2022.115402

PAPER II

Phytochemical characterization and anti-inflammatory activity of a water extract of *Gentiana purpurea* roots

Zhang, Lin; Ulriksen, Emilie Steinbakk; Hoel, Håvard; Sandvik, Lene; Malterud, Karl Egil; Tvete Inngjerdingen, Kari; Inngjerdingen, Marit & Wangensteen, Helle. Journal of Ethnopharmacology. 2023; 301:115818. doi: 10.1016/j.jep.2022.115818

PAPER III

Comparative chemical and immunological profiling of polysaccharides from *Angelica archangelica* subsp. *archangelica* and *Angelica archangelica* subsp. *litoralis*

Ulriksen, Emilie Steinbakk; Esam, Nabila; Butt, Hussain Shakeel; Wangensteen, Helle; Hamre Anne Grethe; Inngjerdingen, Marit & Tvete Inngjerdingen. Kari.
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Manuscript.

Outreach

- Graver i skogen og arkiver på leting etter nygamle legemidler (2020) - Norsk Farmaceutisk Tidsskrift
- News article: Gammal plantekunnskap kan gi ny medisin – Nationen (2021)
- Exhibition: Pharmaceutical Time Travels: From Ancient Plant Remedies to Modern Medicines – Natural history museum (2021)
- Workshop: Organizer of the UiO:Life Science workshop “Pharmaceutical Time Travel” at the Life Science conference 2022
- Science fair stand: Forskningsdagene, Teknisk Museum (2022)
- Exhibition: Plant Medicine: Belief and Knowledge (2023) at the Cultural History Museum, Oslo

Summary

Medicines from natural sources, plants in particular, have a long history of everyday applications to treat ailments in all human cultures around the world. These traditional uses are why many pharmaceutical companies have sought inspiration from local uses of medicinal plants to develop novel drugs. There has been a particular interest in the discovery of drugs for cancer treatment and antibiotic resistance, and in recent years, there is also a focus on how the gut microbiota can be regulated to target different inflammatory and autoimmune diseases [1]. The pursuit of natural products within pharmaceutical industry saw a decline from the 1990s onwards. In recent years however, natural products are gaining increasing interest for their many structures and effects, and are being revitalized as sources for novel drug.

Based on the need for novel drugs, and the still untapped resources in nature, we gathered a group of scientists from different fields and started an interdisciplinary work to trace the use of traditional medicinal plants within Scandinavia as potential sources for new medicines. We relied on historical texts and recollections of traditional uses of medicinal plants passed down from generations as the inspiration for this project. This turned out to be a good starting point for the project, providing information about several medicinal plants that served as a base for further exploratory work. This initial work resulted in one collective paper (**Paper I**) that again served as the basis for several sub-projects within the consortium.

In **paper I** we focused on the work on reviewing and interpreting the historical records found in the archives of the University of Oslo, reading through old firsthand accounts and wisdom passed down from generations. The work provided a detailed list with 79 plants used against stomach ailments and/or conditions interpreted to be related to immunological factors. For the purpose of this thesis, we chose to focus on 23 of these plants for the chemical isolation of substances and immunological screening. Of note, antibacterial and anti-cancer effects were also screened, but the results are not included in this thesis due to proprietary reasons. 21 of the selected plants had previously little to no descriptions of pharmacological studies within immunology. The plants were collected during field work in areas around southern Norway at similar timepoints as was noted in the historical records, and with use of the same plant parts as historically used. From the plant material, we extracted a crude water extract, and fractionated polysaccharide- and polyphenol-enriched fractions. Through the general screen, we discovered that some of the plants had potent immune stimulatory effect, while some showed immune inhibitory effects. There were large variations in responsiveness between different plants that could not be explained by differences in yields. In particular, *Daphne*

mezereum stood out, as the tested fractions showed high immune activation. Based on these results, we concluded that using traditional historical records can serve as good starting point in the search for novel pharmaceutical products.

In **paper II**, we focused on *Gentiana purpurea*, one of the plants where the polyphenol-enriched fraction showed strong anti-inflammatory properties. *G. purpurea* has a long traditional use in Norwegian folk medicine. The plant grows in mountainous regions in Norway, and the only other place where it can be found is in the Alps. It was therefore an important export herb in Norway. Additionally, no bioactivity studies had until now been performed. We isolated 11 low molecular weight compounds from a polyphenol fraction made from the *G. purpurea* root. Some of the isolated compounds, in particular gentiopicrin and amarogentin, and the degraded products erythrocentaurin and gentiogenal exhibited anti-inflammatory responses. We report for the first time the degradation product of *G. purpurea*, showing modifications of plant metabolites through the water-extraction process, with erythrocentaurin found to be the major product and having the most potent anti-inflammatory effect through dose-dependent inhibition of the pro-inflammatory cytokine TNFα.

In **paper III**, we focused on another plant with strong traditional use in Norway, the *Angelica archangelica*. The *A. angelica* root and the plant was exported from Norway to other countries around the 1500s. The use of this plant goes even further back, with mentioning's in the Icelandic sagas. Dedicated angelica gardens, protected by law, existed in Norway up to the late 1800s. The polysaccharide-enriched fraction from roots of *A. angelica* were found to induce strong immune stimulatory effect in **Paper I**. In this paper, we compared the immunostimulatory effects of the subspecies *archangelica* that grows in the mountains with the subspecies *litoralis* that grows along the seashores. It was specifically the *archangelica* subspecies that was used in traditional medicine. Anion exchange chromatography was used to obtain neutral and acidic polysaccharide fractions from polysaccharide enriched fractions of the two subspecies. Monosaccharide composition and linkage analysis found that the neutral fraction represented mainly starch, while the main acidic fractions from both subspecies contained monosaccharides and linkage types characteristic of pectins. The pectic fractions induced strong nitric oxide release from macrophages, induced secretion of both TNFα and IFNγ from peripheral blood mononuclear cells, and was demonstrated to bind the TLR4 receptor. The pectic polysaccharide fraction from *A. angelica* subsp. *archangelica* was more potent than the pectic polysaccharide fraction from *A. angelica* subsp. *litoralis*, which might be due to differences in structural confirmation.

In **paper IV**, we sought to explore how the medicinal plant polysaccharides and/or polyphenols could modulate the gut microbiota and the gut barrier. The rationale was that the traditional use of the plants in treatment of gut-related ailments were through oral intake of a tea. As polysaccharides and polyphenols are complex macromolecules, they are not absorbed by the intestine, but rather metabolized by the commensal bacteria. We thus tested whether polysaccharides and polyphenol fractions could modify the abundance of beneficial taxa, and how this could impact on the gut barrier integrity and the related immune responses. We focused on four plants: *D. mezereum*, *A. archangelica* subsp. *archangelica*, *Hypericum perforatum*, and *Ranunculus acris*. We found that the polyphenol-enriched fraction from *D. mezereum* uniquely induced higher abundance of several beneficial bacteria. Amongst these were bacteria that produces short chain fatty acids that are known to protect the gut epithelium and promote gut immune homeostasis. We also showed that the metabolites induced by culturing the polyphenol-enriched *D. mezereum* fraction with fecal material had protective effects on the intestinal organoids.

Sammendrag

Medisiner fra naturlige kilder, planter spesielt, har en lang tradisjon av hverdagslige applikasjoner i alle land rundtomkring i verden. Disse tradisjonelle bruksområdene er grunnen til at mange farmasøytiske selskap har funnet inspirasjon fra lokale tradisjonelt brukte medisinplanter for å utvikle nye medisiner. Det har spesielt vært interesse for oppdagelse av nye medikamenter mot kreft og antibiotika resistens, og i nyere tid har det også vært økende fokus på hvordan tarmbakteriene kan reguleres for å behandle forskjellige inflammatoriske og auto immune sykdommer [1]. Jakten på naturlige produkter innen farmasiindustrien opplevde en nedgang fra 1990 tallet. I nyere tid har imidlertid interessen for naturlige produkter økt på bakgrunn av deres mange strukturer og effekter, og en nedgang i nyoppdagede medikamenter.

Basert på behovet for nye medikamenter, og de enda uutnyttede ressursene i naturen, samlet vi en gruppe forskere fra forskjellige felt og startet et interdisiplinært arbeid for å spore bruken av tradisjonelle medisinplanter innen Skandinavia som potensielle kilder til nye medisiner. Vi bukte historiske tekster og gjenfortellinger av tradisjonelle bruksområder for medisinplanter, overført fra generasjon til generasjon, som inspirasjon for dette prosjektet. Dette viste seg å være et godt utgangspunkt for prosjektet og ga informasjon om flere medisinplanter som fungerte som grunnlag for videre utforskende arbeid. Det første arbeidet resulterte i en kollektiv artikkel (**Artikkel I**), som igjen fungerte som grunnlag for flere underprosjekter innenfor gruppen.

I **Artikkel I** fokuserte vi på å gjennomgå og tolke de historiske dokumentene funnet i arkivet ved Universitet i Oslo. Vi leste gjennom førstehånds beretninger og kunnskap passert videre gjennom flere generasjoner. Dette arbeidet resulterte i en detaljert liste med 79 planter brukt mot mageplager og/eller tilstander tolket til å være relatert til sykdommer og plager relatert til immunsystemet. For denne avhandlingen valgte vi å fokusere på 23 av disse plantene for kjemisk isolering av stoffer og immunologisk screening. Antibakterielle og anti-kreft effekt ble også undersøkt, men resultatene er ikke inkludert i denne avhandlingen grunnet annen prioritering. 21 av de utvalgte plantene hadde tidligere lite eller ingen beskrivelser av farmakologiske studier innen immunologi. Plantene ble samlet under feltarbeid i områder rundt Sør-Norge ved like tidspunkt som de dokumentert i de historiske kildene, og med bruk av samme plantedel som historisk ble brukt. Fra plantematerialet fraksjonerte vi ut et råekstrakt og polysakkarid- og polyfenol- berikede fraksjoner. Under den generelle screeningen oppdaget vi at noen av plantene hadde potent immunstimulerende effekt, mens andre viste immunhemmende effekt. Det var store variasjoner i responsen mellom forskjellige planter som

ikke kunne forklares ut fra forskjeller i utbytte. Spesielt skilte *Daphne mezereum* seg ut gjennom den høye immun aktiveringen observert i ekstraktene. Basert på disse resultatene konkluderte vi med at det å bruke tradisjonelle historiske kilder kan fungere som et godt utgangspunkt i søken etter nye farmasøytiske produkter.

I **Artikkel II** fokuserte vi på *Gentiana purpurea*, en av plantene hvor det polyfenol-berikede ekstraktet viste sterk anti-inflammatorisk effekt. *G. purpurea* har en lang historie med tradisjonell bruk i norsk folkemedisin. Planten vokser i fjellområder i Norge, og den eneste andre plassen hvor den er funnet er i Alpene. Den var derfor en viktig eksporturt i Norge. I tillegg hadde ingen bioaktivitetsstudier blitt utført på denne planten tidligere. Vi isolerte 11 lavmolekylære stoffer fra et polyfenolekstrakt fra *G. purpurea* roten. Noen av de isolerte stoffene, spesielt gentiopicrin og amarogentin, og de degraderte stoffene erythrocentaurin og gentiogenal viste anti-inflammatoriske effekter. Vi rapporterte for første gang degraderingsproduktene fra *G. purpurea* og viste endringer av plantemetabolitter gjennom vann-ekstraksjons prosessen, med erythrocentaurin funnet å være hoved produktet og ha mest potent anti-inflammatorisk effekt gjennom dose-avhengig hemning av den pro-inflammatoriske cytokinen TNFα.

I **Artikkel III** fokuserte vi på en annen plante med betydelig tradisjonell bruk i Norge, *Angelica archangelica*. *A. archangelica* roten og planten var eksportert fra Norge til andre land rundt 1500-tallet. Bruken av denne planten går enda lengre tilbake hvor den er nevnt i de Islandske sagaene. Dedikerte Angelica hager, beskyttet av loven, eksisterte i Norge helt opp til slutten av 1800-tallet. Det polysakkaridberikede vannekstraktet fra roten til A. archangelica var funnet å indusere sterk immunstimulerende effekt i **Artikkel I**. I denne artikkelen sammenlignet vi den immunstimulerende effekten av underarten *archangelica* som vokser i fjellområder med underarten *litoralis* som vokser langs kystlinjen. Det var hovedsakelig *archangelica* underarten som var brukt i tradisjonell medisin. Anion utbytte kromatografi ble brukt for å isolere en nøytral og to sure polysakkaridfraksjoner fra røttene. Monosakkaridsammensetningsanalyse fant at den nøytrale fraksjonen hovedsakelig representerte stivelse, mens en av de sure fraksjonene inneholdt monosakkarider karakteristisk for pektiner. Denne sistnevnte fraksjonen induserte sterk nitrogenoksid frigjørelse fra makrofager, induserte utskillelse av både TNFα og IFNγ fra mononukleære celler i perifert blod og ble funnet å binde til TLR4 reseptoren. Pektin polysakkaridene fra *A. angelica* subsp. *Archangelica* var mye mer potente enn pektin polysakkaridene fra *A. angelica* subsp. *litoralis*, som kan støtte den selektive tradisjonelle bruken av *archangelica* underarten

I **Artikkel IV** ønsket vi å utforske hvordan medisinplante polysakkarider og/eller polyfenoler kunne endre tarmbakteriene og tarmbarrieren. Begrunnelsen for dette var at tradisjonell bruk av planter i behandling mot mage og tarm-relaterte plager var normalt gjennom oralt inntak av te. Ettersom polysakkarider og polyfenoler er komplekse makromolekyler så blir de ikke absorbert i tarmen, men blir i stedet metabolisert av tarmbakterier. Vi testet derfor om polysakkarid- og polyfenolekstrakter kunne endre mengden av gunstige bakterie arter, og hvordan dette kunne påvirke tarm barriere integriteten og relaterte immunresponser. Vi fokuserte på fire planter; *D. mezereum*, *A. archangelica* subsp. *archangelica*, *Hypericum perforatum*, og *Ranunculus acris*. Vi fant at den polyfenol berikede fraksjonen fra *D. mezereum* hadde en unik evne til å indusere høyere mengder av flere gunstige bakterier. Blant disse var bakterier som produserte kortkjedede fettsyrer som er kjent for å beskytte tarmepitellaget og promotere immun homeostase. Vi viste også at metabolittene indusert av kultivering av den polyfenolberikede *D. mezereum* fraksjonen sammen med avføringsmateriale hadde beskyttende effekt på tarmorganoider.

1 Introduction

1.1. Medicinal plants and new drugs

Local plants have been used as medicine for treating diseases and other ailments for as long as there have been humans, and probably even predating humans, with recordings of plants used as medicine by animals as well [2-9]. The longstanding use of traditional medicinal plants is what makes them interesting for pharmaceutical companies as sources for novel medicines. Ethnopharmacology is a growing field, emphasizing the importance of these traditional uses. It is today defined as "the interdisciplinary scientific exploration of biologically active agents traditionally employed by man" [10].

Of available drugs on the market today, about 40% are derived from a natural origin [11]. Between 1981 and 2019, 1881 new chemical entities were approved as new drugs and among these drugs, 441 (23%) were of natural origin or made from a natural component, while 930 (49%) were either a natural product, a natural product derivative or the structure was inspired by a natural product [11, 12]. As of 2018, terrestrial plants were still the main source of new natural compounds [13]. There are several well-known examples of traditional medicinal plants used in modern medicine. Among the most famous are probably morphine from *Papaver somniferum* (Opium poppy), paclitaxel (Taxol®) from the bark of *Taxus brevifolia* (Pacific yew tree), artemisinin from *Artemisia annua* (Sweet wormwood), and atropin from *Atropa belladonna* (Belladonna/Deadly nightshade) [14-17].

The diversity of plant metabolites is why plants are central in the discovery of novel pharmaceuticals. Genetic analysis estimates that based on the number of genes, a single plant species can have between 5 and 15 000 metabolites, which is much more than what is found in microorganisms (appr. 1500) or animals (appr. 2500) [18-20]. Within these metabolite groups, the diversity can be immense, and even minor changes in the chemical structure like a change in a methyl or hydroxyl moiety can change the pharmaceutical property of the molecule. A small chemical difference can mean a major biological difference [13].

Of the about 310.000 plant species described worldwide, only 6% have been pharmacologically investigated and 15% have been analysed for their phytochemical properties. This leaves many species and compounds yet to be investigated [13, 21].

The need for interdisciplinary work to discover new drugs from plants have previously been stated by several researchers [21-23]. We have in this project investigated historical records of medicinal plants used in Scandinavia for centuries. Based on the plant medicinal

uses, previous pharmacological studies, and occurrence in different historical sources, we selected 23 plants for further study. These 23 plants were collected from publicly available areas in southern Norway, properly identified by botanists in the project team, and chemical extraction and fractionation were performed to give three main plant extracts: a crude water extract, a polysaccharide-enriched fraction, and a polyphenol-enriched fraction. Based on the broad immunological screens performed in **Paper I**, six of the plants were selected for further investigations in this thesis (*Daphne mezereum*, *Hypericum perforatum*, *Angelica archangelica* subsp. *archangelica* (*A. archangelica*), *Angelica archangelica* subsp. *Litoralis* (*A. litoralis*), *Gentiana purpurea*, and *Ranunculus acris*). With primary focus on *D. mezereum*, *A. archangelica* and *G. purpurea*.

1.1.1. Norwegian and Scandinavian medicinal plants

There has been strong reliance on local medicinal plants and traditional uses of these in Norway and other Scandinavian countries. The traditional preparations of ingested medicinal plants were often in liquor, as infusions or decoctions. Either the whole plant or parts of the plant were used as medicine.

Despite common uses, traditional Scandinavian medicinal plants have received little attention for their potential pharmaceutical effects. The immense genetic diversity of plants is the basis for the high diversity of plant metabolites and compounds that can vary depending on location and time of harvest. Because of the harsher colder Nordic climate, the midnight sun with high UV radiation, and the vast amount of mountain areas, it is logical to assume that the chemical composition of Norwegian plants may differ from other plants in other parts of the world. Factors like UV radiation and temperature are previously found to influence plant secondary metabolites [24, 25]. Both differences in ecological location and differences between plants collected at different time points are the reason why so many different plant compounds exists, and why it is necessary to search for new compounds that can serve, directly or indirectly, as new molecules in drug discovery [13].

1.1.2. Angelica archangelica

Angelica archangelica L. (Norwegian: “kvann”) is a perennial plant in the Apiaceae family that grows to be about 0.5-2 meters high. For several years, the plant present as a leaf-plant while gathering nutrients in the root. When the plant has sufficient nutrient storage, the flower stem appears, and the plant is ready to spread its seeds. It can take between 2-7 years before

the flowering occurs. After completing this process and the seeds are spread, the plant dies [26]. Angelica is native in Norway, Sweden, Iceland, Greenland, the Faeroes, Finland, Russia, and eastern parts of continental Europe [27]. In Scandinavia, angelica were one of the first vegetables harvested during early spring and were therefore a valuable vitamin supplement after long winters in these northern colder countries [28].

Around the 1500-1600s, large amounts of angelica were exported from Norway to other countries and angelica is the only vegetable of Nordic origin known to enter the world trade market from Norway [28]. During the last 200 years, the export of angelica from Norway has decreased in favour of increased import of angelica from other countries. Today, the largest market for angelica plants is for the essential oils in the roots [28]

Angelica was a central and important plant in Norwegian households all the way up to the mid-1900s. This is emphasized by the dedicated angelica gardens dating back to the Viking age and the mentioning of this plant in different Norwegian laws dating back to the 11th century AD (e.g. "Gulatingsloven"). By stealing another person's angelica plant, you were declared to have no legal rights and could be punished as the owner saw fit [27, 28]. "Vossakvann" is now the name of the *A. archangelica* subsp. *archangelica* species that have survived from Norwegian angelica gardens [29].

The name angelica stems from the Latin name for angel. The name were allegedly given to the plant after a monk in the 17th century (a time of great disease and death in Europe) claimed to have been visited by the archangel Gabriel in his sleep and the angel told him of the healing powers of the angelica root against the plague and other ailments [28, 29]. The root was said to help against everything from rabies and colic to bad vision and hearing. The name was further amplified by the adding of archangelica- the arch angel [28, 29]. Another testimony to the importance of angelica in Norway is the number of places and topographic landscapes named after this plant. Over 50 names beginning with "kvann-" is found in a Norwegian atlas [27].

In Norway, there are two subspecies of the angelica plant, *A. archangelica* subsp. *archangelica* (defined as *A. archangelica* in this thesis) and *A. archangelica* subsp. *litoralis* (Fr.) Thell (defined as *A. litoralis* in this thesis). *A. archangelica* is the largest, most aromatic and most used of the two. *A. litoralis* on the other hand has a sharper taste and was not widely used [28]. The English name "wild angelica" is misleading regarding these two plant species, as this refers to "sløke" (*Angelica sylvestris* L.). *A. sylvestris* looks like *A. archangelica* and belong to the same *Angelica* genus, but these are two separate plant species. Sløke was not widely used in Scandinavia for medicine or food [27, 28].

The most important substances in angelica is the bitter substances angelicin and essential oils. These substances give the plant a strong aromatic flavour and smell, and are regarded as an important factor explaining why angelica were an important vegetable and medicinal remedy [26]. For medicine, the roots were primarily used.

Today, different *A. archangelica* plants are cultured on a large scale in several countries, including Germany, Belgium, Holland, Poland, and France [30]. Its main uses are in herbal medicine, and in the production of various alcoholic beverages, like vermouth, Bénédictine, Chartreuse, and gin. In France, angelica is cultured for use in confectionery in Niort, and apparently on a lesser scale in Clermont-Ferrand and Apt [31]. *A. archangelica* is also an ingredient in a medical product called Iberogast that is used against gastrointestinal diseases.

1.1.3. Daphne mezereum

Daphne mezereum L. (Norwegian: "tysbast") is a small leafy flowering bush in the Thymelaeaceae family that can grow to be between 0.5-1 meters high. The Daphne genus has over 90 species found to grow wild in forest areas in Europe, North Africa and West-Asia. In Scandinavia, however, *D. mezereum* is the only wild growing species in the Thymelaeaceae family [26, 32].

Similar to other plants in the Thymelaeaceae family, the stem and bark from *D. mezereum* is known for its mild toxicity and bitter smell and taste [26, 33]. Eating the bark and stem raw are documented to leave the person with a burning sensation in the mouth and swelling of the throat. Despite its toxicity, there are recordings of the bark being used for medicinal purposes. When used as medicine, it was diluted in liquor or boiling water. The bark was used against several different ailments like the cold, headaches, toothache, diarrhoea, stomach pain, rickets, pneumonia, tuberculosis and as an abortion remedy [26].

The medicinal uses of other plants from this genus have usually also been the bark, and several phytochemicals like coumarins, flavonoids, lignans, steroids and terpenes have been identified from different daphne species and are thought to be the reason for the pharmacological and biological activities [32]. Analysis of the phytochemicals in *D. mezereum* have found daphnin (a glucoside of daphnetin), coumarins and terpenoids to be the major components [33-38]. Daphne diterpenoids which are the characteristic compounds of the *Daphne* genus, are the compounds found to be responsible for the toxic effect [32]. The plant is known for its toxicity, but no scientific studies are conducted on the biological activities of

D. mezereum from Norway. Previous studies have found several *Daphne* species from different countries and climates to have antioxidant and anti-inflammatory effects. Some *Daphne* species are also found to have analgesic effect, cytotoxic effect against different cancer cell lines (among them colon and rectal), anti-HIV-1 effect, and haemostatic effects [32].

1.1.4. Gentiana purpurea

Gentiana purpurea L. (Norwegian: "søterot") is a perennial plant with dark purple corollas, historically known among Norwegian people for its bitter tasting roots (hence the ironic Norwegian name "sweet-root"). It belongs to the Gentianaceae family and can grow to be 0.2-0.8 m tall. The name comes from "Gentius", a king of Illyria in Europe believed to have discovered the medical properties of the root. Different species of the *Gentiana* plant also has names referring to the bitter taste, like "karru" meaning bitter in Himalaya for the *Gentiana kurroo* Royle found in the Kashmir Himalayas [39, 40]

G. purpurea is not widely distributed in Europe and is only known from mountain areas in southern Norway and the Alps [40]. During the 18th and 19th century, *G. purpurea* was among the most widely used and important medicinal plants in Norway. Among the most common solutions used to prepare extracts of *G. purpurea* roots were water, alcohol (liquor and beer), milk and cream. Diseases commonly treated by these root preparations were stomach related diseases, colic, chest diseases like bronchitis, coughing and the cold [41]. Because of the bitter substances in the plant, it was also regarded as an appetite stimulant.

Despite its significant uses and importance in Norwegian culture, no extensive research is conducted on the Norwegian *G. purpurea* plant. Some studies from other *Gentiana* species have found it to exhibit anti-inflammatory properties through cytokine inhibition and reduction in pro-inflammatory enzymes [42]. This, if true for the Norwegian species, could explain its common uses against chest diseases and for treatment against coughing and the cold [41]. Other studies find *Gentiana* species to have protective effect on the hepatic system, gastrointestinal tracts, and cardiovascular system, influencing vascular disease development, and exert immune modulatory effects [43, 44].

Different compound have been recorded for *Gentiana* species, with the most common being iridoids, xanthones and flavonoids, with iridoids appearing to be present in all species [45]. *G. purpurea* contains a biphenyl iridoid glucoside known as amarogentin (chirantin) that is so bitter that even at 1:58,000,000 dilutions, you can taste the bitterness. Because of this property, amarogentin is commonly used to scientifically measure bitterness [39, 46-48].

1.2. Phytochemicals

Plant extracts are composed of several different compound mixtures that can act synergistically to yield the observed or desired effect. These plant extracts therefore have the ability to hit a diversity of biological targets [49]. An example of this is the red sage (*Salvia miltiorrhiza*) leaves that are used against liver fibrosis, where at least three of the compounds found in the extract need to be present to achieve the desired effect [50].

Plants make primary and secondary metabolites, where primary metabolites include carbohydrates (including polysaccharides), amino acids, proteins and fats that are directly involved in plant growth, development and metabolism. Secondary metabolites on the other hand, are primarily low molecular weight compounds including flavonoids, coumarins and other polyphenols, terpenoids and alkaloids that are not essential for plant growth but are made for defence and protection for the plant to survive in the given environment. These secondary metabolites are commonly thought of as having medicinal and pharmaceutical values, with over 100 000 structures being describes as of 2020 [51].

A water decoction of plants will contain both polar high molecular weight macromolecules like polysaccharides and semi-polar low molecular weight substances like phenolic compounds. Because high molecular weight compounds are more complex and therefore more difficult to purify, identify and test biologically, early research primarily focused on low molecular weight polyphenols as potential active compounds for biological modulation. The advances in modern chemical, immunological and pharmaceutical testing methods have made detailed analysis of low molecular weight polyphenol compounds and analysis of high molecular weight polysaccharide and polyphenols possible. Several studies are now focusing on interpreting the effect these compounds have as immune modulators with potential health benefits.

1.2.1. Plant polysaccharides

Plant polysaccharides are a group of biopolymers consisting of 10 or more single monosaccharide units that in plants have a structural function or serve as energy storage. The plant cell wall consist of several different polysaccharides, among them are cellulose, hemicellulose and pectins, together with other structural proteins, enzymes and the phenolic polymer lignin that modify the physical and chemical properties of the plant cell wall [52]. Polysaccharides are usually composed of either five carbon (pentose) or six carbon (hexose)

monosaccharides bound by an O-glyosidic bond [53]. Though polysaccharides generally are macromolecules, their molecular weight distribution, structure and the molecular size can vary between the different types of and sources of polysaccharides. Another factor that influences polysaccharide characteristics is whether the structure is branched or linear, or if it is a homopolysaccharide consisting of only one type of monosaccharide or a heteropolysaccharide consisting of different monosaccharides. Examples of homopolysaccharides are starch and cellulose that only consist of glucose (Glc) units. Heteropolysaccharides are usually more complex and include polysaccharides like pectin and hemicelluloses [53].

Another important characteristic of polysaccharides is charge. Most commonly, polysaccharides have either a neutral charge, like starch and cellulose, or a negative charge (anionic), which is the case for polysaccharides with acid groups in their monosaccharide units, for example pectins that contain galacturonic acid (GalA). No plant polysaccharides are found to have a positive charge (cationic) [53].

The last decades have seen a surge of research on polysaccharides. Much research is now focused on the potential immunomodulatory properties of these compounds [54, 55]. One of the big advantages of polysaccharide compounds as therapeutics is that they are generally considered biocompatible, biodegradable and non-toxic [56]. Among the many different polysaccharides found in medicinal plants, the pectins with arabinogalactan side chains or pure arabinogalactans are considered among the most biologically active carbohydrates [57]. Pectins are the plant polysaccharides primarily shown to have immune modulatory effects, and previous studies have found structure-activity relationship between these molecules and cells of the immune system [52, 58].

Pectins

Pectins are heteropolysaccharide dietary fibre, primarily found in the plant cell wall where they function as structural component supporting the cellulose in the matrix. Pectins are defined by the presence of GalA and form some of the most complex macromolecule structures in nature [52]. The best known pectin structural units include the galacturonans like homogalacturonan (HG) characterized by a α-1,4-linked GalA backbone, rhamnogalacturonan-I (RG-I) which has a structural backbone consisting of rhamnose (Rha) and GalA residues, with neutral side chains, and the more complex rhamnogalacturonan-II (RG-II). Schematic drawings of HG and RG-I are shown in **Figure 1** [52].

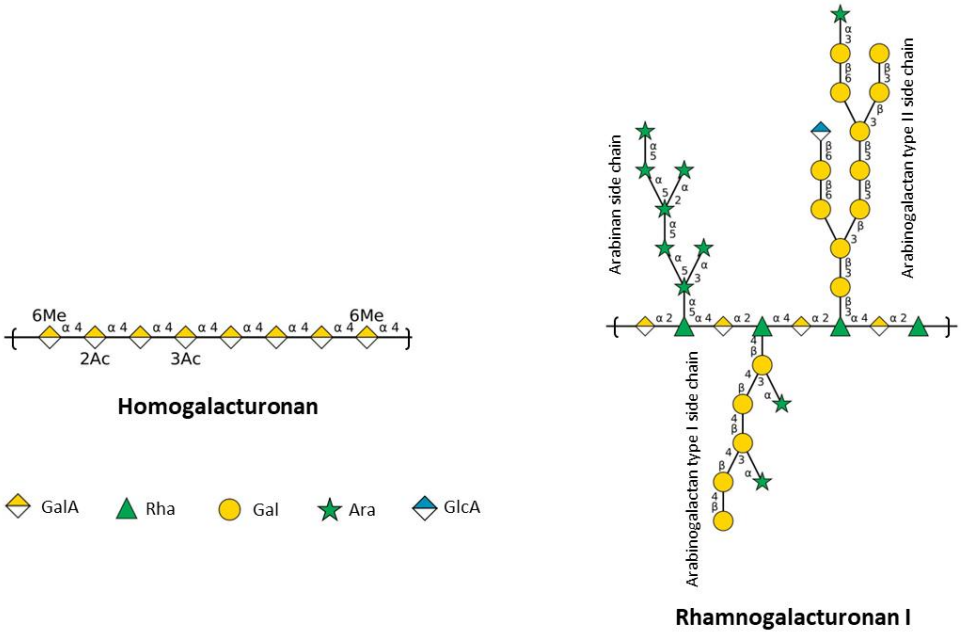

Figure 1. Pectic polysaccharide structure. Pectin polysaccharides are made up of several structural elements, including homogalacturonan and rhamnogalacturonan type I which are depicted here. The structures are drawn using DrawGlycan [59]

The differences in charge and conformation of the side chains in the pectin structure might create different microenvironments within the molecule. This is likely to play a role in the different biological activities observed from pectins of different origin [60]. Another factor that can influence the pectin structural characteristics are methyl-esterification and acetylation of the GalA-units, and the amount of either of the substituted galacturonans RG-I or RG-II [58].

Pectin polysaccharide structures are not only found to influence the immune system, they have also gained much attention for their potential to influence the gut microbiome and improve intestinal barrier function [58, 61-63]. They are thought to have positive effects on the gastrointestinal immune barrier and thus potentially reduce the risk of inflammatory bowel disease (IBD), leaky gut and infections caused by pathogens penetrating the barrier and entering circulation [58]. Bacteria in the gut can break down the pectin structure and produce secondary metabolites called short chain fatty acids (SCFAs) that provide energy to intestinal epithelial cells. Pectins can also favour adhesion of commensal bacteria and inhibiting adhesion of pathogens to epithelial cells, thus protection the gastrointestinal barrier [58]. The direct pectin effect in the gut includes strengthening of the mucus layer, enhancing the epithelial integrity and influencing dendritic cells and macrophages by having an activating or inhibiting effect. The interaction of pectin structures with immune cells are hypothesized to be through

pattern recognition receptors (PRRs) like Toll-like receptor (TLR) 2 and 4 (described further in section 1.3.3) [58].

1.2.2. Plant polyphenols

Polyphenols are compounds consisting of multiple phenol elements and are the largest class of secondary plant metabolites. A phenol consists of a phenyl ring ($-C_6H_5$) with an attached hydroxyl group. The hydroxyl groups are often linked to one or more sugar residues, but direct linkage to the aromatic carbon ring of monosaccharides or polysaccharides also occur. They can also be attached to methyl groups, organic acids or isoprene units. Classification of polyphenols are usually done based on the number of phenol rings and the structural elements that bind the rings together [64]

Polyphenols comes in a variety of sizes and structures. In plants, polyphenols are phytochemicals important in the defence against radiation from UV lights, protection against induced oxidative stress or against pathogens [64]. For humans, the main dietary polyphenols are coumarins, flavonoids (the largest and most studied group), lignans, phenolic acids, stilbenes and tannins (**Figure 2**) [65]. Flavonoids are known for being responsible for the strong colours of flowers, fruits and leaves, and are among the most widespread polyphenols in human food [66, 67]

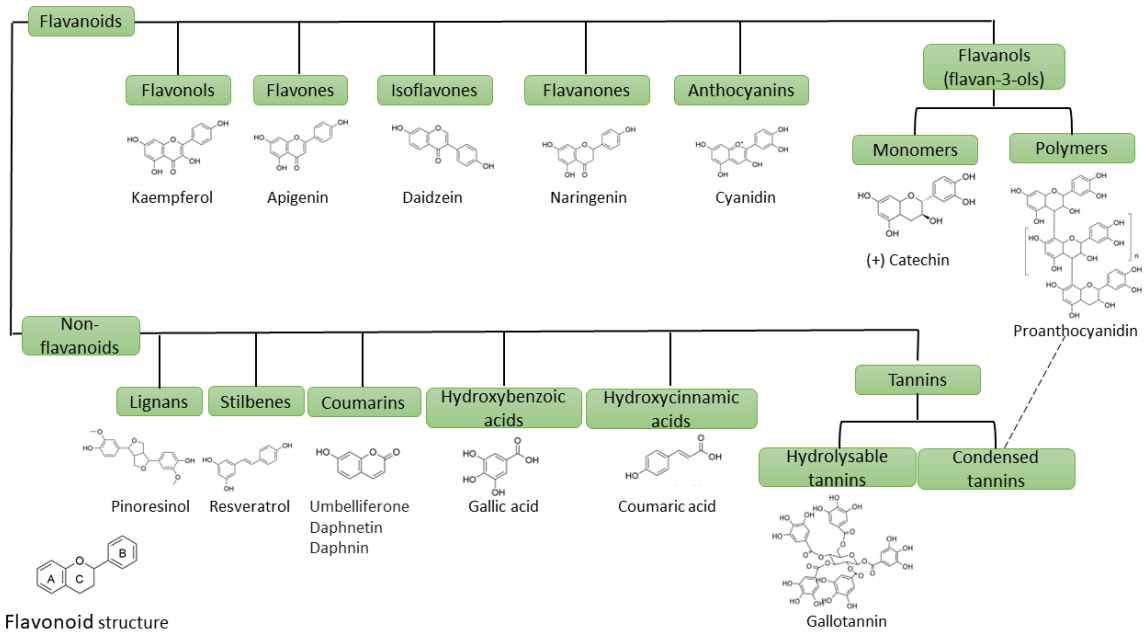

Figure 2: Dietary polyphenols and their structures. Representation of polyphenols commonly found in food and a structure of the basic structure for flavonoids. Dotted line is drawn to represent that proanthocyanidin are the same as condensed tannins

Polyphenols have since the 1990s gained much attention for their antioxidant and anti-inflammatory effects [68]. Increased bodily oxidation without the proper endogenous antioxidant effects will generate high number of free radicals like reactive oxygen species (ROS), that will readily grab electrons from proteins, DNA and lipids, turning these again into endogenous free radicals, creating a chain reaction. Oxidative damage and the subsequent inflammatory responses resulting from excessive free radicals are associated with cell membrane damage, degenerative diseases like Parkinson's disease and Alzheimer's disease, cancer and aging [69-73]. Vitamins have previously received much attention for their antioxidant effect, but polyphenols with their hydroxyl group on the phenolic structure and conjugated double bond give them a structure that better function as antioxidants than vitamins [74].

During the start of 2000, it became clear that the bioavailability of polyphenols and their metabolites after ingestion was quite low and that they therefore might not have an antioxidant activity by directly scavenging free radicals but instead acted by modulating biomarkers of oxidative stress in cells and influencing cell signalling pathways [75]. Increased focus were placed on the link between oxidative stress, inflammation, chronic disease and cancer [76].

The last decade has seen an increasing focus on the effect of polyphenols on the gut microbiota. The bioavailability of polyphenols is low when ingested, only 5-10% of ingested polyphenols reach circulation through absorption in the small intestine [69]. Most polyphenols are thought to reach the large intestine where they are fermented by the gut microbiota. Polyphenol microbiota metabolites include SCFAs and phenolic acids found to have health promoting effects in the gut [77].

1.3. The innate and adaptive immune system

The immune system is an intricate and complex machinery of different cells, chemical signals and molecules acting together to remove potential dangers and keep the body healthy. Dangers to the body can present themselves in the form of pathogens like viruses or bacteria, foreign molecules, and malfunctioning or damaged body cells ("altered self"). All these different potential dangers make the work of the immune system a complex one, and the importance of a well-functioning immune system a potential life-and-death situation. Compounds able to interact with the immune system and up- or down-regulate host immune responses are known as immunomodulators and can potentially be used in treatment to tune the immune response

[78]. Among such modulators we find vaccines that induces specific immune responses towards a pathogen, CRISPR gene editing in cancer immunotherapy, and diverse phytochemicals as described in Section 1.2 [79, 80].

The immune system can be divided into two parts, the innate (general) and the adaptive (specific) immune system, with the constituent innate and adaptive immune cells (**Figure 3**). The innate immune system is the first line of defence that works together with physical barriers like the skin and the mucosal barriers to protect the body against immediate dangers. Innate immune cells can be found within these physical barriers and in near proximity of them. Dendritic cells and macrophages guard these barriers, recognising pathogens through their receptors and initiate immune responses. Dangers arising within the body can also be recognized by innate immune cells. Cells of the innate immune system have a rapid and generalized response towards foreign molecules. The cells of the adaptive immune system have refined specificity towards a particular antigenic structure, this however occurs at the cost of numbers and time. The adaptive immune cells take longer to mount a full response as they need to go through an activation and expansion phase at the first encounter of a pathogen or foreign structure. However, once activated, they provide a tailored and efficient response.

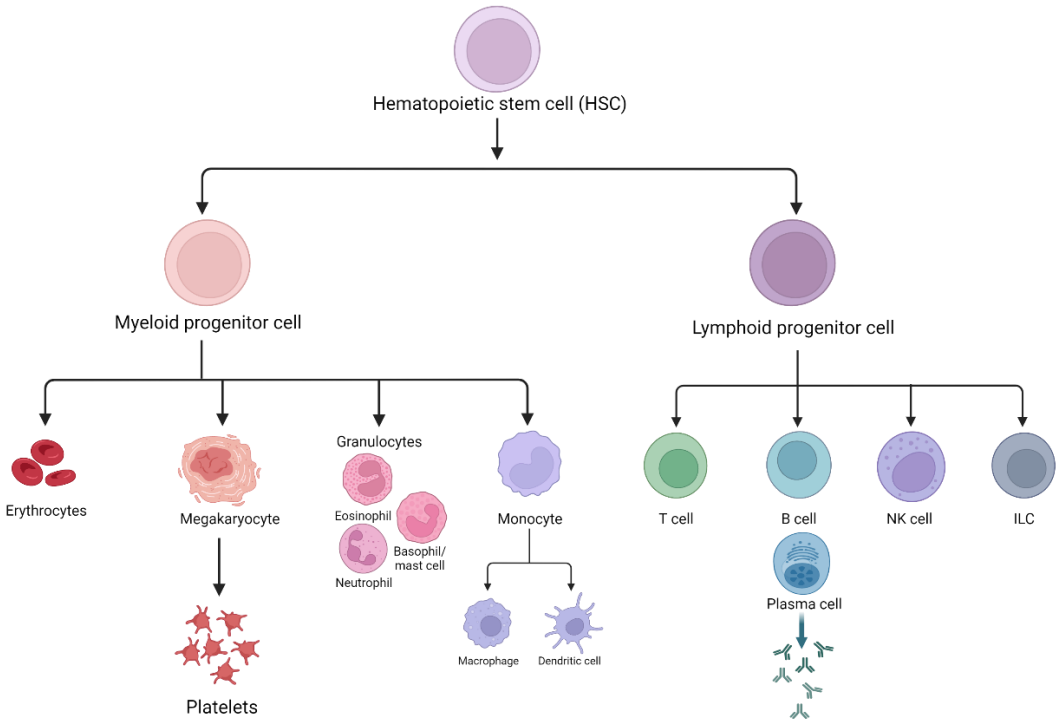

Figure 3: Overview of the main cell types and origins of adaptive and innate immune cells. Immune cells are derived from either a myeloid or a lymphoid progenitor, giving rise to several distinct types of immune cells. The figure was created by BioRender.com.

1.3.1. Innate immune system and innate immune cells

Innate immune cells are characterized by their rapid response (within hours) to perceived foreign molecules that are termed pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). DAMPs are molecular patterns associated with malfunctioning, dying or cancerous endogenous cells and include a large variety of structural patterns. Examples of DAMPs are changes to membrane phospholipids on cells or the presence of molecules in the cytosol that are supposed to be located within phagosomes or lysosomes.

The rapid response of the innate immune cells is due to PRRs found on the surface of these cells that reacts to the molecular patterns of PAMPs or DAMPs. Innate immune cells express a broad repertoire of PRRs. The foreign structures recognized by innate immune cells often include polysaccharide patterns, and PRRs are collectively well equipped for recognizing several different polysaccharide structures. Even though innate immune cells have the genetic makeup for a given set of receptors, they can still up- and downregulate the expression levels of these receptors according to their environment, thus tailoring their responses. The PRRs focused on in this thesis is the well-studied TLRs and the C-type lectin receptors (CLRs).

Cells of the innate immune system include neutrophils, dendritic cells, monocytes, macrophages, innate lymphoid cells (ILCs), and natural killer (NK) cells. Neutrophils develop in the bone marrow and make up the first line of defence of the innate immune cells. Large amounts of neutrophils circulate the blood daily, making up about 70% of peripheral leukocytes in humans. Equipped with large amounts of cytotoxic factors, neutrophils patrol the blood searching for infectious agents or signals from tissues indicating an inflammation. Monocytes are leukocytes originating in the bone marrow that are present in circulation until they are recruited to the site of inflammation where they, depending on the cytokine environment, differentiate to macrophages or dendritic cells and help in tissue repair and inflammation promotion [81]. Dendritic cells function as the main communicators of the innate immune system, they are central antigen presenting cells essential for engaging the adaptive immune system through antigen presentation to helper T cells. Though the main function of dendritic cells is to communicate information about the status quo to T cells, they also contribute to tissue inflammation by secreting pro-inflammatory cytokines [82]. Macrophages are the phagocytic cells of the innate immune system. They are stationed in tissues, and responsible for engulfing and rapidly removing pathogens that interact with the receptors on their surfaces or that are covered with antibodies after the adaptive immune system is engaged. These macrophage receptors are described below in Section 1.3.3. In addition to promoting

phagocytic activity, activating the receptors also elicit secretion of signalling molecules, informing other cells about the state of the environment. These signalling molecules include ROS, nitric oxide (NO) and some cytokines like tumour necrosis factor (TNF) and interleukins (IL-1β, IL-6, IL-8, and IL-12) [83].

Natural killer (NK) cells stem from the same lymphoid progenitor as T- and B cells in the adaptive immune system but are characterized as innate immune cells based on their lack of germline recombinant antigen receptors, and their rapid response time [82]. NK cells specialize not in the killing of foreign pathogens, instead, their task is to identify and kill cells in the body infected with virus or cells that are stressed or become tumorous. They search for changes in surface patterns on cells and destroy altered cells by secreting proteins that are toxic to the cells [84]. Like all the other immune cells, NK cells can also contribute to the immune response environment by secreting cytokines. Most commonly, NK cells release interferon (IFN)-γ, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and different chemoattractant cytokines (chemokines) [85].

When the innate immune cells that patrol tissues discover an unknown molecular pattern, the cells are activated and signalling molecules are sent out to recruit other immune cells. The immune cells release molecules to the surroundings that widens the blood vessels, increasing blood flow, and make the blood vessels more permeable, allowing recruitment of other cells to help clear the infection [82]. This gives the familiar swelling, redness and heat that are characteristic of inflammation. If the inflammatory process prolongs, signals are sent to tell the brain to increase the body temperature (fever). The yellow pus observed after and during an infection is the result of the dying and decaying germs, tissue and immune cells during the immune response and are a normal part of the clearance process [84]. In historical texts, several of these symptoms of infection can be described without specifying a disease or concrete ailment. These symptoms are therefore important to look for when interpreting historical texts on ailments in relation to medicinal remedies.

1.3.2. The adaptive immune system

When the innate immune response is unable to clear the infection, the adaptive immune system, consisting of T- and B cells, is called into action. Though many pathogens encountered by healthy individuals are cleared within days by the innate immune system before the adaptive immune response even gets involved, when the adaptive immune system is engaged, T- and B cells have specialized and efficient immune response against one given pathogen, providing a

more precise and tailored response than the innate immune cells. This specific response also means a given cell with a specific receptor for that pathogen structure need to identify this structure before they can respond. This identification process is why the adaptive immune system has a slower response time (a few days) compared to the innate immune system that already have their arsenal ready [86]. In addition to an efficient response, adaptive immune cells also make specialized memory cells to remember a pathogen they have already encountered. These memory cells already have their arsenal ready and can react fast and strong if they encounter the same pathogen again, providing a much faster adaptive immune response and clearing of the danger. This means you either do not notice that you had the infection or you will have a much milder form of the infection [84]. This smart characteristic of the adaptive immune cells is for instance what vaccines are relying on.

T cells develop in the thymus (hence the name T cells) in an environment that directs cell differentiation, and education through positive and negative selection of the T cells. Lymphoid progenitor cells, developed from hematopoietic stem cells in the bone marrow will migrate to thymus where they, in an antigen independent manner mature to functional T cells. The thymus is also the place where T cells develop their phenotypical specific markers like the T cell receptor (TCR), and the co-receptors CD4 and CD8. Double positive T cells expressing both CD4 and CD8 need to go through both positive and negative selection before leaving the thymus as either T helper (CD4+) or cytotoxic (CD8+) T cells. Positive selection involves the T cells having to bind to either class I (CD8-mediated) or class II (CD4-mediated) major histocompatibility complex (MHC) that also express self-peptides (self-MHC) expressed on epithelial cells in the thymus. Following the positive selection, T cells now has to go through negative selection. During negative selection T cells that interact too strongly with macrophages or dendritic cells carrying class I and II MHC will be eliminated by apoptosis. After surviving the negative selection, T cells leave the thymus as naïve T cells that will be fully activated after interaction with an antigen peptide in combination with a co-stimulatory signal, usually in the form of interaction between CD28 on T cells and members of the immunoglobin B7 family on antigen presenting cells [82].

There are different subsets of T cells with distinct roles. T helper cells secrete chemical signals to activate other cells in the immune system and present peptide antigens to B cells via MHC class II to activate them. Cytotoxic T cells have similar functions as NK cells, they detect and destroy cells in the body that are infected with viruses or are tumorous, this recognition happen through recognition of foreign peptides on the MHC class I [86]. Other T cells important for an optimal and balanced T cell response are subsets of T helper cells, such as T

helper 1(Th1), Th2, Th17 and regulatory T cells (T_{reg}). Th1 cells generally promote cellular immune responses, while Th2 cells promote antibody production from B cells and are associated with allergic reactions. Th17 cells are so named because they primarily secrete the pro-inflammatory cytokine IL-17, in addition to IL-22 and IL-23. This cytokine signalling recruits neutrophils to the site of infection and promotes inflammation. An imbalance of Th17 together with Th1 cell responses are often associated with autoimmune diseases. T_{reg} cells are the main producers of the anti-inflammatory cytokine IL-10, important for supressing immune responses and maintaining immune homeostasis [87].

B cells produce antibodies and develop from precursors in the bone marrow. Naïve B cells migrate to germline centres in lymphatic tissues where they interact with follicular helper T cells. Germline centres are structures within follicles (B cell zone) in secondary lymph nodes where B cells go through somatic hypermutation, differentiate, proliferate, and mature to specialized cells. These specialized activated B cells are called plasma cells and can secrete antibodies/immunoglobulins (Ig) against a specific target motif [86]. The antibodies are secreted into circulation and can then exit circulation when reaching areas of ongoing inflammation.

1.3.3. Toll Like Receptors and C-type Lectin Receptors

At least ten TLRs have been identified in humans, some of them expressed on the cell surface (TLR1/2, TLR2/6, TLR4 and TLR5), and some intracellularly on endosomes (TLR3, TLR4, TLR7/8 and TLR9) (**Figure 4**). In this thesis, we focused on TLR4 and TLR2, as these bind diverse carbohydrate ligands, and thus could serve as receptors for the plant-derived polysaccharides investigated here. A classic example is the endotoxin lipopolysaccharide (LPS) found in the cell wall of Gram-negative bacteria that elicit strong immunes responses following binding to TLR4, which induces early-stage inflammatory signals through the intracellular mediator MyD88 [88, 89]. Lipoteichoic acid, found in the cell wall of Gram-positive bacteria, and peptidoglycan, found in the cell wall of most bacteria are both ligands for TLR2.

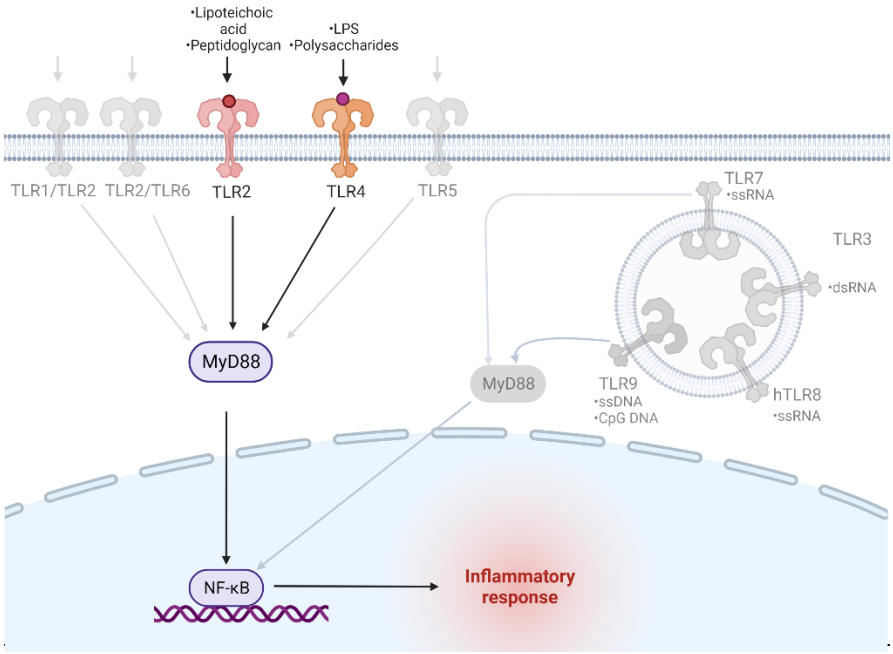

Figure 4: Toll like receptors (TLRs). An illustration of the different TLRs, and their expression on the cell surface or cell interior. Highlighted are the receptors of focus in this thesis with their ligands and main signalling pathway induced. The figure was created by BioRender.com.

TLRs are expressed on cells of the myeloid lineage, including macrophages and dendritic cells, as well as on non-immune cells such as endothelial cells and epithelial cells [90]. Binding to TLRs induce a pro-inflammatory intracellular signalling cascades where inflammatory genes are upregulated, producing pro-inflammatory cytokines and IFNs.

CLRs were among the first animal lectins discovered. These animal lectins (carbohydrate-binding) where in 1988 classified into different groups by Drickamer, where the Ca^{2+}-dependent (C-type) lectins with similar structure as the asialoglycoprotein receptor was classified as C-type lectin receptors [91]. These surface-expressed transmembrane CLRs are expressed on several cells belonging to the hematopoietic lineage, including neutrophils, monocytes, T cells, B cells, NK cells and platelets [92]. The carbohydrate recognition domain of CLRs mediate the carbohydrate binding important for recognising pathogens and for cell-cell interactions [93, 94]. CLRs that lack the conserved Ca^{2+} binding residues, were in 1988 proposed by Weis et al. to be called C-type lectin-like NK receptor domains [94]. These non-classical C-type lectins primarily bind proteins, not sugars [94-96], and are highly expressed by NK cells.

In this thesis, macrophage-inducible C-type lectin (Mincle) and DC-associated C-type lectin (Dectin)-1β and -2 were the primary focus (**Figure 5**). All three receptors are important for the induction of the adaptive immune response, and can bind different glycan structures

[97]. CLRs are grouped into different classes based on their domain structure. Dectin-2 and Mincle belong to Group 2, while Dectin-1 belong to group 5 (the non-classical C-type lectins) [98].

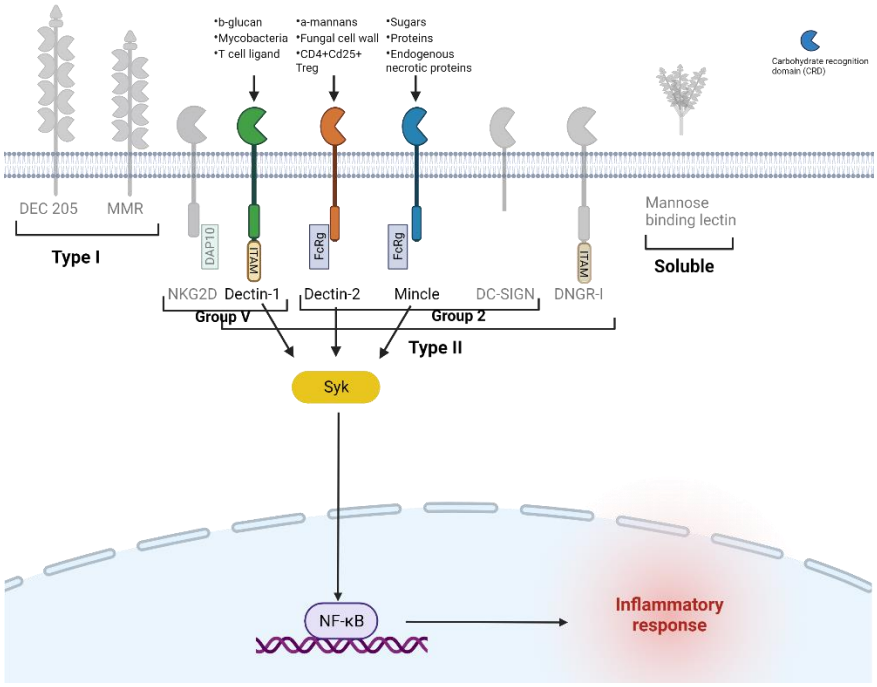

Figure 5: C-type lectin receptors (CLRs). The illustration shows the main types and groups of CLRs. Highlighted are the receptors of focus in this thesis with their ligands and main signalling pathway induced. The figure was created by BioRender.com.

Dectin-1 is found to interact with β-glucans, like zymosan, that comprise a major part of the cell wall of fungi, but can also recognize and bind to mycobacterial species devoid of β-glucans, the exact ligands for this interaction is not known [99]. Dectin-1 is also found to interact with an unidentified ligand on T-cells causing endogenous effects, and through this interaction it is proposed to potentially have a co-stimulatory effect [96]. TLR-4 and Dectin-1 can act synergistically and are found to be necessary to induce Th17 immunity towards fungal infection [100]. Dectin-1 have also been found to act through dendritic cells to induce Th1/Th17 responses to mycobacteria [101] and is together with TLR-2 required to activate macrophages to produce TNFα after mycobacteria infection [102]. In addition to these effects, the response of Dectin-1 to intestinal fungi and their components is found to be important for regulating intestinal homeostasis and regulate the severity of IBD. Deficiencies in Dectin-1 result in reduced production of inflammatory cytokines and reduced Th17 immune responses [103].

Dectin-2 and Mincle have cytoplasmic domains with short tails that lacks known signalling motifs, and instead associate with the signalling adapter molecule Fc receptor γ chain to induce phagocytosis and cytokine production [104-107]. Dectin-2 recognises components of the fungal cell wall, primarily high-mannose structures like α-mannans present on fungal hyphae [105, 108]. Dectin-2 is primarily expressed on tissue macrophages, some dendritic cells and at low levels on peripheral blood monocytes, though expression on these can be transiently increased during inflammation [109]. A study on human Dectin-2 gene expression found it to be upregulated by TNFα, and downregulated by Th2 cytokines like IL-10, suggesting a role of Dectin-2 in T-cell polarization to Th1 or Th2 cells [110].

Like Dectin-2, Mincle have also been found to recognise fungi and induce pro-inflammatory signals like TNFα [111]. Mincle is primarily expressed on macrophages and has been found, in addition to recognizing sugars, to recognise proteins. Mincle have also been found to function in protection against different bacteria including S*treptococcus pneumoniae* and *Klebsiella pneumoniae*, inducing pro-inflammatory and anti-microbial responses, promoting T cell immunity [112-114].

1.3.4. Cytokines in the immune system

Important signalling molecules in the immune system are cytokines. Cytokines are small proteins that help orchestrating the immune response. Different cell types secrete different cytokines in different contexts. Depending on the effect they have on the immune response, cytokines are mainly classified as either pro- or anti-inflammatory [82].

Interferons (IFNs) are a group of cytokines originally defined based on their ability to 'interfere' with viral infections, they have subsequently been found to have antiproliferative and immunomodulatory effects as well. Interferons are subdivided into two groups, Type I interferons that consist of several different cytokines, and Type II interferon that only consist of IFNγ. IFNγ is secreted by activated helper Th1 cells and NK cells and is one of the most potent macrophage activators [86]. It is also important for antimicrobial effects, while the other IFNs primarily are responsible for antiviral states [86].

Tumour necrosis factor (TNF) is a superfamily of 19 members that are found to signal through 29 receptors [115]. TNFs were originally found to be the molecules in serum essential for the haemorrhagic necrosis of tumours found in 1943 by Shear et al. to be induced by LPS, thus the name tumour necrosis factor [116]. Based on several studies, a review in Nature dubbed the TNF superfamily a "double edged sword" as it has proven important for normal

responses, but also are implicated in development of different diseases like tumorigenesis, rejection of transplants, septic shock, rheumatoid arthritis and diabetes [115], emphasizing the importance of a balanced TNF response.

Macrophages, as part of the innate immune response, is recognized as the main producers of pro-inflammatory cytokines important in immune activation. In addition to macrophages, NK cells are known for secreting a variety of pro-inflammatory cytokines including TNFα and IFNγ to engage other cells in the immune system.

Th1 cells stimulate cellular immunity and cell-mediated functions by secreting primarily IFNγ together with IL-2 and TNFα. Th2 cells stimulate humoral immune responses, promoting B cells to proliferate and initiate antibody production, Th2-secreted cytokines are IL-4, IL-5, IL-10 and IL-13 [117, 118]. The cytokines secreted by these cells are referred to as Th1-type cytokines or Th2-type cytokines [118].

Anti-inflammatory cytokines work to reduce inflammation, controlling the pro-inflammatory response and bring the immune system back to balance after an infection. The most common anti-inflammatory cytokines are IL-1 receptor antagonist, IL-4, IL-10, and IL-13.

1.4. The immune system in the gastrointestinal tract

The largest proportion of immune cells in the body are found in the gastrointestinal (GI) tract, containing up to 70% of all immune cells. The immune cells are located in specialized areas called gut associated lymphoid tissues (GALT), as single cells within the gut wall, or as part of the epithelial layer [119]. I will begin by describing the anatomy of the GI tract to provide a better understanding of the importance of the immune system in this organ.

The intestinal epithelium is a single cell layer that makes up the largest mucosal surface of the body with a surface area of about 400 m^2. Intestinal epithelial cells do not only play a role in water, ion and nutrient absorption, they are also a critical part of the immune protection in the gut. These cells together with the mucus layer are the only physical and biochemical barrier that separates host tissue from the gut lumen containing digested products, commensal and pathogenic bacteria, and bacterial metabolites (**Figure 6**). Intestinal epithelial cells have a critical function in maintaining intestinal homeostasis, preventing pathogen interaction with the host, and are important for controlling mucosal inflammation and mucus production [120]
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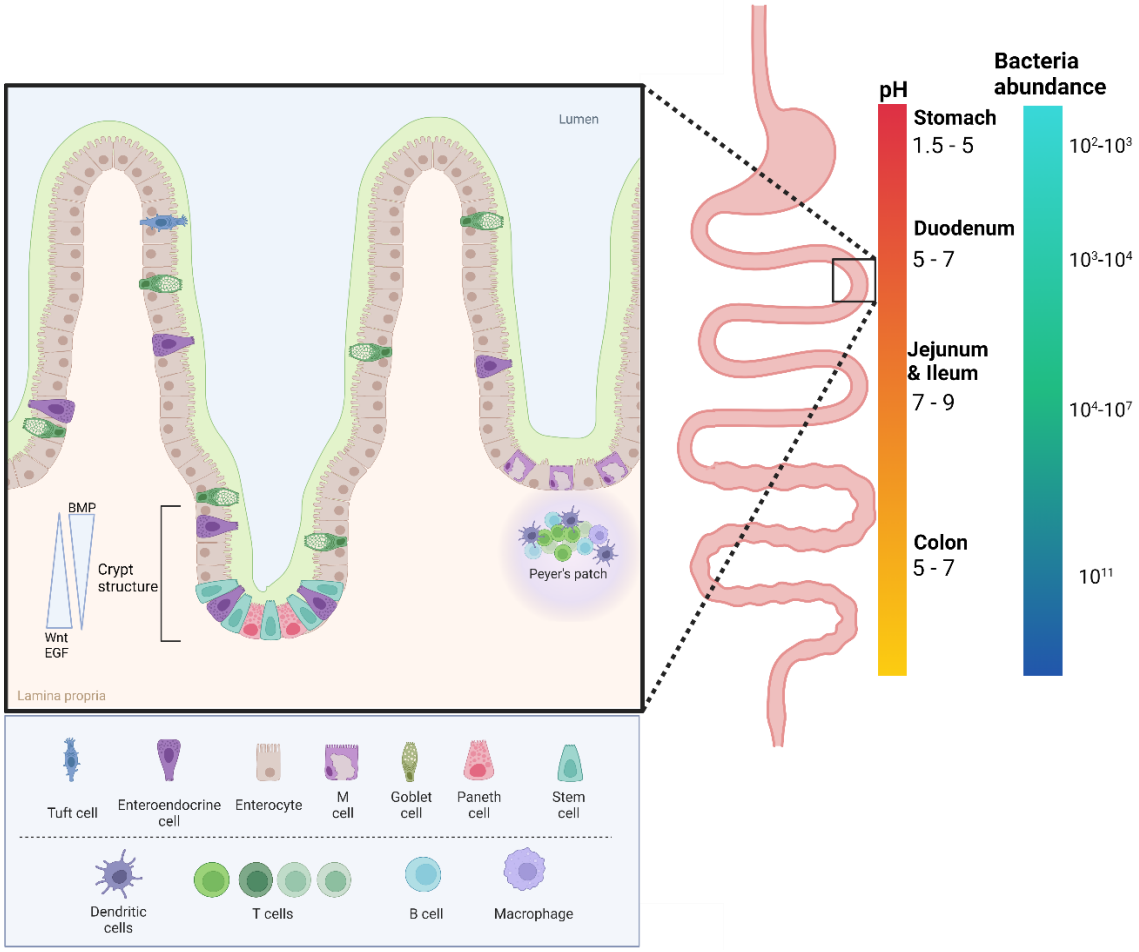

Figure 6: Structural overview of the small intestine, immune cells in the lamina propria, pH and bacteria abundance in the GI tract. Illustrates the small intestinal structure with mucus layer, crypt structures with essential factors, epithelial cells and immune cells found in lamina propria. Also show pH gradient and bacteria abundance along the GI tact. The figure was created by BioRender.com.

The GI tract varies from region to region in line with the varying functions of each region. The small intestine is primarily responsible for the absorption of nutrients, which requires a large surface area and is therefore covered with villi structures to increase this surface area and the absorption effect. The main function of the colon is reabsorption of fluids and water, which does not require a larger surface area, no villi structures is therefore present in the colon. In the small intestinal epithelium, the villi reach into the lumen where they interact with food and other luminal components. The main cells that make up the mature epithelial layer in both the large and small intestine are enterocytes that absorbs nutrients and function in metabolism, enteroendocrine cells that help regulate nutrient metabolism by secreting hormones to the lumen site, goblet cells that are essential for producing highly glycosylated mucus that line and protect the epithelium, and the chemosensory tuft cells [121]. The turnover rate of intestinal

epithelial cells is quite high with regeneration happening every 5-7 days. Paneth cells, that secrete defensive antimicrobial peptides, are only found in the small intestine and differ from the other epithelial cells in that they survive longer (about 20 days) and do not migrate up the crypt during differentiation. They remain in the crypt base where they produce intestinal stem cell niche factors, promoting stem cell proliferation [122, 123]. In the colon, cells expressing regenerating islet-derived family member 4 (Reg4) are the Paneth cell equivalents, secreting antimicrobials and stem cell niche factors [124]. Stem cells are an essential part of the GI tract, responsible for regeneration of epithelial tissue.

1.4.1. Intestinal stem cells and regeneration

Crypts of Lieberkühn (often only referred to as crypts) are invaginations of the epithelium and structures containing intestinal stem cells important for intestinal epithelial cell regeneration in both small intestine and colon [125]. The crypt bottoms represent the stem cell niche where cell proliferation is high due to the high amount of stem cells and the specific composition of molecules present to promote this stem cell state. The signals provided in the stem cell niche by Wnt (acronym for wingless/integrated), and epidermal growth factor (EGF) reduces bone morphogenic protein (BMP) and provides stem cells the ability to self-renew and regenerate. Cells proliferate at the bottom of the crypts, move towards the top where they differentiate as Wnt and EGF signals decreases and BMP increases [121, 125].

EGF binds to the EGF receptor (EGFR) to induce a signalling cascade that convey information about intestinal epithelial cell proliferation and maintenance in the stem cell niche. Binding of Wnt to the Wnt receptor of the frizzled (FZD) family activates a signalling cascade that promotes stem cell maintenance. R-spondin is another protein that strongly potentiate Wnt and β-catenin downstream of the Wnt receptor signalling cascade, increasing the Wnt response [126]. Knowledge about these stem cell mechanisms and necessary components for stem cell renewal and differentiation in the intestine has provided the basis for generating intestinal organoids able to regenerate, proliferate and differentiate to “mini guts” [127]. These organoids contain cells seen in the original intestine from which the crypts are collected.

1.4.2. Barrier protective structures in the intestine

Stem cell renewal is essential for GI health and protection of these cells and structures comes in the form of the mucus layer shielding the cells from the lumen. The mucus layer varies along the GI tract, with a single layer of mucus in the small intestine and two mucus layers in the

colon. The single mucus layer in the small intestine is easily penetrable and loosely attached to the epithelium, likely reflecting the lower bacteria abundance and the function of the small intestine in nutrient absorption. The two mucus layers in the distal colon consist of one sterile, stratified and adherent inner layer, and a loosely attached outer layer that harbours the intestinal microbiota [128].

What surpasses the mucus layer reaches the epithelial cells where tight junctions function as another protection mechanism. Tight junctions are important structures between both epithelial and endothelial cells that control paracellular permeability between these cells. Tight junctions maintain the cell polarization of epithelial cells by forming a border between the apical cell surface located towards the lumen and the basolateral cell surface located away from the lumen, reducing mixing of components from these separate environments [129]. The tight junction barrier is a selective gate that control the passage of molecules and solutes that for some compounds, depending on their size and charge, can pass through this barrier. This enables tight junctions to transmit signals to and from the cell interior and tight junctions can through these signalling networks control cell function (proliferation, migration, differentiation), behaviour and survival [129]. What passes the intestinal barrier structure then need to surpass the protective gut immune cells.

1.4.3. Gut immune cells

Gut immune cells are located either within the epithelium, as single cells in the lamina propria, or within lymphatic tissue structures of the lamina propria. The lamina propria forms the gut wall, and consists of connective tissue, located beneath the single intestinal epithelial layer [130, 131]. These lymphatic tissues consist of both isolated and aggregated lymphoid follicles and are important for protective immune responses in the gut [130-132]. An important structure are the Peyer's patches. Peyer's patches are structures found in the small intestine and consists of lymphoid follicles covered by follicle-associated epithelium. In this epithelium are found specialized cells called microfold cells (M-cells) [121, 133]. These M-cells, together with dendritic cells, connect the luminal environment to the underlying immune cells in lamina propria by delivering luminal antigens or bacteria components that either activate or inhibit immune responses. This communication is essential for developing immune tolerance towards commensal bacteria or initiating a systemic immune response towards pathogenic components in the gut environment, especially during early years of life [134-139]. Adaptive immune cells are important parts of Peyer's patches structures in the mucosa and submucosa in the GI tract.

Especially in the large intestine, where the differentiated IgA producing plasma cells in lamina propria are important for secreting IgA to the lumen. Here, IgA can bind to and coat bacteria antigens, hindering bacteria translocation across the epithelial layer and cause infection, or help innate immune cells in clearing pathogenic bacteria. Most bacteria in human faces are found to be covered with specific IgA [140, 141]. Shift from IgG to IgA producing plasma cells are important for maintaining the intestinal mucosa and promoting commensal bacteria populations. Studies have found that modifications of IgA specificity alter the gut microbiota composition in a negative way. IgA alternations are found to reduce the number of *Bifidobacterium*, a commensal health promoting bacteria, while simultaneously increasing the number of the more pathogenic *Enterobacteriaceae* [142]. Other studies investigating patients with Crohn's disease (chronic inflammation extending through the entire GI tract) or ulcerative colitis (chronic inflammation with abnormal inflammation and ulcers on the inner lining of the large intestine), found these patients to have increased mucosal secretion of IgG antibodies towards commensal bacteria and IgG were also found to activate the complement system and initiate a cascade of inflammatory mediators, this was not observed in normal IgA responses [131, 143].

One popular theory explaining the development of diseases in relation to exposure to commensal or pathogenic bacteria is increased gut wall permeability and the resulting "leaky gut". This gut barrier permeability is also believed to be a key factor in determining the pro-inflammatory load that reach the liver [144]. CD4+ T helper cells are thought to be important for regulating cell wall permeability and proper development of these cells in Peyer's patches is therefore essential [133]. Ulcerative colitis is associated with increased gut wall permeability and disease flare-ups are associated with penetration of bacteria into the mucus layer. Patients with active disease present with a thinner layer than normal [145]

The constant exposure to microbes in the GI tract means endogenous cells in this environment have a particularly tricky task of distinguishing between pathogenic and non-pathogenic agents and ensure proper gut homeostasis and a tolerant immune environment. Intestinal epithelial cells and immune cells balance this task in collaboration with the commensal microbiota to function properly [146, 147].

The effect on immune response is thought to be the result of bacteria components or bacteria secondary metabolites interacting with the host cells. With bacteria secondary metabolites being especially important for colonocytes and their health, helping in the production of crypt cells and cell proliferation in the intestine by ensuring healthy epithelial cells [148, 149].

The first colonisation of the GI tract in new-born infants are thought to be important for shaping the later more permanent gut flora that follows into adulthood. The first bacteria to settle can modulate gene expression in host epithelial cells, creating a favourable habitat for themselves, preventing growth of other bacteria [130]. Supporting this is the finding that specific bacteria LPS exposure early in life have been associated with the development of autoimmune diseases. A study found *E. coli* LPS, a potent immune stimulator, to be the main LPS in infants from areas with low early onset autoimmune diseases development compared to areas with high early onset of autoimmune diseases. In areas with early onset autoimmune diseases, the infants were found to have higher abundance of *Bacteroides* LPS, a less potent immune stimulant. The authors behind the study suggest that the early occupation of microbiota that activate innate immune responses might benefit immune education and that lack of this education from species that silence immune function might result in the development of autoimmune diseases like type 1 diabetes [150].

The microbiome can influence host health in many ways, but host cells can also interact with and influence bacteria in the intestine. Innate immune cells can phagocytose and kill pathogenic bacteria and intestinal epithelial cells can produce pro-inflammatory molecules, relying information to the underlying cells in lamina propria and coordinate host responses [146].

A healthy gut microbiota, facilitated by host immune cells and their actions, can help promote host health in several different ways. Gut microbiota are found to help increase intestinal barrier function by modulating mucosal homeostasis, increase epithelial tight junction integrity and maintain gut homeostasis [147]. A dysregulated gut barrier leads to bacterial translocation of viable bacteria or endotoxins from the GI tract through the epithelial mucosa and entering circulation where they might cause local or systemic inflammation [130]. An increasing number of studies find the gut microbiota to be an essential and previously underestimated part of human health.

1.4.4. Gut microbiota

The “gut microbiota” is defined as the community of microorganisms (bacteria, viruses, fungi and protozoa) that lives in our gut, while the “gut microbiome” refers to the genetic and functional profile of these microbes [151-154]. The complexity and genomic diversity that resides in this community, with about 100 trillion micro-organisms, is the reason why this community is thought of as a virtual organ in the body [155-158]. Where the human genome

consists of about 23.000 genes, the microbiome together encodes over three million genes [159]. These genes are involved in producing a variety of different proteins, many of which are capable of supplementing host functions, and some exert functions that the host is not capable of [146, 160, 161]. The microbiome is sufficiently equipped to influence and modify host fitness and health [162]. Among the microbiota colonizing the gut, the bacterial composition is the most studied and will be the focus in this thesis when discussing the gut microbiota

The numbers of bacteria vary along the GI track with abundance increasing towards the colon [163, 164]. Two bacteria species found to dominate the microbiota composition are the Gram-negative *Bacteroidetes* and the Gram-positive *Firmicutes* phyla, with more than 90% of the bacteria species belonging to either of these two phyla [146, 156, 158, 165]. The gut microbiota is gaining increasing attention as a contributing factor in the connection between genes, environment and the immune system in heath and disease [146].

Both hereditary components and environmental factors influences the microbiota composition. Environmental factors include food, medicines and other components the person is exposed to, while hereditary or individual factors include genetics, diseases, age and weight/BMI [166]. The gut microbiome have also been linked with several non-gut related aspects of human health like neural development, immune responses, endocrine effect, and metabolic traits [162, 167].

The general lack of species-specific differences in disease studies relating to the gut microbiota indicate that maybe not specific bacteria, but the collective effect of the bacteria present and the avoidance of bacterial dysbiosis, is what plays the most important role in disease development associated with the gut microbiota. Dysbiosis of the microbiota has been associated with many diseases [168, 169]. Type 2 diabetes and colorectal cancer are two diseases that are on the rise and are linked to increased gut dysbiosis with no clear taxa signatures, indicating a “functional dysbiosis” [170-172]. Functional redundancy on the other hand is the capacity of one microbial group to carry out the same responses in the same time as another group [173]. This redundancy, especially of SCFA butyrate producing bacteria, seems to be an important factor for disease prevention and for normal gut homeostasis [172, 174, 175]. This also explains why different people can have different microbial composition without compromising on basic functions.

Some bacteria found to play a part in maintaining gut homeostasis and promote beneficial effects in the gut are *Bifidobacterium* and *Slackia isoflavoniconvertens* belonging to the Actinobacteria phylum, and *Faecalibacterium prausnitzii* and *Clostridium butyricum* belonging to the Firmicutes phylum, emphasising the importance of gut microbiome diversity.

Among these, *Faecalibacterium* and *Bifidobacterium* are associated with reduced C-reactive protein (CRP) and IL-6 levels, both known inflammatory markers [176]. *F. prausnitzii* is a Gram-positive bacterium that in recent years has gained much attention for its anti-inflammatory properties and important role in promoting gut health, with reduced *F. prausnitzii* abundance found in different intestinal disorders. This bacterium is therefore proposed to potentially function as a biomarker to assist in discriminating ulcerative colitis and Crohn's disease [177].

Bifidobacterium and *S. isoflavoniconvertens* are Gram positive bacteria important for the bioconversion of isoflavones, in particular genistein and daidzein found in soya, to equol [178, 179]. Equol are primarily found to have beneficial effects by binding to estrogenic receptors and reducing menopausal symptoms in some women. The ability to produce equol varies greatly among people, assumed to be because of variation in bacteria composition capable of converting these isoflavones to estrogen-like molecules [180]. As humans does not have the enzymes necessary to break down isoflavones, the anti-oxidant and anti-inflammatory health benefits associated with isoflavones in relation to cardiovascular diseases, multiple sclerosis and cancer are probably due to the metabolic activity of bacteria in the gut [181].

Bifidobacterium is a well-known probiotic. It has been found to function in protection against pathogens, produce antioxidants and Vitamin B, in addition to stimulating the immune system [182, 183]. Reduced levels of *Bifidobacterium* are associated with IBD, atopic disease/allergy, irritable bowel syndrome (IBS) and autism. Some of the factors associated with reduced levels of *Bifidobacterium* are increased age, caesarean section and formula feeding of infants [183].

Clostridium butyricum is a Gram-positive butyrate producing bacteria that has been recognised and used as a probiotic for decades [184, 185]. It has been found to alleviate symptoms of IBD, intestinal injury and gut acquired infection, in addition to possess immunomodulatory and anti-inflammatory properties, and alleviate metabolic diseases and colon cancer [185]. *C. butyricum* is found to be non-pathogenic, with no pathogenic markers, lacking in toxic genes associated with some other bacteria in the clostridial order and is therefore considered a safe probiotic [186]. The localised production of SCFAs, especially butyrate, is proposed to be one of the mechanisms behind the health promoting effects of this bacterium. Other suggestions include the ability of *C. butyricum* to modulate the gut microbiome, increasing the abundance of beneficial bacteria like *Bifidobacterium* [187-189].

While some bacteria are strongly associated with health benefits, other bacteria like *Bacteroides* induce an effect strongly dependent on the location where they reside. Generally,

Bacteroides are found to have a beneficial relationship with the host while being retained in the gut. They can however cause severe pathology like bacteremia and abscess in multiple body sites if they escape the colon [190]. One study identified *Bacteroides salyersiae* in abdominal cavity liquid (peritoneal fluid) of post operative patients with colon or rectal cancer [191].

Other bacteria like *Clostridium perfringens* are considered pathogenic. *C. perfringens* consist of Gram-positive pathogenic bacteria grouped into five groups, where each group show slightly different disease picture. The diseases found to be caused by C. *perfringens* include a variety of GI diseases like food poisoning and non-food bourn diarrhoea (antibiotic-associated and sporadic diarrhoea), Chron's disease, and gas gangrene or Clostridial myonecrosis that can spread through the blood from the GI tract and is followed by muscle and tissue destruction, multiple organ failure and eventually death [192-198]. The bacterium is also found to cause necrotizing enterocolitis that leads to extensive bleeding and serious ulceration or mucous necrosis [197, 199]. The large variety in disease picture related to C. *perfringens* probably relates to its ability to produce an arsenal of toxic proteins with different effects, making it a serious pathogen to humans [200, 201].

Enterococcus, also a group potential pathogenic Gram-positive bacteria. *Enterococcus* does not produce toxins, but secrete and produce different virulence factors like bacteriocins, cytolysin, gelatinase, serine protease and toxic oxygen metabolites that can cause cell injury. *Enterococcus* is defined as an opportunistic pathogen and are the leading cause of nosocomial infections [202, 203]. The most common infections caused by *Enterococcus* are bacteraemia, with the bacterium translocation from the intestine and to the lymphatic system and bloodstream, inflammation of the heart (endocarditis), urinary trat infections and intra-abdominal and intra-pelvic infections [202, 204-206]. Vancomycin-resistant enterococci are becoming an increasing concern because of the high mortality rate associated with this bacterium [202, 207, 208]. *Enterococci* have a particular high virulence because of their resistance to antibiotics and high resilience, being able survive under a broad range of conditions like different pH, temperatures and hyper- and hypotonic conditions [209-211]. The infections are also often associated with biofilm formation, causing persistent infection and making them harder to get rid of [206].

1.4.5. Bacterial secondary metabolites

Bacteria in the large intestine are important for breaking down and fermenting indigestible substrates that are not absorbed in the small intestine. These substrates include dietary

oligosaccharides and fibres like oligofructose, inulin, pectin and arabinoxylan. Microbial fermentation results in secondary metabolites, of which the most studied are SCFAs, such as acetate, butyrate and propionate [212, 213]. SCFAs are defined as free fatty acids with less than 6 carbons. Acetate has two carbons (C2), propionic acid has three carbons (C3) and butyric acid have four carbons (C4). Because of their short hydrophobic chains and their hydrophilic carboxyl group, SCFAs are soluble in water and easily transported by epithelial cells [214]. This enables SCFAs to function as an energy source for the colonic epithelium, and they are also found to modulate inflammation [212, 215-218]. Dietary fibre can, through these three SCFAs, affect several different immune cells, including macrophages, dendritic cells and T_{reg} cells [219-222]. SCFAs are also important for regulating gut motility and ion absorption, and for promoting mucin and peptide production important for maintaining the gut barrier function [214].

Increasing number of studies have been conducted that support the important role of SCFAs in influencing both peripheral and local immune responses. In addition to serving as an energy source, butyrate also suppress pro-inflammatory signals in colonic epithelial cells [223-225], and inhibit the pro-inflammatory effects of IFNγ [226]. In clinical trials, butyrate is found to improve clinical and histological cases of distal ulcerative colitis and improving clinical outcomes or remission in patients with Chron's disease. In lamina propria, butyrate is found to reduce the responsiveness of macrophages to commensal bacteria, inducing tolerance to these bacteria [227].

Bacteria belonging to the *Firmicutes* phylum that is known for producing butyrate to generate their health promoting effect include *F. prausnitzii* and *C. butyricum* [185, 228, 229]. *F. prausnitzii* is a butyrate-producing bacterium that have gained much attention in recent years for its association with reduced inflammation [230-234]. Studies find *F. prausnitzii* associated to reductions of pro-inflammatory cytokines, increased frequencies of T_{reg}, and increased production of the anti-inflammatory IL-10 cytokine [235, 236]. Additionally, supernatants from *F. prausnitzii* cultures were found to contain metabolites that enhanced intestinal barrier function, reducing paracellular permeability [234, 237]. Several studies have implicated a depletion of *F. prausnitzii* in gut diseases like IBD (Crohn's disease [235, 238, 239] and ulcerative colitis [240, 241]) [232, 236, 242-247], and the low grade inflammatory state observed in obesity and diabetes [248, 249]. Many of them are linking the subsequent reduction in butyrate production as a possible role in disease development.

Bifidobacterium have been found to primarily produce acetate and through this function in gut homeostasis and host health. Acetate were found to be important for the interaction

between *Bifidobacteria* and different butyrate producing bacteria like *F. prausnitzii* in humans [182].

1.4.6. Effect of diet on the gut microbiota

The effect of diet on the microbiota is a hot topic in gut microbiota research. Studies have found that high-fat diets and westernised diets containing high cholesterol are associated with a higher degree of Gram-negative bacteria and LPS leakage across the epithelial barrier [144, 250-253]. Higher LPS in the intestine can result in chronic low-grade inflammation, associated with obesity and a variety of diseases like type 2 diabetes, cardiometabolic disorders, and liver diseases [253-256]. Despite the connection between Gram-negative bacteria, LPS, and inflammation, Gram-positive bacteria can also function as opportunistic pathogens to induce inflammation [257], as is the case for *C. perfringens* and *Enterococcus*.

Fibre intake is necessary for the production of SCFAs from the microbiota, and a low fibre diet have been shown to shift the metabolism of the microbiota towards less favourable nutrients, resulting in the production of damaging metabolites and potentially increase the risk of colonic diseases [258-260]. Low fibre diets might also starve the bacteria that then utilizes mucus as an energy source, degrading the colonic mucus barrier, which could result in translocation of microorganisms or microbial components and increase pathogen susceptibility which again could lead to inflammation [261, 262]. While higher fibre intake are associated with decreased probability of early mortality and cardiovascular disease [263]. These findings provide a possible link between the increase in chronic diseases and allergies observed in countries with westernized lifestyles and diets with little fibre intake.

Several studies show that environmental factors and the food we eat can influence the gut bacteria composition and highlights the importance of a good bacteria composition in promoting health. Prebiotics and probiotics are two methods gaining increasing attention for their suggested ability to influence the gut microbiome.

1.4.7. Prebiotics and probiotics

Probiotics refers to live micro-organisms that are introduced to an organism to confer health benefits to the host, though documentation of this effect is varying [264, 265]. Examples of probiotics that are widely used are *Bifidobacterium* and *Lactobacillus* species [162], and the aforementioned *C. butyricum*. A study found oral administration of the probiotic *Bifidobacterium* to have antitumour immunity [266].

Prebiotics, which is most relevant for this thesis, is the term used for compounds not absorbed in the small intestine and not metabolized by intestinal enzymes, that instead travel to the large intestine and selectively nourish beneficial colon residing organisms [267]. The concept of prebiotics was first coined in 1995 by Glenn Gibson and Marcel Roberfroid, describing prebiotics as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [268]. This definition was considered a bit narrow, including only a few carbohydrate compounds like short and long chain β-fructans, lactulose, and galactooligosaccharides [269]. The definition was altered in 2008 following the 6th Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) to include other compounds as well [270], and again in 2017 defining a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit", thus also including noncarbohydrate substances like polyphenols as prebiotics [271]. For prebiotics to reach the large intestine and exert an effect on the gut microbiota, they need to be resistant to the acidic pH in the stomach, they can not be metabolized by mammalian enzymes, and they can not be absorbed in the GI tract. The promotion of good microbiota, selectively simulating their growth and limiting the growth of pathogenic bacteria is an essential part of a prebiotic effect.

The last decade has seen a steep increase in studies analysing the effects of prebiotics on health. Prebiotics are, after ingestion, degraded by the gut microbiota to produce metabolites like SCFAs that either act locally on colonocytes in the intestine or reaches the circulatory system to exert peripheral effects. The prebiotics that have gained most attention for their health promoting effects in humans are β-fructan oligosaccharides (inulin and oligofructose) and galactooligosaccharides. Inulin are larger molecules consisting of several fructose units joined by β-glycosidic links with a degree of polymerisation of ≤60, oligofructose are the degradation product of inulin with a lower degree of polymerization ≤10 [272]. Galactooligosaccharides are carbohydrates usually containing one molecule of glucose and one to five molecules of galactose (Gal) [273, 274].

Studies are usually focusing on the ability of these prebiotics to influence and increase the abundance of primarily *Bifidobacterium* and *Lactobacillus* [269, 275-286]. By inhibiting the growth of pathogenic bacteria and increasing the abundance of beneficial bacteria like *Bifidobacterium* and *Lactobacillus*, prebiotics can improve epithelial barrier integrity, reduce endotoxin production and translocation, increase conversion of primary bile acids (that are colon cancer promoters) to secondary bile acids and through this help maintain gut immune

homeostasis and promote gut health [287]. Some studies indicate that galactooligosaccharides are preferred by a wider range of *Bifidobacterium* spp compare to the other prebiotics [288]. In addition to the direct effects, Scott et al. found in 2013 that the prebiotic effect can be enhanced by cross-feeding, meaning that the product of one species is consumed by another [289-291]. An example of this is *Ruminococcus bromii* that are needed to degrade resistant starches, providing fermentation products that can be used by several other bacteria species [292].

Studies on prebiotic effects have primarily focused on the effect of the immunomodulatory roles of dietary fibres, but recent years have seen an increase in the potential prebiotic effect of phytochemical antioxidants like dietary polyphenols [65, 77, 293, 294]. High molecular weight polyphenols are not absorbed in the small intestine but reach the large intestine virtually unchanged. In the large intestine polyphenols can both alter the gut microbiota and are broken down, often undergoing deglycosylation, to yield smaller, more lipophilic molecules that can exert a local effect in the intestine or be absorbed through the GI tract, reaching systemic circulation [295]. Though flavonoids and phenolic acids are often associated with antioxidant and antimicrobial effects [296-301], polyphenols are also found to influence the gut microbiome, promoting growth of good bacteria and the production of SCFAs and phenolic acids [65, 302, 303].

The more recent discovery of the prebiotic effect of dietary polyphenols means clinical trial studies are still scares. For saccharide compounds, clinical studies and human trials, have found different prebiotics, primarily fructo-oligosaccharides and inulin, to have beneficial effect against IBD and mild Chron's disease by reducing pro-inflammatory markers and increase *Bifidobacteria* levels in the faces [304-307]. Other studies have however found that treatment of Chron's disease with fructo-oligosaccharides or oligofrusctose-enriched inulin show no clinical benefit [308].The effect of prebiotics on IBS also show some inconsistent results, with some studies seeing no effect on IBS after prebiotic intake of oligofructose or fructo-oligosaccharides [309, 310]. These findings could indicate that the microbiota composition or the specific compounds in the prebiotic administrated are important factors for influencing colon health.

Though many studies have been conducted on the effect of prebiotics in the intestine and the body, it is important to note that there is no accepted standard protocol for measuring microbiota activity in faecal samples. This means that reported studies see big differences in sample size, prebiotic doses, duration of the study, how faecal samples are collected and how the microbiota are quantified.

2 Aims of the thesis

The overall goal of this thesis was to identify potentially novel compounds from Scandinavian medicinal plants that may be exploited in the future for clinical applications.
To reach this goal, the following aims were defined:

Aim 1: To identify candidate medicinal plants that could serve as sources for the identification of novel medicinal substances

This aim was addressed in **Paper I.** To reach this aim, we worked in a highly interdisciplinary manner together with culture historians, botanists, archeobotanists, biologists and pharmacists. Emphasis was placed on selecting plants with mentioned use against gastrointestinal related ailments or symptoms interpreted to have immunological relevance, and that have few reported pharmacological studies.

Aim 2: To characterize immunomodulatory effects of polysaccharides and polyphenols

This aim was addressed in **Paper II** and **Paper III**. Polyphenols contained in *G. purpurea* was isolated and identified, and their anti-inflammatory activities reported in **Paper II**. The immunomodulatory properties of pectic polysaccharides of *A. archangelica* and *A. litoralis* were investigated in **Paper III.**

Aim 3: To investigate prebiotic effects of polysaccharides and polyphenols on the gut microbiota

This aim was addressed in **Paper IV**. Selected polysaccharide and polyphenol fractions were tested for effects on abundances of microbial taxa related to beneficial effects and studied for their potential effects in gut barrier integrity and immune homeostasis.

3 Summaries of the individual papers

Paper I: The discovery of novel immunomodulatory medicinal plants by combination of historical text reviews and immunological screening assays

There are several different pharmaceutical compounds yet to be discovered and new inspirational sources are needed. The need for novel medicines is ever increasing, new compounds should preferably have been found yesterday. By combining different research fields, we have discovered several potential novel sources for compounds to cover some of the medicinal needs. Through thorough review of written records from the 19th century, we selected 23 plants to screen for immunomodulatory activities. The plant extracts were fractionated into a polysaccharide-enriched fraction or a polyphenol-enriched fraction that was compared against the crude extract. Each extract and fraction were tested for immune stimulatory and anti-inflammatory effect by use of ELISA human TNFα and IFNγ on PBMCs and measurement of NO secretion from the J774A.1 murine macrophage cell line. The ability to induce cell proliferation were measured by use of CFSE staining and flow cytometry. The monosaccharide composition and total phenolic content were determined. The *D. mezereum* solid-phase extraction (SPE) polyphenol enriched fraction was the only fraction inducing T-cell proliferation, this fraction also induced NK-cell proliferation and strong IFNγ and TNFα response. The TNFα and IFNγ response from *D. mezereum* also displayed a strong synergistic effect with the ConA stimulant used to test the anti-inflammatory effect of the fractions. For *Angelica*, the hydrophilic PS enriched fraction displayed potent TNFα secretion and NO secretion. The anti-inflammatory effect on IFNγ and TNFα were generally, except for *D. mezereum*, most prominent in the SPE fraction.

Paper II: Phytochemical characterization and anti-inflammatory activity of a water extract of *Gentiana purpurea* roots

G. purpurea is a perennial plant with limited distribution in Europe, only found in the Alps and in the mountain areas in southern parts of Norway. The root from this plant was widely used in Norway during the 18th and 19th century, primarily against gastrointestinal and airway related diseases. It was regarded as one of the most important Norwegian medicinal plants during this time. Because of its restricted distribution, limited research has been conducted on *G. purpurea*. This despite its wide use as a medicinal herb in the local areas where it is found and reported anti-inflammatory properties of *Gentiana* species in general. In this study, we conducted hot-water extraction to imitate preparation of the roots. To obtain the low molecular weight compounds from this water extraction, a Diaion HP20 column, followed by fractionation with Sephadex LH20, reverse phase C18 and normal phase silica gel was performed. To analyse the structure of these fractionated compounds, 1D NMR, 2D NMR, and ESI-MS were used, while HPLC-DAD were used to quantify the compounds. To evaluate the potential anti-inflammatory properties of each compound, secretion of TNFα from ConA stimulated PBMCs was investigated using ELISA. Phytochemical analysis of the hot water extract of *G. purpurea* roots revealed eleven compounds. Some compounds previously known to be present in the plant, and some compounds never previously reported for the species nor the *Gentiana* genus. Different composition was found in the different parts of the plant with gentiopicrin (gentiopicroside) being the major component in the roots and sweroside being most abundant in the leaves. The highest content of bitter substances, a preferred component, was found in the plants collected during October, which were also the preferred time of harvest when using the plant for traditional medicine in Norway. From the anti-inflammatory analysis, at the highest concentration, erythrocentaurin and gentiogenal displayed highest inhibition of TNFα secretion, while at the lowest concentration, erythrocentaurin displayed the most potent anti-inflammatory effect. Previous studies have found erythrocentaurin to inhibit NO production in macrophages, while this was the first study showing anti-inflammatory activity of gentiogenal. The observed anti-inflammatory properties in water-extracted compounds from *G. purpurea* can possibly contribute to the described health benefits of this plant.

Paper III: Comparative chemical and immunological profiling of polysaccharides from *Angelica archangelica* subsp. *archangelica* and *Angelica archangelica* subsp. *litoralis*

Angelica archangelica subsp. *archangelica*. (*A. archangelica*) L. and *Angelica archangelica* subsp. *litoralis* (*A. litoralis*) are the two angelica species growing in Norway. *A. archangelica* have a longstanding tradition in Norwegian folk medicine, being used to treat, amongst other diseases, the cold, throat- and chest diseases and infections. The widespread use of this plant in Norway for several centuries dating back to the Viking Age, prompted us to investigate the composition of the immune modulatory polysaccharides found in the roots of this plant, and compare against *A. litoralis* that were not commonly used in the traditional medicine. From the main polysaccharide-enriched water extract of both angelica species, one neutral and two acidic fractions were isolated by use of anion exchange chromatography. The neutral fraction and one of the acidic fractions consisted mainly of α-1,4- and 1,4,6-linked glucose (Glc), assumed to be due to the presence of starch. The other acidic fraction was rich in GalA, along with Gal, Ara and rhamnose (Rha). Subsequent glycosidic linkage analysis indicated the presence of pectins constituting both homogalacturonan (HG), rhamnogalacturonan type I (RG-I) and arabinogalactan type II (AG-II) structural elements. The most potent immune stimulant was found to be the pectin containing fraction, which induced NO secretion from macrophages, and TNFα and IFNγ secretion by human PBMCs and NK cells. Interestingly, the pectic polysaccharide fraction obtained from *A. archangelica* was more potent than the pectic polysaccharides of *A. litoralis*. The study conducted here is the first study reporting on isolation and immunomodulating effects of polysaccharides from the *A. archangelica* species.

Paper IV: A polyphenol fraction from *Daphne mezereum* exert prebiotic effects and protect the gut epithelial barrier

Polysaccharides and polyphenols found in plants are complex macromolecules not absorbed in the small intestine, but are instead found to reach the large intestine, interacting with and being metabolized by the gut microbiota. The medicinal plants investigated in this project were primarily consumed through ingestion of water decoctions, suggesting the polysaccharides and polyphenols found within these plants would have reached the large intestine before exerting their main effector functions. We therefore tested the ability of a selection of plant extracts, found to have strong immune modulatory effects in our previous papers, and with reported usage against stomach ailments in historical texts. The plants selected were *D. mezereum*, *A. archangelica* subsp. *archangelica*, *Hypericum perforatum*, and *Ranunculus acris*. The polyphenol enriched fraction from *D. mezereum* were found to induce a unique skewing of the microbiota profile with increases of commensal health promoting bacteria and reduction in assumed opportunistic pathogenic bacteria. Many of the bacteria found to be increased after culture with the polyphenol fraction from the *D. mezereum* extract were SCFA producing bacteria that are documented to function in protection of the intestinal barrier. Using a 3D intestinal organoid model system, we show that the supernatant collected after fecal cultures with the polyphenol fraction of *D. mezereum* conferred protective effect of organoids, compared to the other fecal supernatants. This observed holistic effect on the gut microbiota and protective effect on intestinal organoids indicate a modulatory effect of the components within the polyphenol fraction of *D. mezereum* that could be exploited to modulate and skew the gut microbiota towards a more health promoting composition, increasing barrier integrity and reducing immune activity.

4 Methodological considerations

4.1. Literature review and basis for plant selection

We primarily reviewed written sources from first-hand accounts of people living in rural areas in Norway and Sweden during the 19th and 20th century. The primary source used were from Ove Arbo Høeg, a professor in botany at the University of Oslo, and his questionnaires asking about the traditional uses of plants from people living in different areas in Norway, collected between 1925 and 1973. Many of the people answering the questionnaire were elderly people recollecting the plants used by their grandparents. The questionnaire therefore reflected the long traditional uses of these plants. The people using these plants would vary, but included farmers, lay practitioners and local healers, reflecting the use of these plants locally by everyday people. This material was supplemented with and confirmed by the questionnaire conducted by medical doctor and medical historian Ingjald Reichborn-Kjennerud in 1920, describing medical terminology and local medicinal traditions from southern Norway, focusing on herbal medicine. We chose to focus on these sources as these were well documented and collected with the aim of informing about people's use of plants in everyday life. The use of medicinal plants by these people were usually anchored in longstanding traditional uses, thus indirectly providing information about the historically commonly used medicinal plants in Scandinavian countries.

When selecting what sources to include, we chose sources written by scholars within different fields, including doctors, botanist and historians. The reason for selecting a broad range of authors was to get a good picture of how medicinal plants were used and how different diseases were described. Understanding how this could be related to our interest in the medicinal plant effect on the immune system and/or GI related diseases and ailments. The reason for combining so many different sources, was an early realisation of the inconsistencies in disease description and treatment, and general inconsistency in documentation of traditionally used medicinal plants.

People primarily seemed to use medicinal plants based on the availability where they lived, relying on information about local plants passed down through generations. This made us aware that when selecting plants for screening, we should select plants mentioned in several different sources, indicating a parallel recognition of the medicinal properties of those plants across different Scandinavian countries, thus strengthening their credibility as medicinal plants. It was also important to link the vernacular names with the scientific names. Two books published in 1975 and 1985 by professor Høeg were used for this work, as he was invested in

tracing and discovering what plants the people were referring to and linking these to their scientific names.

The literature review provided a list of 74 medicinal plants used across Norway and Sweden against immunological and/or gastrointestinal related diseases and ailments. We focused on these ailments as the effect on immune system are related to many diseases and several proposed health promoting compounds in plants are thought to reach the large intestine before exerting their effects. From these plants, we wanted to further focus on the plants with few documented pharmacological studies, as one of our main aims was to identify potential novel compounds. For this, we combined PubMed and SciFinder in the search for previously studies related to pharmacology, immunology and/or phytochemical characterizations. SciFinder was included as it has a broader database than PubMed, also encompassing information on chemical structures and patents. This filtering narrowed the list down to 21 understudied plants. We also chose to include two well studied medicinal plants. *Hypericum perforatum* L. [Hypericaceae] were included as a comparison to the less studied *Hypericum maculatum* Crantz [Hypericaceae], and *Juniperus communis* L. [Cupressaceae] was included as it has a long history as an essential medicinal plant in Scandinavia against some of the same indications as the plants on the filtered list.

4.2. Plant collection and extraction

The plants included in this study were collected from locations in the southern parts of Norway outside of protected areas, either around the Oslo area or in mountain regions. All plants were identified by a botanist in our team, and air dried prior to extractions.

The plants were initially extracted by hot water extraction by a PhD student at the Department of Pharmacy. This preparation method has been the most common way to prepare plant-based medicines, and by choosing this method we were able to have a close similarity between the preparations used in traditional medicine and the extracts obtained in the laboratory. By chemical fractionation, dried extracts were separated into two separate fractions, a polyphenol enriched fraction (SPE-fraction) and a polysaccharide enriched fraction (PS-fraction). Polyphenols and polysaccharides are two of the main bioactive phytochemicals in plants. The two fractions, in addition to the crude water extract, were included for screening in **Paper I**, sorting out the potential immune modulatory effect of the extracts and fractions. The reason for always including the crude extract together with the PS- and SPE-fractions, was to

observe if effects were potentially only contained in the main crude extract, and to control for potential synergistic or antagonistic effects within the crude extract.

All extracts and fractions tested in **Paper I** were dissolved in PBS, to prevent the solvent from influencing the immune readouts. For **Paper II**, some of the more purified compounds had to be dissolved in DMSO due to their low polarity, but we ensured that the final concentrations in readouts were within 0.5% DMSO final concentration. In tests where DMSO were used a solvent, DMSO was also added to negative and positive controls.

4.3. Cell viability testing via MTT-assay

Prior to assessing any immunological effects of the isolated plant extracts and fractions, we tested in **Paper I** their potential cytotoxic effects against the J774A.1 macrophage cell line. As several of the downstream tests in **Paper I** and the following papers were done on primary PBMCs, it would have been more optimal to have done this test also for PBMC. However, for screening purpose and reproducibility of data, it was more convenient to screen against a cell line. We chose to use the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to determine the cell toxicity of all extracts and fractions (crude, PS, SPE) as this is a commonly used assay to determine cell viability during drug screening [311]. The principle behind the MTT assay is that only live cells have the active metabolism required for the enzymatic reduction of the yellow tetrazolium salt MTT to purple formazan crystals. By measuring purple colour intensity using a spectrophotometer at wavelength 570nm (the maximum absorbance of formazan), the intensity of the purple colour (i.e., the amount of formazan produced) will be proportional to the number of live cells in the sample. It is important to stress that the assay only detects living cells and the readout are dependent on the degree of cell activation, dead cells are not detected.

The MTT assay offers a fast, high throughput readout with little manual handling, reducing the likelihood of human errors when performing the assay. However, the assay does have some drawbacks. The assay does not seem to be as sensitive in detecting small changes in cell numbers [312], but since this was a screen, the exact changes in cell numbers was not the main focus. As MTT is taken up and reduced by enzymes in mitochondria and endosomal/lysosomal compartments, the MTT formazans are transported to the surface of cells where they form spike-like structures. The uptake of MTT have not been found to damage the cells, but this metabolism and exocytosis of the formazan needle-like crystals have been found

to potentially damage cells and could initiate apoptosis related factors like caspase-8 or caspase-3, causing underestimation of cell viability [313].

Another aspect of concern regarding the MTT assay, is the finding from some studies that polyphenols may interfere with the readout of the MTT assay and thus yield false positive results and lead to erroneous conclusions that the SPE-fractions are not toxic, when they may indeed be. Some of the SPE-fractions had strong colours in themselves, so any colorimetric reading could be affected by this [314, 315]. In the screening, we could clearly see loss in viability from with some of the SPE-extracts (e.g. from *Sanicula europaea*), so the interference of polyphenols with the MTT assay is at least not universal for all polyphenols. The polysaccharide-enriched fractions generally showed high viability. We also considered that an eventual loss of viability could be a secondary effect of cellular activation, rather than a direct toxic effect.

Other assays that could have been considered instead or as a complementary approach, is the traditional methods of using the trypan blue dye. This is also a simple and inexpensive test to microscopically evaluate cell death. However, for screening purpose, this test would take a long time with respect to the number of samples in each experiment (23 plants, 3 different extracts in duplicates and 3 different concentrations). Another option was fluorometric assays, for instance measuring propidium iodide incorporation into dead cells via flow cytometry, but this is also not high throughput. A high-throughput alternative could have been the CytoGLO assay, which is a luminometric assay measuring adenosine tri-phosphate (ATP). ATP is the most essential energy reservoir in cells and thus a very sensitive measurement of cell viability. However, this assay may also suffer from interference from polyphenolic compounds.

4.4. Immune cell assays

The preliminary screenings in **Paper I** included ELISA to test for cytokine release from PBMC, the measurement of NO secretion from the macrophage cell line J774A.1, and testing proliferation of T cells and NK cells within PBMCs. In **Paper III**, we additionally assayed purified T cells and NK cells. We chose these assays as they are easy to use, precise in their measurements, reproducible and high throughput, being ideal for the amount of plant extracts we had to screen. The screening in **Paper I** formed the basis for selection of plants and extracts for further in-depth studies in **Papers II-IV**.

4.4.1. Choice of cells

To test the immunological effect of the plant extracts, we chose primary immune cells from blood as the main test cells. These were either PBMCs, or purified NK cells and T cells. We chose NK cells and T cells as they are main effectors against infected cells, and as their response can be monitored via the same type of assays (IFNγ and TNFα via ELISA, and proliferation assay). Primary cells are the closest one comes to testing realistic cellular effects *in vitro* and are therefore ideal when testing immune response of novel compounds or plant extracts. A disadvantage with primary cells is that they are difficult to sustain in culture over time as they require a delicate balance of medium components to proliferate and stay viable. These conditions can also in theory alter the cell phenotype. For experiment replication, different donor cells were used to ensure that any observed effects were not donor specific.

Activity of myeloid cells were monitored via NO release from the J774A.1 murine macrophage cell line. This cell line is easy to use and was already well established in our lab for this test. J774A.1 has previously been used as a model to test effects on NO release from macrophages with success [316-318]. In an attempt to use primary myeloid cells, we tried differentiating dendritic cells from PBMCs by culturing for five days with supplementation of IL-4 and GM-CSF on day 1 and day 3 but failed to generate sufficient amounts for our screening purposes.

4.4.2. Cell proliferation using CFDA-SE

To determine the effect the extracts had on cell proliferation, we utilized an assay based on carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) staining, whereby cellular proliferation is measured as dilution of CFDA-SE in daughter cells via flow cytometry. CFDA-SE is a modified version of carboxyfluorescein succinimidyl ester (CFSE) where two acetates have replaced two hydroxyl croups, making CFDA-SE more hydrophilic and cell permeable. Upon entry of CFDA-SE into cells it is cleaved by esterases to form CFSE. Once inside the cell, CFSE is covalently coupled to lysine residues and amine sources on molecules within the cell. Because of this stable interaction, once CFSE is incorporated into the cell interior, it will not be transferred to other neighbouring cells [319]. For each cell division, the CFSE fluorescence halves, leaving the daughter cell with an approximately 50% weaker CFSE stain compared to the parent [320]. This quality makes CFSE ideal to measure cell proliferation. If the labelling concentration is optimal, the dye can measure 7-8 cell divisions before the dye becomes too weak to be measured [321].

An alternative assay to CFSE could be the thymidine incorporation assay or ethynyl-labeled deoxyuridine (EdU), that both measures DNA synthesis. The thymidine incorporation assay uses the radioactive nucleoside 3H-thymidine that incorporates itself into new chromosomal DNA strands during cell division (mitotic stage). The readout of this assay is the radioactivity in DNA that are recovered from the cells by use of a scintillation counter that detect and measure ionizing radiation, thus involving radioactivity. The EdU assay relies on incorporation of EdU into DNA during *de novo* DNA synthesis instead of the naturally incorporated thymidine. The EdU assay though easy, have been found to induce toxicity to the cells because of the generation of copper in this assay during labeling. An alternative, copper-free version is available, the Vinyl-2'-deoxyuridine (VdU) assay, incorporating VdU instead of EdU into the DNA [322]. The reason for using CFSE is this is non-radioactive compared to the thymidine assay, cheaper than VdU, and it is a well-established method in our lab.

4.4.3. Enzyme linked immunosorbent assay for measurement of cytokines

ELISA is a rapid antibody-based assay used to quantify specific molecules in a sample. In this thesis, ELISA was used to measure secretion of TNFα and IFNγ from PBMCs, as well as from purified primary NK and T cells. In addition, we measured the concentration of TNFα upon Transwell co-cultures of PBMCs with the Caco-2 cell line.

When addressing pro-inflammatory immune responses, the focus is usually on bacterial or viral infections. We therefore chose to focus on TNFα and IFNγ that are two of the cytokines known to play crucial roles in the immune response against these threats, and that are released in abundance by PBMCs upon activation. When testing the ability of the extracts to activate immune cells to secrete TNFα and/or IFNγ, it was decided to use a positive control that could compare to the extracts. Concanavalin A (ConA) is a plant lectin that have been shown to induce strong immune stimulation by binding to the mannose residues of glycoproteins and through this activate lymphocytes. Studies have also found that TNFα does not have a mitogenic activity with ConA, meaning that no additional effect should be observed because of this reaction [323].

After an inflammatory response, it is also important to counteract the immune stimulatory effect to bring the immune system back to equilibrium and a normal state. Also, there are some instances, for example in context of autoimmune diseases where the immune response must be suppressed or reduced. We therefore also tested the anti-inflammatory effect of the extracts. As a model for anti-inflammatory activity, we exploited the pro-inflammatory effect of ConA,

and tested whether any of the extracts could reduce cytokine release in response to ConA upon co-cultures of ConA and the extracts.

4.4.4. NO secretion measurements

In order to test NO secretion from macrophages, we used the murine monocyte/macrophage J774A.1 cell line obtained from ATCC. The J774A.1 cell line is a commonly used cell line when testing NO secretion and is often used in initial screening of natural products to check their bioactivity. Because of their nature, cell lines are, by definition, cancerous cells and therefore some caution is needed when interpreting results directly from experiments using such cells. In addition, when cell lines are passaged several times, this is known to cause phenotypic and genetic changes to the cells. The J774A.1 cells were therefore not passaged for more than a month before alternatively thawing a new vial.

When testing NO secretion, LPS from *Pseudomonas aeruginosa* was used as a positive control. LPS is known to be a potent stimulant of macrophages to induce NO secretion. Surprisingly, we experienced that LPS from *P. aeruginosa* had a more potent effect on the J774A.1 macrophage cell line compared to LPS from *Escherichia coli.* These two bacteria have similar LPS structures and are normally used interchangeably to stimulate NO secretion from macrophages. A reason for the observed effect could still be due to the variation in structure of LPS from these two species. LPS structure variation are known to influence their receptor potency and biological activities [324]. When testing the ability of the extracts to suppress NO secretion, we added the extracts together with the same concentration of LPS as were used for the positive control and compared NO release against the LPS positive control alone.

4.5. Reporter cell assay to test receptor binding (HEK-Blue)

Based on the findings from the immune stimulatory assays, we also wanted to investigate how the polysaccharide fractions interacted with receptors on innate immune cells. We chose to test the binding capacity of the fractions on a few selected receptors with conserved structures that are known to be important in recognition of especially glycosylated compounds and polysaccharide structures. The tested receptors were not relevant for the polyphenol enriched SPE fraction, as these are assumed to have another mode of action than directs interaction with these receptors. Based on the several receptors known to bind polysaccharide patterns, we chose to focus on TLR-2 and TLR-4 that are expressed on several immune cell subsets, and the CLRs Dectin-1β, Dectin-2 and Mincle that are primarily expressed on myeloid cells.

The HEK-Blue cell lines were commercially available, containing co-transfected human TLR-2 or human TLR-4 with secreted embryonic alkaline phosphatase (SAEP) genes in HEK293 cells. For TLR-2, a CD14 co-receptor gene had also been transfected into the same cell line to enhance the TLR-2 response. For TLR4, the co-receptor genes MD-2 and CD14 was co-transfected, as TLR-4 will not signal without these co-receptors. When using reporter cell assays, it is important to also include a negative control since the compounds tested can, in theory, interact with some of the other receptors still present on the reporter cells. For the HEK-Blue cells, we used the commercially available CD14 reporter cell to function as a negative control to make sure we had no unspecific binding to other components on the cell surface. Also, since we were working with plant extracts, we tested the extracts for LPS contamination to make sure that the observed effect on the reporter cells were not due to LPS interacting with the receptors.

4.6. Transwell assay to test gut wall permeability

To investigate intestinal barrier integrity, we chose the well characterized human colonic Caco-2 cell line, originally obtained from ATCC. The use of transwells for testing cell permeability is an established protocol, dating back to 1961 and Dr. Stephen Boyden's transwell migration assay or Boyden chamber assay first used to quantify cell migration [325]. Using Caco-2 cells cultured in transwells to represent the epithelial barrier of the small intestinal epithelium was first described in 1989 and have since been acknowledged as a representative *in vitro* model for experimental models for measuring transport and crosstalk across the intestinal epithelium [326, 327].

Although this is a well-established *in vitro* model, the setup has some drawbacks. The use of the Caco-2 cell line means not all differentiated cell types in the intestinal epithelium are represented in this model, the cell line consists of a homogenous cell population of only enterocytes. The Caco-2 enterocytes will however over time express morphological and biochemical properties like those of absorptive enterocytes *in vivo*. The differentiation is found to happen in a mosaic pattern, with some cells being fully differentiated after about 14 days, while others are not. Studies have found that homologous differentiation of Caco-2 cells can be reached after 21 days with cells coupled together by tight junction protein complexes [328, 329].

We let the Caco-2 cells grow for 21 days in order to achieve full epithelial cell differentiation, continuously measuring transepithelial electrical resistance (TEER) as they

grew to ensure a tight monolayer formation. At day 21, the extracts were added to the Transwell insert, representing the luminal area in the intestine. At the basolateral side, in the bottom well, we added PBMCs to test the effect that the test samples might have on the immune system in the "lamina propria" through the epithelial barrier (**Figure 7**).

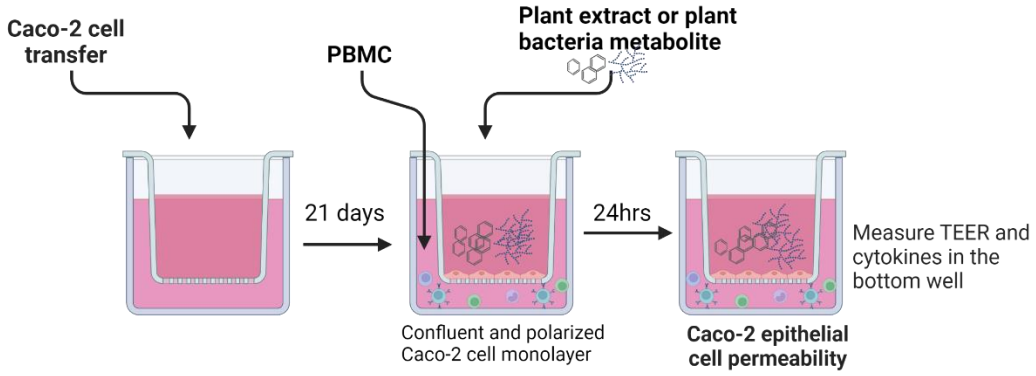

Figure 7: Caco2 transwell assay. Schematic representation of the Caco2 transwell assay used in this thesis. The figure was created by BioRender.com.

When using the Caco-2 assay, it is important to make sure that the polarized monolayer is intact. TEER measurements using an ohmic resistance measuring device represents the easiest and most applicable way of measuring this. The TEER measurements did yield some varying results, but the average TEER value was over 200 $\Omega.cm^2$ at day 21 when the extracts were added to the wells. Caco-2 monolayers usually generate a TEER between 150 and 400 $\Omega.cm^2$ which is deemed sufficient to restrict diffusion across the barrier [329]. Measuring TEER allows for easy tracking of barrier integrity over time without causing cellular damage. These measurements are however quite sensitive to temperature, medium composition and cell passage numbers. When doing the TEER measurements, we made sure the temperature was the same each time, but the age of the medium was not the same each time and the number of cell passage varied between the experiments as the same Caco-2 batch were used to seed different transwell experimental setups in subsequent weeks. These factors could be the reason for the varying TEER values observed. As we generally obtained values over 200 $\Omega.cm^2$ at the day of the experiment, some fluctuations in the measurements were accepted.

A potential drawback of using the Caco-2 cells is that they do not utilize SCFA as energy source, in contrast to primary enterocytes. For this reason, it is also important to study the effect of the different extracts in a more relevant assay. We are currently developing methodology to culture mouse intestinal organoids in the Transwell system. An added advantage of using

organoids for this purpose is that they also contain the different cells normally found within the intestinal epithelium.

4.7. Intestinal organoids (enteroids) as 3D models of the intestine

Organoids are 3D representations of an organ either made from pluripotent stem cells (PSC), induced pluripotent stem cells (iPSC) or primary tissue/adult stem cells (ASC) from the given organ, with advantages and disadvantages to the different approaches. PSC and iPSC are good models for studying the endogenous organ developmental processes, germline related diseases, and testing the importance of different genetic alterations in organ development [330]. PSC and iPSC organoids do, however, take a long time to develop and after differentiation, the organoids will not regenerate or continue to divide [331-333]. ASC collected from the given organ will not undergo the full developmental process, as these stem cells are already semi-determined. The organoids will have similar characteristics as the tissue of origin, they are self-expanding and maintain genetic and functional stability over time [127, 334-336].

We chose to use ASC from the small intestine to establish intestinal organoids (enteroids) that we used to test the effect of compounds, fractions and extracts on the intestinal epithelial cell layer and its differentiation, and the effect of the plant-bacteria metabolites on the intestinal wall structure. The epithelial cells of the organoids surround the fluid-filled luminal cavity that mimics the intestinal interior. This interior is where digested food, food particles and external components interact with the host cells and where the gut microbiome resides. Many food components and medicines are taken up in the small intestine, while the large intestine harbours the most abundant composition of different gut microbiomes, acting on unabsorbed medicines and harvesting extra energy from undigested food. Enteroids have the same characteristics as those seen in the intestine with mucus production, absorption and general secretion functions, in addition to the polarized epithelial layer with the apical site facing the luminal centre [335, 337, 338]. The epithelial layer of these enteroids harbour all the cells found in the small intestine *in vivo*, making them ideal models for studying the effect the extracts can have on different cells in the intestine and in a more *in vivo* like setting.

Many of the components tested in the organoid model are thought to reach the large intestine, interacting with the gut microbiota. Since we also tested some of the bacteria supernatants from the microbiota screen, we could have included an organoid model from the large intestine, the place with most abundant bacteria populations, to specifically test the bacteria metabolites from the plant extracts in this model. However, we used some time

optimising the protocol for culturing organoids, and the protocols for making small intestinal organoids (enteroids) is more established. As many of the cells in the GI epithelium are similar for the small and large intestine, we decided to start with making enteroids and may in the future continue with colonic organoids.

When culturing intestinal organoids from ASC, Wnt or Wnt agonist R-spondin and EGF are essential components added to the medium at the start of organoid formation from isolated crypts. After immature circular organoids take form, Wnt and EGF becomes less important and higher concentrations of BMP becomes important for cell differentiation into specialized epithelial cells. Noggin is a protein that binds to and antagonizes BMP proteins, thus helping suppress differentiation and are important for maintaining undifferentiated stem cells. As new organoids are made from stem cells after splitting the organoid cultures, it is important to maintain a relatively high concentration of stem cells in addition to the differentiated cells. The medium needs to be supplemented with the right balance of EGF, Noggin and R-spondin which allows long-term expansion of intestinal epithelium and the ability of the stem cells to differentiate into different cell types [334, 335].

The protocol for culturing human and mice intestinal organoids is well established, with human organoids requiring slightly more components compared to mice enteroids to grow and differentiate. We first aimed to make rat enteroids based on different combinations of the protocols used for mice and human intestinal organoids. We did manage, by using culture mediums and steps most resembling that used for human intestinal organoids, to culture some rat enteroids with very good intestinal assembly and many crypt structures (**Figure 8**). This however proved difficult to sustain and we therefore decided not to continue working on this protocol. We also considered human intestinal organoids, as these would be good models since most of the immunological and bacterial screenings were done with human material. Human intestinal samples are however much more difficult to come by for ethical reasons, and since we were just starting up with organoid experiments and still need to optimise culture conditions and experimental setup, we decided to focus on mice enteroids in this project.

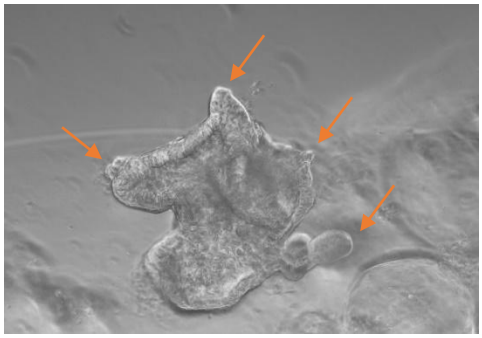

Figure 8: Brightfield image of rat organoid: Rat intestinal organoid taken with 4x zoom showing differentiated enteroids with crypt structures (arrows).

4.8. Microbial culturing and 16S rRNA sequencing

The gut microbiome is gaining increasing attention for its ability to influence health and diseases. The microbial community and the anaerobic conditions in where they live are however difficult to recreate outside the intestine. To better understand how the gut microbiome responded to a selection of the plant extracts in our project, we cultured human faeces together with the selected extracts and fractions in a self-engineered anaerobic chamber placed in a cell incubator to keep the bodily 37°C. The bacteria culture was homogenised in an anaerobic basal broth before adding to the sample and the chamber was made anaerobic by adding two bags of AnaeroGen. An anaerobic indicator was added to make sure the chamber was sealed properly, and the environment were kept in an anaerobic state. The anaerobic basal broth contained components that would support growth of anaerobic bacterial assumed found within the intestine. The broth is however formulated to optimize growth of most anaerobic organisms believed by the manufacturer (ThermoFisher) to be of clinical importance. This medium has obvious drawbacks as it would favour growth of specific bacteria species and the findings would be skewed towards this. There are however few *in vitro* alternatives to this method. There are not many alternative basal broth mediums on the market, and the ones available contain the same components supporting the same conditions.

We chose to focus on the bacteria composition of the gut because these are the most abundant and are, so far, the ones that have received most attention for their potential influence on host health. We used 16s rRNA sequencing to determine the bacteria composition as this is the most effective way to analyse and identify strains of bacteria that are poorly described, rarely isolated or have unusual phenotypes. The 16S rRNA is part of the small ribosomal subunit that play an essential role in mRNA decoding. This essential role means the 16S rRNA is universally conserved among prokaryotes. Because it is so conserved, some researchers

argue that the 16S rRNA gene is too conserved to be able to separate prokaryotes at lower more specific levels [339]. For the purpose of our experiments this was not a major concern.

We selected to include plants where we had seen a distinct immune modulatory effect and that had been used against stomach related ailments in the historical texts. We included all the three samples (crude water extract, PS-enriched and polyphenol enriched SPE fraction) from *D. mezereum* as this plant was of particular interest based on the immunological screening and described to be used against different stomach ailments in historical texts. In addition to *D. mezereum*, we included the crude water extract and PS fraction from *A. archangelica* and *R. acris*. Polysaccharides serves as energy source for the commensal bacteria, and we therefore chose to include two of the most bioactive polysaccharide fractions (**Paper I**). Also, the root of *A. archangelica* had a reported usage against various ailments of the stomach. We also included *Hypericum perforatum* (crude water extract and SPE fraction) that it is sold as an herbal remedy for its anti-depressant effect and that is documented by the literature sources to be used against stomach pains. The mechanism behind its anti-depressant effect is not clear, and we thought, with the growing interest of the effect of microbiome on the brain [340, 341] that this would be an interesting plant to include.

For each test sample we chose two concentrations (1 mg/mL and 0.1 mg/mL). These were two of the concentrations used in the immunological screens. Other *in vitro* studies often use levels above physiological relevance (e.g. 150 mg/mL [342, 343]), we wanted to test concentrations that we assumed were more realistic to be in the intestine. A previous study performed on polyphenol rich plant fractions used concentrations between 0.25 and 10 mg/mL, showing most promising results with concentrations at 1 mg/mL or below [344]. Ideally, we would have included lower concentrations than 0.1 mg/mL as well, but because of the cost of the assay, we had limited resources to test the extracts. As it turned out, we found most effects with the highest concentration.

When analysing the effect of the extracts on the gut microbiota, we decided to focus only on bacteria where we had phylogenetic definition down to at least genus levels, as bacteria from the same order, or even sometimes family can display very different effects in the intestine. An example of this is the pathogenic *Clostridium perfringens* and *Enterococcus* that both belong to the Firmicutes phylum often associated with health promoting bacteria, and with *C. perfringens* even belonging to the same order as the health promoting *F. prausnitzii* and *C. butyricum*

5 General discussion

5.1. Discovering novel pharmaceutical compounds

Setting out to find inspirational sources for discovering novel drug compounds can be a daunting task. The development of high throughput methods and advanced data technology means that screening of medicinal plants for their potential medicinal effects is a more manageable task than it was only two decades ago. It has long been known that medicinal plants are able to influence immune responses and the gut microbiome, but the novelty of analytical tools means it is now feasible to investigate the actual mechanisms behind such reported effects.

There is a growing trend towards seeking back to nature and relying on local food and sustainable resources. We wanted to empirically test some of the traditionally used herbal medicines to see if they have scientific foundation for their historical uses. It is well known that some medicinal plants found to have an effect against a certain ailment, ended up as a panacea thought to be able to cure everything from headache, stomach pain and rashes, to the plague. We observed several references to such panaceas during the literature search, making empirical testing even more important to determine their actual effects. For people living in rural areas in Norway and the rest of Scandinavia, which were the majority up to the mid-1900s, medicines were mainly based on plants, with instructions about specific plant parts, collection time and preparation method being passed down for generations. Because of the longstanding use of these medicinal plants, we decided to focus our search here.

We assumed, based on their wide and longstanding use that these plants could contain components with actual immune modulatory effect and/or effect on gut health based on their described uses. Based on the preliminary screening done in **Paper I** where we compared polysaccharide and polyphenol fractions, our assumption proved valid as some, but not all, of the selected plants did show effects on immune cells. The effects we observed were independent of the yield of the substances tested, indicating plant-specific effects. We also showed an effect on the gut microbiota (**Paper IV**). Among the plants found to be extensively described and widely used but that did not show any potent effect in our screens were *Juniperus communis* (juniper) that was regarded as a universal panacea, *Cochlearia officinalis* (scurvygrass) though to have anti-inflammatory properties and *Vaccinium vitis-idaea* (cowberry) used against the cold and lung infection. Interestingly, some of the plants where we did not observe any significant effects, also did not have extensive descriptions in the literature,

like *Polygonatum multiflorum* (solomon's seal) and *Antennaria dioica* (catsfoot). And some, like *Sanicula europaea* (Sanicle), were found to be quite toxic. In the methods used, based on SPE and ethanol fractionation, we could deduce whether the observed effects were in the phenolic enriched or polysaccharide enriched fractions, providing a preliminary idea of the compounds involved in the observed effects.

Among the plants selected for our study (**Paper I**), we observed that the immunological activities differed in terms of the bioassays they were subjected to. Some of the plant extracts showed potent immunomodulatory activities (e.g. *D. mezereum, A. archangelica* and *G. purpurea*), others had effects in anti-tumour assays as potential cytostatics or as novel sources for antimicrobials. The latter results are not included in this thesis but are currently on-going projects.

5.2. Selection of plants for further analysis

One of the main challenges after the screenings in **Paper I**, was to decide which plants to continue investigating in the frame of this thesis. We decided to put emphasis on plants showing the strongest effects in the immunological assays, and selected *G. purpurea* due to anti-inflammatory activity of the polyphenol-enriched fraction (**Paper II**) and *A. archangelica* based on strong activity of their polysaccharide-fraction (**Paper III**). Both these plants are also unique medicinal plants to Norway with strong roots in Norwegian cultural history. *D. mezerum* was the plant demonstrating the highest bioactivity in all assays tested, and we therefore also selected this plant for further in-depth studies (**Paper IV**), as well as two studies not included in this thesis that are focussed on structural elucidation of compounds.

5.2.1. Gentiana purpurea

For **Paper II**, we focused on the anti-inflammatory responses of the SPE-fraction from *G. purpurea*. We purified several compounds from the root of this plant, including secoiridoids, one flavonoid and one lignan. The major compounds in the extract, as well as the (assumed) metabolites formed by enzymatic degradation during water extraction were selected for activity screening, of which four displayed significant anti-inflammatory effects. The compounds found to have anti-inflammatory responses in our study are consistent with the anti-inflammatory properties reported for *G. purpurea.*

5.2.2. Angelica archangelica and Angelica litoralis

For **Paper III**, we chose to focus on the immune stimulatory effects observed by the polysaccharide enriched fractions from two subspecies of *A. archangelica*. Interestingly, in **Paper I** we had observed that the measured bioactivity differed between the subspecies *A. archangelica* and *A. litoralis*, and we therefore undertook a study to compare the polysaccharides from these two plants in more detail. The study concluded that isolated pectic polysaccharides from *A. archangelica* were more potent at stimulating immune cells than those obtained from *A. litoralis*, despite similar monosaccharide and linkage type compositions. We speculate that the degree of branching of the polymers or their three-dimensional structure could be different, and further structural elucidations are currently ongoing. Our data thus support that the subspecies *archangelica* is the traditionally referenced in historical texts. For **Paper IV**, we also chose to include the polysaccharide fraction from *A. archangelica* for the microbiome screen, as *A. archangelica* have historically been used to treat stomach ailments like diarrhoea, gas and stomach-aches, and since the polysaccharides from this plant had one of the highest bioactivities amongst the polysaccharides tested (**Paper I**). We saw an effect of the polysaccharide fraction from *A. archangelica* on the butyrate producing *Clostridium* cluster XIVa, increasing the abundance of this bacterium (**Paper IV**).

5.2.3. Daphne mezereum

The polyphenol enriched SPE fraction from *D. mezereum* was in the immunological screening in **Paper I** found to have potent immune stimulatory effect, and an additional synergistic effect with all the positive controls used (ConA, LPS, and IL12/15/18). For **Paper IV**, the SPE-fraction from *D. mezereum*, with its distinct immunological effect, were of interest. *D. mezereum* were also in historical texts frequently mentioned to be used against stomach ailments. We therefore chose to include both the crude water extract as well as the polysaccharide and polyphenol fractions of this plant in the microbiota screen in **Paper IV**. The polyphenol fraction, but not the polysaccharide fraction, from *D. mezereum* displayed remarkable effects on the gut microbiota, promoting growth of healthy bacteria and suppressing growth of pathogenic bacteria. We see the prebiotic value that some components within this fraction could have and are in the process of determining the effector substance in this fraction.

5.3. Purified plant compounds

5.3.1. Daphne mezereum

After observing such strong immunomodulating effects, as well as a unique effect on the gut microbiota of the *D. mezereum* polyphenol fraction, we decided to isolate its major compounds. New bark samples of *D. mezereum* were collected and extracted with 50% ethanol. This extraction method was chosen in order to obtain higher yields of the compounds of interest. The hot water extract contains high amounts of carbohydrates and other highly hydrophilic compounds that were not present in the SPE-fraction, and that would not be present in a 50% ethanol extract. This purification process was performed by a master student at the Department of Pharmacy and revealed two major polyphenolic compounds in the plant; the flavonoid neochamaejasmin A and the coumarin daphnin. HPLC-DAD and ^{1}H NMR analysis confirmed that these two compounds were the major components of the original SPE-fraction as well.

Neochamaejasmin A is a biflavanone that have been found to induce apoptosis in different cancer cell lines like human liver carcinoma cell line (HepG) and human prostate cancer cell line (LNCaP) [345, 346]. Neochamaejasmin A is also found to induce growth of specific bacteria in both female and male rats, increasing the abundance of *Ruminococcaceae*, *Lachnospiraceae* and *Pseudoflavonifractor* [347]. In our microbiota experiment, we observed similar results for *Ruminococcus*, both in the crude extract and the polyphenol enriched fraction, but the opposite effect was observed for *Pseudoflavonifractor*. As we tested the polyphenol enriched fraction and not pure compounds in the microbiota screen, this could explain differences in the effect on the gut microbiota compared to pure compounds. The potential effect of neochamaejasmin A on these other bacteria can be masked by the presence of other compounds in the extract or differences in bacteria composition between the experiments. In the anti-inflammatory assay, neochamaejasmin A induced strong anti-inflammatory effect through suppressing TNFα secretion from PBMCs in the presence of ConA, consistent with the anti-inflammatory effects reported in literature.

Daphnin is associated with antibacterial effect and fever relief. In addition, a daphne flower extract were found to have antioxidant and anti-inflammatory effects and promote neuroprotective effects both *in vitro* and *in vivo* [348, 349]. The antibacterial activity of daphnin have been found to be potent against *S. aureus*, MRSA, *E.coli*, and *P. aeroguinosa* [350]. Daphnin (daphnetin 7-O-glucoside) is the glucoside of daphnetin (7,8-dihydroxy coumarin), made through enzymatic glycosylation inside the plant [351]. The additional glycosyl group means daphnin have higher water solubility while daphnetin is more lipophilic

and can more easily be absorbed through the lipid bilayers in cells. As the gut microbiota can influence deglycosylation, daphnin may be hydrolyzed by intestinal beta-glucosidases to give daphnetin.

Daphnetin is found to have anti-inflammatory effects and target different molecular and cellular pathways to protect against a variety of inflammatory diseases. Among these diseases are neurological disorders, cardiovascular diseases, rheumatoid arthritis, psoriasis, systemic lupus, kidney disease, various cancers (kidney, liver, ovary, leukemia), lung infections, intestine related diseases and oxidative stress. The mechanism for these effects is through the NF-kB, MAPK and Nrf2 pathways. Daphnetin suppresses TLR-4, NF-kB, MAPK and pro-inflammatory cytokines like TNFα and IL-6, inducing anti-inflammatory effects. Daphnetin is also found to enhance Nrf-2 expression by increasing nuclear translocation of Nrf2, increasing the production of antioxidant enzymes, suppressing ROS and inhibiting oxidative stress [352-371]. We observed with our anti-inflammatory tests that daphnetin was a more potent anti-inflammatory compound than daphnin (**Paper IV**).

The reported anti-cancer effect of daphnetin is most likely through its ability to inhibit protein kinases intracellularly, and inactivation of the Wnt/β-catenin pathway, reducing or inhibiting cell proliferation [372, 373] In organoid models, Wnt is required for crypt proliferation and for maintaining the undifferentiated crypt progenitor state. Loss of Wnt or Wnt activator R-spondin leads to disruption of intestinal epithelial integrity as a result of eliminated proliferation capacity. When testing daphnin on the organoid models, we observed no significant inhibition of cell proliferation, indicating either a need for this parent compound to be converted to daphnetin to exert its anti-proliferative effect, or possibly that the effect is more pronounced in cancerous states where overactive Wnt/β-catenin signaling is a factor [374].

Locally in the colon, daphnetin is found to suppress colonic inflammation, improving intestinal barrier integrity, and functioning in restoring immune and metabolic homeostasis in animal colitis. A study found daphnetin to increase the abundance of SCFA producing bacteria, increasing T_{reg} cell development and IL-10 production, and reduce pro-inflammatory Th17 cell differentiation [375-377]. Even though daphnetin is not found to directly affect estrogen activity [378], our microbiota screen identified increased abundance of two estrogen producing bacteria, *Bifidobacterium* and *S. isoflavoniconvertens,* by the *D. mezereum* SPE-extract that could indicate a possible indirect estrogenic effect in addition to the anti-inflammatory and anti-oxidant activities previously reported.

In vivo and *in vitro* toxicological studies have found no mutagenic or allergenic effects, no mucosal irritation and no mortality from daphnetin, indicating no toxic effects of daphnetin at pharmacological relevant concentrations [379]. Daphne polyphenol compounds could therefore potentially have therapeutic anti-inflammatory and antioxidant roles in humans. We are currently testing the composition of the fecal culture supernatants, where one of the aims is to identify the abundances of daphnin versus daphnetin.

The high secretion of TNFα induced by the *D. mezereum* crude water extract and polyphenol fraction observed in **Paper I** does suggest a potential role in lymphocyte activation for some of the compounds in the polyphenol fraction. In contrast, after isolating compounds from the polyphenol fraction of *D. mezereum*, it was observed that the compounds were anti-inflammatory with exception of daphnin. The nature of the pro-inflammatory component is unknown at the moment. We considered fatty acids that are present in the fraction, however these are present in very low abundance. Either there is an as yet uncharacterized pro-inflammatory compound in the fraction, or there is a synergistic effect between several low-abundant pro-inflammatory compounds.

5.3.2. Gentiana purpurea

The most potent anti-inflammatory compounds isolated from *G. purpurea* was gentiopicrin (gentiopicroside), gentiogenal, erythrocentaurin and amarogentin, showing a dose-dependent inhibition of TNFα secretion. In animal models, studies have found gentiopicrin from other Gentiana plants to have anti-inflammatory activities, anti-rheumatic effects and antioxidant activity [380-382]. The anti-rheumatic and anti-arthritis effects were associated with suppression of the NF-kB pathway [382]. The anti-inflammatory effects previously observed for this compound is consistent with our findings, where a reduction in TNFα secretion likely could be the result of NF-kB pathway suppression.

Not many studies are conducted on the effect of gentiogenal, a derivative of gentiopicrin. The studies conducted have focused on its antimicrobial activity against penicillium [383, 384]. Erythrocentaurin is not well studied either, but the studies conducted have fond it to inhibit α-amylase, an enzyme important for digesting starch and glycogen, with an IC50 of 1.67 ± 0.28 mg/mL and to show antibacterial effects [385, 386].

Amarogentin is one of the most potent bitter compounds we know and binds to bitter taste receptors. Though the correlation between bitter taste and toxicity are not conclusive, bitter taste receptors are believed to have functioned in preventing intoxication of mammals,

carnivores in particular, by avoiding intake of poisonous foods that usually taste bitter [387]. Amarogentin is found to affect tuft cells in the upper airway by binding to these bitter taste receptors, resulting in signaling to the surrounding epithel cells to secrete antimicrobial peptides like β-defensins 1 and 2 [388]. These bitter taste receptors are also found throughout the GI tract and can function in pathogen protection by secretion of antimicrobials like a-defensins 5 and regenerating islet-derived protein 3 α (REG3A), in addition to regulating the expression of immune factors like mucins and chemokines that affect pathogenic growth [389]. Amarogentin is also found to have high affinity to spike glycoprotein and Angiotensin-converting enzyme 2 (ACE2) attached to membrane cells in the intestine and show a good pharmacokinetic profile with little predicted toxicity level. Amarogentin was found to target the same pathways as those altered during Covid-19, among them the TNF pathway, ion transport and G-protein coupled receptor pathway [390]. The observed inhibitory effect on TNFα secretion by amarogentin in our studies indicate a potential therapeutic effect like those observed in the forementioned Covid study. These anti-inflammatory effects and the interaction with bitter taste receptors and subsequent production of antimicrobials and immune modulators are also consistent with the traditional use of *G. purpurea* against airway diseases and the cold that most likely stem from viral or bacterial infections. Suppression of TNFα production is particularly important for people showing cytokine storm symptoms after infections, where too many cytokines, in particular TNFα, are produced. Excessive TNFα production is also associated with chronic inflammation and autoimmunity, making suppressing and regulating this cytokine important.

5.4. Receptors for plant polysaccharides

Receptors of the innate immune system have evolved alongside pathogens for the recognition of structures on the pathogens, amongst these are polysaccharide structures. Plant polysaccharides, which contain a variety of different polysaccharide structures, are likely to bind these receptors. TLRs and CLRs are two conserved families of PRRs found on several different innate immune cells (Section 1.3.3). Among the polysaccharide fractions tested, the PS fraction from *A. archangelica*, and one of its purified fractions containing pectins, showed potent binding to TLR-4, but no binding to TLR-2 or Dectin-1β. Also, polysaccharides from *D. mezereum* bound TLR-4, as well as Mincle and Dectin-2, the latter tested using a BWZ assay (unpublished data). In a separate study, the polysaccharides of *D. mezereum* was subjected to enzymatic degradation of the homogalacturonan region of the pectin. This version

showed potent TLR-4 binding, indicating that the interaction with TLR4 occurs through the RG-I regions (Ulriksen and Butt, unpublished data). All the receptors tested are found expressed on macrophages and is found to activate the NF-kB signalling pathway. Binding to these receptors can therefore induce pro-inflammatory responses and could explain the potent NO secretion from macrophages stimulated with the polysaccharides (**Paper I**).

5.5. The pro-inflammatory effects of polysaccharides

Both TNFα and IFNγ are important pro-inflammatory cytokines at the centre of many immune activating mechanisms. On the other side of the scale are anti-inflammatory cytokines important for suppressing an active immune system and bringing the immune response back to balance. Balancing these responses are important for proper immune function.

In **Paper III,** we tested IFNγ and TNFα production from PBMC and purified primary NK cells and T cells in response to polysaccharides isolated from *A. archangelica*. For PBMCs, the acidic fraction containing pectins was more potent in inducing TNFα and IFNγ secretion compared to the neutral fractions containing starch and hemicellulose. Dietary fibre like pectins are found to directly interact with intestinal cells and mucosal immune cells by binding to TLRs and influencing immune cell responses [391]. Pectins are also found to bind LPS, reducing LPS binding to TLR-4 leading to reduced endotoxin-induced inflammation [392]. We observed minimal cytokine release from naïve T cells with polysaccharides from *A. archangelica*, but somewhat more TNFα than IFNγ (**Paper III)**. Naïve $CD8^+$ cytotoxic T cells are found to secrete TNFα [393], while IFNγ is primarily produced by effector $CD8^+$ T cells [394]. In our 24-hour assays of PBMCs, we therefore only measured responses from naïve T cells. On the other hand, robust release of both IFNγ and TNFα was observed from NK cells. We have not pinpointed the receptors on NK cells mediating this effect, and NK cells do not express TLR-4. We can therefore not exclude that there are some contaminating monocytes remaining in the NK cell isolate, and that these are the primary responders to the polysaccharides and induced a secondary TNFα response from NK cells.

Macrophages are avid producers of TNFα in response to diverse activating signals. We did not directly test TNFα secretion from macrophages/monocytes in this thesis, only NO, but it is reasonable to assume that monocytes contribute to the TNFα response measured from PBMCs. A potential mechanistic effect can be suggested from the results of *D. mezereum* polysaccharides that interacted with several of the CLR and TLR receptors, inducing NO secretion from macrophages and display relatively potent TNFα secretion from PBMC. As

activated macrophages secrete TNFα, polysaccharides found in *D. mezereum* could bind to either of these macrophage activating receptors, inducing production of pro-inflammatory molecules like NO and TNFα.

In addition to testing the ability of the extracts to induce NO secretion from macrophages, we also tested their ability to suppress NO release from the same macrophage cell line after stimulating with LPS. NO have many important roles in the body, influencing platelet function, function in mitochondrial respiration and as a vasodilator, increasing blood flow and lowering blood pressure [395]. However, overproduction of NO is found to function in circulatory failure, it can be toxic to surrounding tissue and can result in systemic inflammation and septic shock [396]. As with every other component in the immune system, a balanced NO release is essential.

5.6. The anti-inflammatory effects of polyphenols

Polyphenols are associated with strong anti-inflammatory and antioxidant abilities, having gained much attention for these effects over the last thirty years. To begin with, it was believed that polyphenols exerted their effects through direct scavenging of free radicals and chelating metals. Later, it was found that the low bioavailability of polyphenols and subsequent low plasma concentration meant it was unlikely for polyphenols to exert a direct antioxidant effect [397]. During the last decade we have seen an increase in studies focusing on the effect of polyphenols in the intestine, finding polyphenols to exert anti-inflammatory and antioxidant effect through this route, reducing intestinal inflammation [294].

Several TNFα inhibitors are in clinical use (blocking antibodies such as Infliximab) in the treatment against inflammatory and autoimmune diseases like rheumatoid arthritis and Chron's disease [398-400]. Potentially, the anti-inflammatory polyphenolic compounds could be further exploited for their effect on suppressing TNFα release. Of note, suppressive immunotherapies such as TNFα blockade will over time lead to side effects such as increased susceptibility of infections [401, 402]. Understanding how the polyphenols mechanistically influence TNFα-responses, will therefore be important to understand their potential medicinal potential. Of note, the polyphenol fraction from *D. mezereum* induced strong cytokine release, which is a non-typical effect of these compounds considering that the other polyphenol fractions were generally anti-inflammatory and suppressed TNFα and IFNγ production.

Altered gut microbiome and gastrointestinal inflammation is associated with mitigation of systemic and local chronic diseases like cardiovascular disease, diabetes, neurodegenerative

diseases like Alzheimer's disease and Parkinson, IBD and cancer [403-409]. Polyphenols are associated with ameliorating these diseases [397, 410-413]. The systemic effects observed as a result of intestinal inflammation can be the result of excess ROS linked to gut inflammation, damaging of the cell membrane and tight junctions in the intestine, resulting in "leaky gut" and increased intestinal permeability [294, 414].

Oxidative stress is found to activate the NF-kB and MAPK pathway, both leading to expression of pro-inflammatory cytokines like TNFα and IL-6 that can cause cellular damage and disease if in excess [415-417]. Polyphenols negatively regulate these NF-kB and MAPK inflammatory effects both at protein and gene levels, reducing pro-inflammatory cytokine production and inducing an anti-inflammatory state. Here the structure, rather than the concentration seems to be an important factor for the effect [418]. Thus, differences in polyphenol structures from different plants can explain why we observed distinct effects on immune responses and the gut microbiota from different plants.

In the lamina propria, mesenteric lymph nodes and spleen, polyphenols influence T cell differentiation, increasing T_{reg} cells and a regulatory Th17 population [419]. Polyphenols also directs macrophages towards the anti-inflammatory M2 phenotype, leading to increased secretion of the anti-inflammatory cytokine IL-10 [419, 420].

In addition to suppressing the pro-inflammatory signalling pathway, the anti-inflammatory and antioxidant activity of polyphenols are also due to their ability to inhibit enzymes involved in ROS production and upregulate endogenous antioxidant enzymes [421]. Interaction of polyphenols with the cytosolic aryl hydrocarbon receptor (AhR), induce a signalling cascade leading to translocation of Nrf2 and expression of antioxidant enzymes [422, 423].

A study using Caco-2 cell line found only polyphenols of molecular weight of less than 10 kDa to show strong cellular antioxidant and anti-inflammatory activity on this epithelial cell line, indicating a possible need for processing of the compounds before they exert their anti-inflammatory effects [73]. Studies find complex polyphenols, especially those with oligomeric and polymeric structures to reach the colon almost unchanged where they interact with and can be metabolized by the gut microbiota [424]. However, once they reach the colon, the interindividual differences in gut microbiota composition can influence how these polyphenols are processed and what potential health effects they might provide [342]. This is speculated to be a reason for differences observed in studies with participants consuming polyphenol enriched food or polyphenol compounds.

5.7. Modelling of the intestinal epithelium

The intestinal epithelium plays a central role in regulating inflammatory and immune responses, and it is the place for absorption and transport of ingested compounds. To test the ability of the plant extracts and bacterial metabolites to influence epithelial cells and immune cells in the lamina propria, we used the well-established human intestinal epithelial cell line Caco-2 in Tsranswell inserts. Caco-2 have previously been used to test the bioavailability and anti-inflammatory effects of polyphenols [73, 425, 426]. Caco-2, once differentiated, consists of a polarized cell monolayer that have apical and basolateral membranes and express many of the intestinal enzymes, mirroring enterocytes in the intestine. However, the full complexity of the intestinal layer is not recapitulated in this model. Alternatively, we could have co-cultured Caco-2 with the cell line HT-29, which is a mucus-producing cell line. We tested co-cultures of HT-29 and Caco-2 but did not test their differentiation in a systematic manner. This will be subject for future experiments.

Compared to the traditionally used immortalized Caco-2 cell line, intestinal organoids are emerging as new model systems with several advantages. Intestinal organoids contain all the different cell types found naturally in the intestine, making them physiological relevant. The ability to passage organoids several times without genetic changes also makes them good models for studying the effect of medicines, diseases or other factors able to affect the intestine. Still, Caco-2 cell monolayers serve as good models for high-throughput screening purposes, both for testing direct effects on the epithelial cells but also on underlying cells. The intestinal organoids used here were derived from mice, however, the intestinal epithelium is considered relatively similar in mice and humans, with intestine of one month old mice resembling that of the developed intestine in humans [427]. We observed a potent proliferative effect in the organoids with both the polyphenol fraction from *D. mezereum* and the isolated compounds daphnin and neochamajaesmin A.

The bacterial metabolites from faecal cultures with the *D. mezereum* polyphenol fraction yielded higher number of organoids and more intact organoid structures after three days compared to the other faecal supernatants. This effect was akin to observations made in the Caco-2 assay with faecal cultures with *D. mezereum* polyphenol fraction displaying higher TEER values than faecal cultures with water control. This indicates a similar beneficial effect of the *D. mezereum* polyphenols on human Caco-2 enterocytes and enterocytes present in the murine intestinal organoid model. As many of the receptors along the GI tract can interact with similar compounds, the effect observed of the bacterial metabolites in the small intestinal

organoid models could also be true for colonocytes. Indicating an additional positive effect in the colon for the *D. mezereum* polyphenol fraction.

5.8. Microbiota and prebiotics

The gut microbiome is gaining much attention for its influence on host health and disease, with research focusing on the effect that different compounds, medicines or food have on bacterial species found to have health promoting effects. Most extensively studied are the probiotic bacteria *Bifidobacterium* and *Lactobacillus*. Health promoting bacteria are assumed to exert their effects either through physically occupying space in the intestine so pathogenic bacteria do not have anywhere to settle and/or through production of secondary metabolites, of which SCFAs are the most studied. In addition to these effects, the gut microbiome is important in shaping and influencing the immune system and immune responses, both locally in the gut and in circulation/peripheral organs.

Much of the focus on pectic polysaccharides have in recent years been on their effects in the intestine through fermentation of beneficial microbiota to produce SCFAs associated with increased bacteria community diversity and abundance, and function in reducing mucosal inflammation, and intestinal inflammation associated with IBD, improve cardiovascular health, carcinogenesis, and allergy [428-430]. Preclinical evidence in animal studies also generally find polyphenols to stimulate growth of prebiotic targeted microorganism and induce production of SCFA. The exact pathway for this effect is not known, but polyphenols are found to increase *Lactobacillus* ssp., *Bifidobacterium* ssp., and *Faecalibacterium* ssp. among others, all of which are potent SCFA producers [294]. These studies are consistent with our observation of potent increase in both *Bifidobacterium* ssp., and *Faecalibacterium prausnitzii* in the polyphenol enriched *D. mezereum* fraction.

In the microbiota screen (**Paper IV**), we generally observed increased abundance of health promoting bacteria like *Faecalibacterium prausnitzii*, *Slackia isoflavoniconvertens*, *Clostridium butyricum* and *Bifidobacterium,* and decreased abundance of pathogenic bacteria like *Clostridium perfringens* and *Enterococcus*. We did however also observe effect of the *D. mezereum* polyphenol fraction on *Bacteroides,* one of the main lineages of bacteria, but that display pathogenic effects if found anywhere other than the intestine.

Another genus found to be enriched in fecal cultures with *D. mezereum* polyphenol fraction was *Oscillibacter*. Though not extensively studied, one species of this bacterium, *O. valericigenes*, was found to be present in over 90% of healthy people compared to only 12.5%

of Chron's disease patients [431]. Another study found *O. ruminantium* to be increased in older patients (over 60 years) with alcohol dependency, ulcerative colitis/colonic adenocarcinoma or Alzheimer dementia and risk factors associated with chronic liver disease [432]. The other health conditions observed in these patients does however indicate that *O. ruminantium* were probably at least not the sole cause for the reported diseases. *Oscillibacter* together with *Clostridiales* are found to be enriched in individuals able to metabolize ginsenoside [433]. This indicates a potential role of *Clostridiales* and *Oscillibacter* in producing the health promoting effects associated with this natural product, including beneficial effect on nervous system diseases, inflammatory responses, cardiovascular disease, viral infections and oxidative stress [434].

Clostridium cluster XIVa is recognized as one of the main players in the microbiome, accounting for over 50% of mucus-adherent bacteria. These butyrate producing bacteria are though to enhance butyrate bioavailability by increasing butyrate concentration close to the mucus layer. Butyrate producing mucin-adherent bacteria, like *Clostridium* cluster XIVa, are because of this, of interest for treatment of diseases like IBD [435]. In the microbiota screen, the PS enriched fractions from *D. mezereum* and *A. archangelic* induced growth of *Clostridium* cluster XIVa. As *Firmicutes* are known for utilizing carbohydrates to produce butyrate, the abundance of *Clostridium* cluster XIVa in the microbiota analysis indicates that this bacterium has the enzymes necessary for utilizing polysaccharides found in these plant fractions for their growth.

We also observed a decrease in the abundance of *Pseudoflavonifractor,* a bacteria that has not received much attention in microbiome studies. One study has however found *Pseudoflavonifractor capillosus* to be decreased in a cohort of proton pump inhibitor users, where long term proton pump inhibitor use is found to alter the gut microbiota and is associated with increased risk of *Clostridium difficile* infection [436]

Based on several studies, an understanding of some of the mechanisms behind how the gut microbiome help shape and collaborate with the immune system are emerging. Some components of bacteria, like polysaccharide A on Gram-negative *Bacteroides fragilis* and the Gram-positive bacteria class *Clostridia* are found to interact with TLR-2, engage MyD88 and induce the differentiation of CD4+ T-helper cells to T_{reg} cells in the lamina propria and in circulation [437-439]. T_{reg} cells produce anti-inflammatory cytokines like IL-10 and can suppress pro-inflammatory responses initiated by Th17 cells [439].

Prebiotics are emerging as a therapeutic intervention that can skew the microbiome towards health promoting bacteria populations. Previously, much focus was placed on

polysaccharides as the components influencing the gut microbiome to produce health promoting SCFAs, but recent studies find the anti-inflammatory and antioxidant effects of polyphenols to likely result from interactions with the gut microbiome. In our studies, the most striking effect where in the polyphenol enriched fraction from *D. mezereum.*

As there are no standard way of measuring prebiotic effects and active components can vary greatly between different sources and studies, the variation when analyzing prebiotic effects of polyphenols are quite large. A review from 2019 evaluating *in vitro*, *in vivo* and clinical trials of polyphenols as prebiotics found big variations between the studies [342]. Generally, the *in vitro* studies tended to use quite high concentrations (up to 150mg/mL) and the *in vivo* studies varied in concentrations form 4g/kg body weigh in mice to 1 mg/kg body weight in rats, concentrations not thought to be physiologically relevant for consumed polyphenols. Clinical trials were also found to vary with regards to dosing and duration of the study, making comparison difficult. From the studies conducted, there seems to be little general consistencies, though healthy subjects receiving cocoa flavanoles or red wine seemed to have an increase in the abundance of *Bifidobacterium* and a reduction in triglycerides and inflammation marker CRP levels after 4 weeks of daily intake, with red wine also reducing blood pressure and total cholesterol. What seemed to be the most consistent finding from both *in vitro, in vivo* and clinical trials were an increase of *Bifidobacterium* after intake of different polyphenols at different concentrations (reviewed in [342]). In the microbiota screen, we found similar effects on *Bifidobacterium* for the polyphenol containing SPE fraction from *D. mezereum*. Interestingly, this effect was not observed for any of the other SPE fractions from different plants, emphasising the importance of chemical composition and structure for optimal prebiotic effects. In addition to the polyphenol source, interindividual differences in the microbiome composition, influenced by endogenous and environmental factors, are also likely to affect the prebiotic impact.

Of the documented effects prebiotic intake can have, animal studies have found prebiotic intake to improve intestinal barrier integrity by stimulating growth of healthy bacteria and reduce colonization of pathogenic bacteria. TNFα and IFNγ are two pro-inflammatory cytokines found to increase epithelial permeability at tight junctions, increasing translocation of endotoxins and bacteria antigens [272]. Colitis animal models have found bacteria promoted by prebiotics to function in epithel defence mechanisms, protecting against inflammation [440, 441]. SCFA butyrate and propionate are proposed as the effector compound in the anti-cancer effects prescribed to prebiotics. Butyrate as an important energy source for epithelial cells are thought to be of particular importance for intestinal epithelial health and the protective effects.

Prebiotics, through their effect on good bacteria, are also found to alter the immune response and stimulate production of anti-inflammatory cytokines and secretion of antibacterial substances.

The importance of prebiotics, both dietary fibre and polyphenols, are evident in the association between different prebiotic intake and improvements of different intestinal diseases like IBD, IBS and colon cancer [442], and also systemic/peripheral diseases like cardiovascular diseases, type 2 diabetes and neurological related diseases like depression and Alzheimer's disease [69]. Despite documented effects on different diseases, there are some varying results, indicating an effect of the different prebiotics used, and the high interindividual differences observed in the microbiota composition and proportion of bacteria between people [342]. This is most likely also true for animal models. These differences are important to consider when evaluating prebiotics and their health promoting effects.

6 Conclusions

Historical text and traditional uses can serve as inspirational sources for the rediscovery of potential novel pharmaceuticals. We discovered several effects from different traditional Scandinavian medicinal plants and show scientific backing for the traditional uses of some of these plants.

In **Paper I** we find some of the traditionally used medicinal plants to have potent immune modulatory effects. The selection criteria for the plants were the same, but not all plants were found to have effects, emphasizing the need for scientific investigation. For *G. purpurea*, *A. archangelica* and *D. mezereum* we find scientific evidence that could explain their historical described uses.

In **Paper II**, *G. purpurea*, a plant used against stomach related diseases and chest diseases, were found to have potent anti-inflammatory effects. Isolation of pure compounds within the SPE fraction revealed the anti-inflammatory effect observed in the main fraction were likely from the bitter compounds within the roots, either from the most potent erythrocentaurin or the most abundant gentiopicrin.

In **Paper III**, pectic type polysaccharides isolated from *A. archangelica*, were found to have potent innate immune stimulatory effects, likely mediated via an effect of innate myeloid cells. *A. archangelica* was traditionally used against the cold, coughing and to prevent infection.

In **Paper IV**, the polyphenol enriched SPE fraction from *D. mezereum* show a distinct prebiotic profile in human microbiota culture and positive effect on intestinal organoids. We find that specific plant fractions influence the gut microbiome and that this effect is determined by specific compounds or combinations of these. We also observe similar positive effects on both intestinal epithelial cells and gut microbiota composition for the polyphenol enriched *D. mezereum* fraction, indicating a good prebiotic effect of compounds within this fraction.

7 Future perspectives

As the need for novel pharmaceuticals are growing, we find that looking back at traditional medicinal plants can help in future discovery of pharmaceutical compound and medicines. Future aspects for the results found during this interdisciplinary collaboration will be to look deeper into some of the plants collected, but not investigated in this thesis. Like the ones found to have potent anti-bacterial and anti-cancer.

We are further working on isolating the active compounds within the *D. mezereum* polyphenol enriched fraction. We will test these isolated compounds on different microbiota cultures, including cultures from adult people. A possible synergistic effect in the main fractions, a phenomenon previously reported for prebiotic effects of some plant compounds, also need to be tested. If discovering the active compound and confirming the results in a different microbiota model and *in vivo*, this compound can be used as a prebiotic for improving gut health and could potentially be used against IBD. Based on previous research finding polyphenol compounds to function against colon cancer, an active compound within the polyphenol enriched fractions could potentially also be used as a preventative compound against this type of cancer.

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The discovery of novel immunomodulatory medicinal plants by combination of historical text reviews and immunological screening assays

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ABSTRACT

Ethnopharmacological relevance: With the advent of immunotherapies against cancers, autoimmune diseases and infections, there is a steady demand for novel medicines. New sources for discovery of potentially novel immunomodulatory compounds are therefore needed. Nature contains a large and diverse reservoir of novel compounds that can be exploited for their potential as new drugs, and exploring the pharmaceutical potential of medicinal plants used in traditional medicine is highly relevant.

Aim of the study: We aimed with this study to explore usage of medicinal plants in Scandinavian folk medicine against diseases interpreted to involve the immune system, and to further screen water extracts from previously overlooked medicinal plants in order to discover potential new sources of immunomodulatory compounds.

Materials and methods: We systematically investigated historical records dating back to the 1800s with an emphasis on plants used as treatment for wounds or diseases interpreted to be inflammatory. Of 74 candidate plants, 23 pharmacologically under-studied species were selected for further characterization. The plants were collected from their natural habitats in Southern Norway, air-dried, and subjected to boiling water and accelerated solvent extraction. The crude extracts were separated into polysaccharide-enriched fractions and C-18 solid phase extracted fractions. Immunological screenings were performed with all extracts and fractions. Monosaccharide composition and total phenolic content were determined and compared across all species.

Results: We identified 10 species with clear immune activating effects and 8 species with immune inhibitory effects by comparing cytokine production by human peripheral blood mononuclear cells, primary human T- and NK-cell proliferation, and nitric oxide production from macrophages.

Conclusions: With this study, we provide a comprehensive overview of Scandinavian medicinal plants and their usage, and our findings support an approach of combining historical sources with modern pharmacology in the discovery of plant sources containing potentially new pharmacological compounds.

1. Introduction

As far back as we can trace human activity and medical treatment, plants have been used in folk medicine for their medicinal benefits (Conrad et al., 2008; Newman et al., 2000; Pan et al., 2014; Petrovska, 2012; Porter, 1999), and self-medication with plants has also been recorded in other animals (de Roode et al., 2013). The close relationship between people and plants, which provide oxygen, food, shelter,

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clothing, ornamentals, and medicine is essential for human life and culture (Teixidor-Toneu et al., 2020). People tended to use plants found in near proximity of where they lived, and these plants inspired herbal medicines. Knowledge about medicinal plants and their uses has been preserved and passed on both orally and in written in different cultures and social groups all over the world (Chen et al., 2020; Colombo and Ammirati, 2011; Laroche, 2009; Rankin, 2013). Based on this long history of medicinal plant use, it is logical to assume that nature still retains a large potential for the discovery of new pharmacologically active compounds (Cravens et al., 2019; Li, Li and Smolke, 2018; Scannell and Bosley, 2016). While current pharmaceutical research is focused mainly on development of new synthetic compounds and exploration of pre-discovered molecules, there is a steady decline in the discovery of novel compounds. In light of ever increasing demands of novel, effective immunomodulatory drugs against cancer, infections and autoimmune diseases, this calls for new efforts into the discovery of novel compounds from natural resources (Atanasov et al., 2015; Grabley and Thiericke, 1999).

About a third of modern day essential medicine derives from natural products and 25% from medicinal plants and fungi (Calixto, 2019; Newman and Cragg, 2020). Examples are morphine from the opium poppy and paclitaxel from the bark of the Pacific yew tree. A Scandinavian example is the accidental discovery of cyclosporine from the fungus *Tolypocladium inflatum* W. Gams. found in the mountains of Southern Norway. Cyclosporine is now on the WHO list of essential medicines. Advances within the field of modern medicine and pharmacology have made it possible to investigate, in detail, the diversity of chemical structures available from natural sources and plant compounds. These new advances make it possible to discover bioactive compounds from medicinal plants and generate valuable pharmaceutical leads.

Traditionally, medicinal plants were prepared with liquor or as infusions or decoctions, and administered orally or applied externally. In this study we have chosen to focus on water extracts where phenolics and polysaccharides are abundant constituents, and can mediate potent immunomodulatory or anti-cancer effects (González-Gallego et al., 2014; Inngjerdingen et al., 2008; Ramberg et al., 2010). Polysaccharides are complex biological macromolecules, and are a structurally diverse class of molecules. They are composed of sugar monomers linked together with glycosidic bonds, with different degrees of branching, molecular weight and structural confirmation. In the last decades, a range of studies have shown that polysaccharides have promising immune modulating and anti-tumor effects (Meijerink et al., 2018). Polysaccharides are promising candidates for novel therapeutics as they are generally non-toxic, biocompatible, and biodegradable (Yin et al., 2019). Phenolic compounds are produced in plants as secondary metabolites with protective functions against e.g. ultraviolet radiation, pathogen aggression, and oxidative stress. They are the largest class of bioactive compounds found in plants (Annunziata et al., 2020), including flavonoids, hydrolysable tannins, lignans, stilbenes, coumarins, as well as smaller phenolic compounds. Several *in vivo* and *in vitro* studies have found polyphenols to have anti-inflammatory properties in animals and humans (Yahfoufi et al., 2018).

In this study, Norwegian and Swedish primary written sources from the 19th and 20th centuries were studied to comprehensively identify medicinal plants used in Scandinavian folk medicine against immune-related diseases, infections, or wounds in order to identify new sources of compounds with immunomodulatory activity. From the identified plants, water extracts were prepared from a selected set of previously understudied medicinal plants and screened for immunomodulatory activities.

2. Materials and methods

2.1. Literature review of primary written sources from rural Norway and Sweden

To identify Scandinavian medicinal plants, we reviewed primary written sources focusing on firsthand accounts from farmers, including lay practitioners and local healers, in rural Norway and Sweden during the 19th and 20th century. These accounts give recollections of what these societies and possibly their ancestors used as medicinal plants. Divided into sections for different illnesses and their traditional treatments, or by describing symptoms, these sources give accounts of what people did when sickness struck, what type of plants and other remedies they collected and how they prepared them for use. The sources have been systematized and made available in printed collections by scholars of various academic backgrounds and these collections form the basis of this study. The main collections documenting these firsthand accounts were published by Ingjald Reichborn-Kjennerud (1865–1949), medical doctor and medical historian, Ove Arbo Høeg (1898–1993), professor in botany at the University of Oslo, and Erling Christophersen (1898–1994), Norwegian botanist, geographer and diplomat (Christophersen and Hjort, 1960; Høeg, 1975, 1985; Reichborn-Kjennerud, 1922, 1944, 1947). These collections were supplemented with works from the Swedish authors Lars Hammarin and Kristina Frølich (Frølich and Wille, 1921; Hammarin, 2013). Collectively, the sources made available from these authors were the focus of this study. During this study, it became obvious that plants for medicinal use were inconsistently documented during this period. People used what they had available and different common/vernacular names were often given depending on where in the country the plant was used. Most of the sources include scientific names, matched to the vernacular names used by people in the countryside. We used two books published by Høeg as the main references when linking vernacular and scientific names (Høeg, 1975, 1985). Høeg has done extensive work in trying to track these different vernacular names and gathering them under their standard scientific name. Accepted botanical names were confirmed by consulting the Catalogue of Life Checklist (Bánki et al., 2021; Roskov et al., 2020). The following traditional therapeutic indications were chosen for the selection of plant species: Gastrointestinal related diseases interpreted to be inflammatory, other inflammatory related diseases, infections, and inflamed wounds. These conditions were identified from vernacular diseases like gastrointestinal-related problems (aches, cramps, constipation, and diarrhea), skin wounds and rashes.

2.2. Selection of study plants

From 74 plants identified from review of historical sources (Table 1), plants with few pharmacology or chemistry search results (<50) in PubMed or SciFinder were selected for further studies in order to test immunological activities in under-studied plant species. The search term used to find publications in PubMed was the scientific plant name + "Pharmacology". In SciFinder, only the scientific plant name was used as a search term, and the search hits were then refined by the category heading "Biology". For the search of chemical compounds, the search was further refined by "Substances in biology", while the search for immunological related publications were refined by the category "Immunology". SciFinder has a broader database compared to PubMed, and therefore resulted in a higher number of search hits. Thus, the publications encountered were assessed individually for each plant to determine the degree of relevance towards immunology and infections. *Hypericum perforatum* L. [Hypericaceae] was included amongst the short-listed plants as comparison against the pharmacologically less studied *Hypericum maculatum* Crantz [Hypericaceae]. *Juniperus communis* L. [Cupressaceae], which is also well studied, was included due to its long-standing role as a Nordic medicinal plant, probably since the Viking Age (Teixidor-Toneu et al., 2021).

Table 1
Collection sites of studied plants.

FAMILY	SPECIES	HABITAT	GPS COORDINATES	PLANT PART COLLECTED	VOUCHER NUMBER
Apiaceae	*Angelica archangelica* subsp. *archangelica*	Mountain area, amongst juniper bushes	61°1′47″N 8°45′28″E (Vestre Slidre)	Roots	RL-20200814-aa
	Angelica archangelica subsp. *litoralis* (Whalenb.) Thell.	Coastal drift line	59°2′13″N 10°12′59″E (Larvik)	Roots	RL-20190929-aal
	Pimpinella saxifraga L.	Cultural landscape	59°54′38.1″N 10°40′11.5″E (Oslo)	Roots	RL-20200911-ps
	Sanicula europaea L.	Deciduous forest	59°4′13″N 10°18′44″E (Sandefjord)	Leaves	RL-20200713-se
Asparagaceae	*Polygonatum multiflorum* (L.) All.	Deciduous forest	59°2′6″N 10°13′12″E (Larvik)	Roots	RL-20200809-pm
Asteraceae	*Antennaria dioica* (L.) Gaertn.	Cultural landscape	61°05′37″N 8°42′54″E (Vang i Valdres)	Flowers	RL-20200720-ad
Brassicaceae	*Cochlearia officinalis* L.	Rocky coastal zone	59°2′3″N 10°13′12″E (Larvik)	Aerial parts	RL-20200407-co
Betulaceae	*Alnus incana* (L.) Moench	Mixed forest	59°58′03″N 10°43′12″E (Oslo)	Bark	RL-20200421-ai
Crassulaceae	*Sedum acre* L.	Mountain side	59°54′47″N 10°41′32″E (Oslo)	Aerial parts	RL-20200611-sa
Ericaceae	*Phyllodoce caerulea* (L.) Bab.	Dry mountain area above the treeline	60°59′25.1″N 8°49′56.8″E (Vestre Slidre)	Aerial parts	RL-20200719-pc
	Vaccinium vitis-idaea L.	Coniferous forest	59°58′10″N 10°43′15″E (Oslo)	Leaves	RL-20200421-vv
Gentianaceae	*Gentiana purpurea* L.	Willow thicket	60°59′29.5″N 8°37′41.7″E (Vang i Valdres)	Roots	RL-20200723-gp
Hypericaceae	*Hypericum maculatum* Crantz	Cultural landscape	61°9′19″N 8°47′58″E (Vang i Valdres)	Aerial parts	RL-20200814-hm
	Hypericum perforatum L.	Cultural landscape	59°52′27″N 10°28′23″E (Asker)	Aerial parts	RL-20190708-hp
Menyanthaceae	*Menyanthes trifoliate* L.	Lake	59°57′56″N 10°42′25″E (Oslo)	Leaves and stem	RL-20200515-mt
Orobanchaceae	*Euphrasia officinalis* L.	Mountain	61°14′14.2″N 10°27′50.1″E (Øyer)	Aerial parts	RL-20200808-eo
Cupressaceae	*Juniperus communis* L.	Cultural landscape, meadow	61°05′37″N 8°42′54″E (Vang i Valdres)	Twigs with needles	RL-20201001-jc
Polygonaceae	*Rumex longifolius* DC.	Roadside	59°53′56,5″N 10°40′27″E (Oslo)	Roots	RL-20200429-rl
Polypodiaceae	*Polypodium vulgare* L.	Deciduous forest, on rocks	59°2′5″N 10°13′11″E (Larvik)	Rhizome	RL-20200407-pv
Ranunculaceae	*Ranunculus acris* L.	Meadow	59°56′52″N 10°42′48″E (Oslo)	Aerial parts	RL-20200626-ra
Rosaceae	*Agrimonia eupatoria* L.	Meadow	59°57′3.1″N 10°42′45.5″E (Oslo)	Leaves	RL-20200807-ae
	Potentilla erecta (L.) Raeusch	Cultural landscape	59°59′43″N 10°45′26″E (Oslo)	Roots	RL-20200529-pe
Thymelaeaceae	*Daphne mezereum* L.	Mixed forest	59°57′55″N 10°42′30″E (Oslo)	Bark	RL-20200421-dm

2.3. Plant material and study area

Plant material was collected in Southern Norway; primarily in the Oslo region (0–100 MASL) and mountain areas in Southern Norway (800–1000 MASL) (Table A1). Collection occurred during March to September 2020, depending on the historically described collection time points for each plant, or when the plants were in flower. The collection was focused on plant parts with reported usage in the historical sources (see Table 1 and Table A1). Plant materials were dried at room temperature and powdered with a blender machine (Vitamix Acenti 2300i). Plant identities were confirmed by botanists at the Natural History Museum, University of Oslo, and herbarium specimens were collected and deposited at the Natural History Museum herbarium (voucher numbers are given in Table A1).

2.4. Accelerated solvent extraction

Powdered plant materials (ca. 10 g) were extracted with an Accelerated solvent extraction ASE 350 instrument (Dionex, Sunnyvale, CA, USA) with 100 °C dH_2O as solvent. The water was removed by freeze-drying to give the crude extracts which were divided into three equal parts.

2.5. Polysaccharide precipitation

The crude extracts were precipitated in ethanol (75%, 48 h, 4 °C), followed by centrifugation (4400 rpm, 20 min), and filtration using a Büchner funnel. The precipitate was washed with ethanol, centrifuged (4400 rpm, 5 min) and filtrated again. The precipitate was washed with acetone, dissolved in water, and freeze-dried for 48 h to give the polysaccharide-enriched fraction (PS-fractions).

2.6. Solid phase extraction (SPE)

Solid phase extraction (SPE) to obtain fractions enriched in phenolics was performed as previously reported with small modifications (Ho et al., 2017). A Phenomenex strata C-18 2 g column (Phenomenex, Macclesfield, UK) was washed with methanol and conditioned with water. The third part of the crude extract was applied to the column and eluted with water, 50% methanol and 100% methanol. The methanol fractions were combined and evaporated under reduced pressure to give the SPE-fractions.

2.7. Monosaccharide composition analysis

PS-fractions (1 mg) were dried in a desiccator 24 h prior to methanolysis, which was performed as previously described (Chambers and Clamp, 1971; Wold et al., 2018). In short, the samples were subjected to methanolysis with 3M hydrochloric acid in anhydrous methanol for 24 h at 80 °C. Trimethylsilylated derivatives of the methyl glycosides obtained after methanolysis were analyzed using a Restek RTx-5 silica column (30 m, i.d. 0.25, 0.25 μm film thickness) coupled to a Focus GC (ThermoFisher Scientific, Waltham, MA) with flame-ionization detection. The parameters used for the gas chromatography were identical to previous studies (Ellefsen et al., 2021; Wold et al., 2018). Helium was used as carrier gas and Chromelion software v.6.80 (Dionex Corporation, Sunnyvale, CA) was used to analyze the results. Mannitol was used as an internal standard.

2.8. Total phenolic content

Total phenolic content was determined using Folin Ciocalteu reagent (Singleton and Rossi, 1965). The SPE-fractions were dissolved in DMSO (0.1 and 0.5 mg/ml), and 20 μL added to a 96-well plate along with dH_2O (50 μL). Folin Ciocalteu reagent (10%, 100 μL) was added to the

samples, mixed and left for 5 min. Na_2CO_3-solution (20%, 30 μL) was added to the wells, followed by shaking and the plate was allowed to stand for 2 h. Finally, the absorbance was measured at 765 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, San Jose, CA). The values were reported as milligrams of gallic acid equivalents per grams of extract (mg GAE/g extract).

2.9. Cells and cell cultures

The J774.A1 mouse macrophage cell line was cultured in complete RPMI medium (cRPMI; RPMI1640 with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 1% penicillin/streptomycin (all from ThermoFisher) at 37 °C and 5% CO_2. Cells were passaged three times per week.

2.10. Peripheral blood mononuclear cell (PBMC) isolation

Buffy coats were obtained from the blood bank at Oslo University Hospital upon informed consent according the declaration of Helsinki, and the study was approved by the South-Eastern Norway Regional Ethical Committee. PBMCs were separated by density gradient separation on Lymphoprep (Axis-Shield, Oslo, Norway) and spinning for 20 min at 650 *g* with no brakes. The mononuclear cells were collected and washed in PBS with 2% FBS, and viably frozen at −80 °C.

2.11. NK cell and T cell proliferation assay

To assess cellular proliferation, PBMCs were stained with 5- (and 6)-carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher) (Muul et al., 2008). In brief, 1×10^7 cells/ml in PBS with 2% FBS were stained with 5 μM CFSE for 10 min at 37 °C in the dark. Cells were washed, and plated in 24-wells at 1×10^6 cells/ml (5×10^5 cells/well), and incubated with test samples at a final concentration of 100 μg/mL, 10 μg/mL or 1 μg/mL in duplicates. Concavilin A (ConA) served as positive control and reference value for T-cell proliferation at 5 μg/mL, while 500 IU/mL of IL-2 served as positive control and reference value for NK-cell proliferation. Cells were incubated for 6 days at 37 °C in a 5% CO_2 cell incubator, then stained with the following antibodies to distinguish T cells and NK cells: CD56-AF647, CD3-AF700, CD14-PerCP-Cy5.5 and CD19-PerCP-Cy5.5 (all from BD Biosciences, San Jose, CA). Cells were acquired using a BD LSRFortessa Flow Cytometer (BD Biosciences), and analyzed by FlowJo (v10.5.3). Proliferation was calculated as loss of CFSE signal in either $CD14/CD19^-CD3^-CD56^+$ NK cells or $CD14/CD19^-CD3^+CD56^-$ T cells. Results are presented as the percentage $CFSE^{low}$ cells of test sample-treated cells relative to the reference values of the positive controls (Table A.2).

2.12. MTT cytotoxic assay

Toxicity of extracts was tested with the J774.A1 macrophage cell line via the MTT assay (Ferrari et al., 1990). Briefly, 100 μL (1×10^6 cells/ml) J774.A1 cells were plated in duplicates in 96-well flat bottom plates, and incubated overnight with samples at concentrations of 100 μg/mL, 10 μg/mL or 1 μg/mL. Medium alone served as negative control and reference value for live cells, and LPS (10 ng/mL) as positive control. Cells were next incubated with 10 μL MTT reagent (Roche Diagnostics, Germany) for 4 h at 37 °C, followed by addition of 100 μL MTT detergent solution (Roche Diagnostics). Cells were incubated in a 37 °C cell incubator overnight, then colorimetric detection was performed at OD570. Data are presented as percentage live cells relative to live cells in medium alone, calculated as the fraction of OD570 values in test samples versus medium alone (Table A.2).

2.13. Nitric oxide (NO) release

NO release was measured from the macrophage cell line J774.A1 using the Griess reagent assay (Griess, 1879). Briefly, 100 μL (1×10^6 cells/ml) J774.A1 cells were plated into a 96-well flat bottom plate. Plant extracts were added to duplicate wells at 100 μg/mL, 10 μg/mL or 1 μg/mL. Medium alone served as negative control and LPS (10 ng/mL) as positive control and reference value. Cells were incubated overnight at 37 °C in a 5% CO_2 cell incubator, the supernatant was harvested, spun at 350 *g* for 2 min, and 50 μL supernatant was mixed with 50 μL Griess reagent A (1% sulfanilamide and 5% phosphoric acid) and incubated for 10 min at room temperature in the dark. Afterwards, 50 μL Griess reagent B (0.1% N-(1-napthyl)ethylenediamine in sterile water) was added to each sample and colorimetric detection was measured at OD_{570}. The amount of NO secreted was calculated based on a $NaNO_3$ standard curve, and NO-concentration of test samples were normalized against NO released from negative and positive control, and data presented as % NO release in test samples relative to LPS (Table A.2).

2.14. Enzyme-linked immunosorbent assay (ELISA)

Human TNF-α or human IFN-γ ELISA were run using a kit from Mabtech, Sweden. PBMCs were stimulated in duplicates for 16 h in 96-well round-bottom plates at 1×10^6 cells/ml with 1, 10, or 100 μg/ml of test samples, medium alone as negative control, or 5 μg/ml ConA as positive control and reference value. Plates were coated overnight (4 °C) with capture antibodies in PBS using 96-well EIA/RIA Flat Bottom High Binding Plates (Corning). Plates were blocked for 1 h at room temperature with 200 μl/mL PBS with 0.05% Tween-20 and 0.1% BSA (Incubation buffer). Assay standards or test samples were added to appropriate wells and incubated for 2 h at room temperature. 100 μL/well of human TNF-α or human IFN-γ monoclonal detection antibody (Mabtech) diluted in incubation buffer (1 μg/mL) were added. After washing, streptavidin-HRP (Mabtech) was added, and the plates were developed with TMB substrate for 15 min followed by 0.2 M H_2SO_4. All washes were done using a BioTek ELx405 plate washer with 0.05% Tween-20 in PBS. Absorbance at 450 nm was measured using a Molecular Devices FlexStation 3 Reader. Data are presented as the concentration of cytokines in test samples relative to concentrations of cytokines measured in response to ConA (Table A.2).

2.15. Bioinformatics analysis

Hierarchical clustering was done using R studio version 4.1.0 using Euclidean distance and complete linkage using the agglomeration method (Warnes et al., 2020). For each analysis, the average normalized value of each sample was used.

3. Results

3.1. Comprehensive analysis of Scandinavian medicinal plants with usage against immune-related diseases and infections

The available historical records were reviewed with a focus on obtaining information on medicinal plants used in rural Norway and Sweden to treat ailments of skin and gut that were interpreted as wounds, infections or immune-related disorders. This led to the identification of 74 medicinal plants. A comprehensive overview of their traditional usage and the historical context of their usage is provided in Table A1, and is based primarily on the written sources made available by Høeg, Reichborn-Kjennerud and Hammarin (Hammarin, 2013; Høeg, 1975, 1985; Reichborn-Kjennerud, 1922, 1944, 1947). We further focused on plants with few past pharmacological studies, as assessed by the number of pharmacological studies identified by literature searches in PubMed (February–April 2020) and SciFinder (March–May 2020, December 2020) for each plant, Table 1 and Table A1. All 23 plants included in the study were subjected to water extraction to yield a crude extract, a polysaccharide enriched fraction, and a solid phase extracted fraction as described in the methods section. The yields of all extracts

and fractions related to dry plant material are presented in Table A.3. Chemical and immunological characterizations were performed with all extracts and fractions.

3.2. Chemical characterization

Total phenolic content of the SPE-fractions was determined using the FC-reagent. The total phenolic content of the SPE-fractions is shown in Fig. 1, and presented as mg GAE/g. Among the analyzed plants, *Alnus incana* (L.) Moench [Betulaceae] had the highest phenolic content, with 150 mg GAE/g followed by *H. maculatum* and *Vaccinium vitis-idaea* L. [Ericaceae], while the SPE-fraction from *Polygonatum multiflorum* (L.) All. [Asparagaceae] contained very low amounts (4.2 mg GAE/g).

The monosaccharide compositions of the PS-fractions were analyzed by methanolysis and GC. The data are presented as percentage values of each monosaccharide related to the total amount of carbohydrate in the sample (Fig. 2). The 23 plants cluster into three main groups, driven primarily by differential percentages of glucose (Glc) versus galacturonic acid (GalA), arabinose (Ara), and galactose (Gal). Some of the PS-fractions, such as those from *V. vitis-idaea*, *Polypodium vulgare* L. [Polypodiaceae], and *Pimpinella saxifraga* L. [Apiaceae], contained mainly Glc, which is most likely due to the high abundance of starch. PS-fractions that consisted of considerable amounts of GalA were *Daphne mezereum* L. [Thymelaeaceae], *P. multiflorum*, and *Agrimonia eupatoria* L. [Rosaceae], which could indicate the presence of pectins. The more unusual monosaccharide 4-*O*-methyl glucuronic acid (4-*O*-Me GlcA) was found in relatively high amounts in *Antennaria dioica* (L.) Gaertn [Asteraceae].

3.3. Comparison of cytotoxic effects of plant extracts on cells

Prior to testing immunomodulatory effects of the samples, their potential cytotoxic effect was tested using the MTT assay that measures

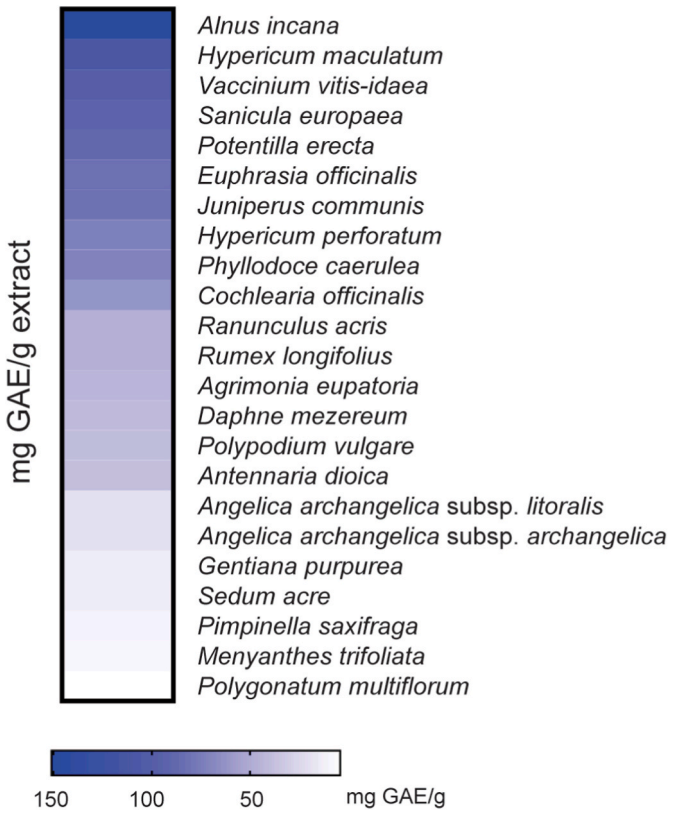

Fig. 1. Total phenolic content in SPE-fractions expressed as gallic acid equivalents (mg of GAE/g of extract).

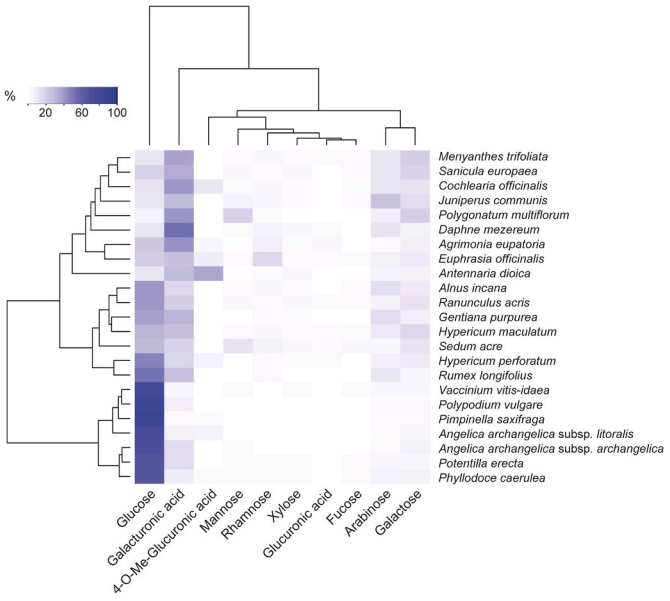

Fig. 2. Illustrative representation of the monosaccharide composition of PS-fractions obtained from each plant. Data are presented as the percentage of each monosaccharide in relation to total amount of carbohydrates in the sample, and hierarchical clustering of monosaccharides versus plant species.

cellular viability. J447.1 mouse macrophages were incubated overnight with 1, 10, or 100 μg/ml of either crude extracts, PS-fractions, or SPE-fractions. Cell viability was measured relative to J447.1 cultured in medium alone. The SPE-fractions from *Sanicula europaea* L. [Apiaceae] and *P. saxifraga* showed high cytotoxic effects at both 10 and 100 μg/ml (only 7.9% and 4.3% viability with 100 μg/ml) (Fig. 3). A relative high cytotoxic effect was also observed with the highest concentration of the PS-fraction from *P. vulgare* (25.3% viability). For the remaining plant extracts and fractions, the overall cell viability was within acceptable limits (well above 50%).

3.4. Immune-activating effects of plant extracts

To assess the immune activating effects, NO-secretion from macrophages was assessed. This test has been commonly used to assess pro-inflammatory activities of plant constituents (Wink et al., 2011). A

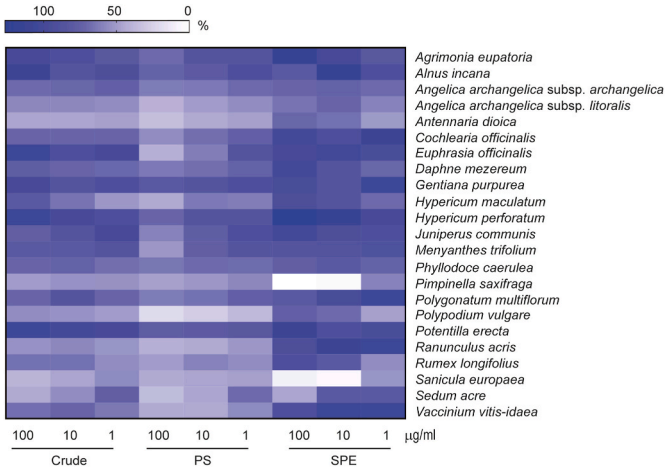

Fig. 3. Assessment of cellular toxicity of extracts. Cell viability of the J774.1 macrophage cell line, measured by the MTT assay for each plant extract and fraction. Data are presented as percentage live cells of treated cells relative to untreated cells.

dose-dependent increase in NO in cell supernatants was generally observed in response to both PS- and SPE-fractions from many study-plants (Fig. 4A), but with only minor response from *Potentilla erecta* (L.) Raeusch [Rosaceae] and *Rumex longifolius* DC [Polygonaceae]. The strongest NO release was observed with PS-fractions from *Angelica archangelica* L. subsp. *archangelica* [Apiaceae], *D. mezereum*, *Ranunculus acris* L. [Ranunculaceae], and *Sedum acre* L. [Crassulaceae] (83%, 90%, 170% and 168% relative to LPS stimulation with 100 μg/ml). For *S. acre*, we also observed high NO release induced by the crude extract at 10 or 100 μg/ml. For *J. communis* and *V. vitis-idaea* a high NO-release induced by the SPE-fractions was observed (80% and 67% relative to LPS stimulation with 100 μg/ml).

Next, proliferative effects on T cells and NK cells, both important mediators of immune responses, were tested. T cells classically respond to specific antigens through the T cell receptor, while NK cells express receptors that can recognize complex biomolecules. For T cells, a striking proliferative response was observed with the crude extract and the SPE-fraction from *D. mezereum* (79% and 84% relative to ConA stimulation at 100 μg/ml) (Fig. 4B). High proliferative activity was also observed with the PS-fraction from *A. archangelica* subsp. *archangelica* (71% relative to ConA stimulation at 100 μg/ml). As for T cells, NK-cell proliferation was observed for the PS-fraction from *A. archangelica* subsp. *archangelica* (249% relative to IL-2 stimulation at 100 μg/ml), but less strongly with the SPE-fraction from *D. mezereum* (Fig. 4C). Instead, proliferative effect was observed for the SPE-fractions of *P. saxifraga* (232% relative to IL-2 stimulation at 100 μg/ml) and *Cochlearia officinalis* L. [Brassicaceae] (227% relative to IL-2 stimulation at 10 μg/ml) and PS-fractions from *P. saxifraga* and *H. maculatum* (265% and 453% relative to IL-2 stimulation at 100 μg/ml) (Fig. 4C).

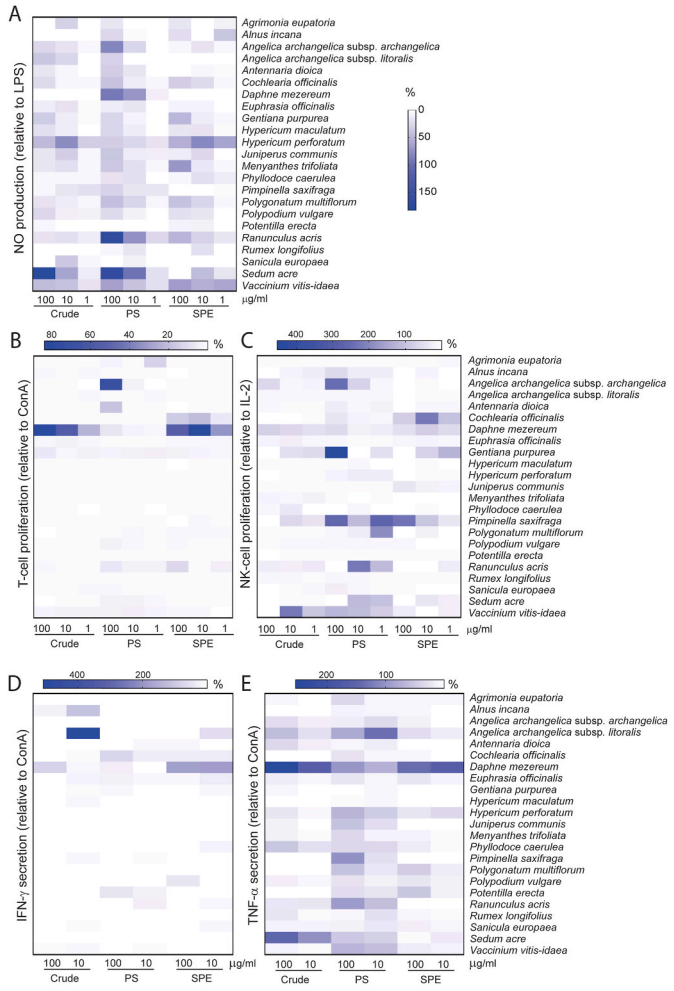

Fig. 4. Immune activating effects of plant extracts and fractions. A) NO-secretion from J477.1 macrophages for each plant extract or fraction, presented as percentage values relative to LPS stimulation. B) T-cell and C) NK cell proliferation after 6-day cultures of CFSE-labelled PBMCs with indicated plant extracts and fractions. Data presented as percentage values relative to ConA stimulation for T cells and IL-2 stimulation for NK cells. D) IFN-γ and E) TNF-α secretion measured via ELISA in supernatants from overnight cultures of PBMCs with indicated plant extracts and fractions. Data are presented as percentage relative to PMA/ionomycin stimulation.

Further, secretions of TNF-α and IFN-γ from PBMC were assessed. We found generally a higher secretion of TNF-α compared to IFN-γ (Fig. 4D and E). IFN-γ secretion was observed in response to crude extracts from Angelica *archangelica* subsp. *litoralis* (Fr.) Thell. [Apiaceae] and *A. incana* (257% and 57% relative to ConA, respectively at 10 μg/ml), the SPE-fraction from *D. mezereum* (both at 10 and 100 μg/ml), and very weakly in response to the PS-fraction from *C. officinalis* (95% and 37% relative to ConA, respectively at 100 or 10 μg/ml) (Fig. 4D). For TNF-α, there was generally secretion in response to the PS-fractions derived from all the plants, except *H. maculatum* and *Gentiana purpurea* L. [Gentianaceae] (Fig. 4E). The strongest production was observed in response to PS-fractions from *A. archangelica* subsp. *litoralis*, *P. saxifraga* and *R. acris* (272%, 204% and 189% relative to ConA at 100 μg/ml). For *D. mezereum* we observed a higher TNF-α secretion with the crude extract and the SPE-fraction (507% and 324% compared to 195%, relative to ConA at 100 μg/ml) (Fig. 4E).

3.5. Immune-inhibitory effects by plant extracts

We next evaluated the immune inhibitory effects of the extracts and fractions, by testing their ability to inhibit LPS-mediated NO-release from macrophages, or ConA-mediated cytokine release from PBMCs. An inhibitory effect on NO-release from macrophages were observed in response to the SPE-fractions of *A. incana*, *A. archangelica* subsp. *litoralis*, *A. dioica*, *S. acre, Phyllodoce caerulea* (L.) Bab [Ericaceae] and *S. europaea* (91%, 127%, 98%, 105%, 55% and 182% reduction compared to LPS alone at 100 μg/ml) (Fig. 5A). Low or negligible inhibitory activity was observed with the PS-fractions. In contrast, rather synergistic effects were observed for PS-fractions from *P. multiflorum*, *S. acre*, and *R. acris* (55%, 50% and 86% at 100 μg/ml). Measuring inhibition of ConA-mediated cytokine release, a higher inhibitory effect was again observed for SPE-fractions compared to PS-fractions (Fig. 5B and C). Of note, samples from *D. mezereum* showed synergy with ConA for both TNF-α and IFN-γ secretion (855% and 1162% relative to LPS alone at 100 μg/ml), but this synergistic effect was not observed for LPS-mediated NO-release from macrophages.

Finally, we performed an unsupervised hierarchical clustering of all samples with the immunological assay datasets (Fig. 6). This analysis demonstrated that *D. mezereum* exhibited the most potent immune stimulatory effect, while samples from *A. incana*, *H. perforatum* and *S. europaea* displayed the most potent immune inhibitory effects.

4. Discussion

We describe here the traditional use and pharmacological properties of pharmacologically under-studied medicinal plants used in Scandinavian folk medicine against inflammatory related diseases, infections, wounds, or diverse gastrointestinal ailments. Chemical characteristics with respect to polysaccharide and phenolic contents were matched against assays measuring immune modulatory activities in order to assess whether our approach successfully could select plants for further in-depth pharmacological studies.

Water extraction of medicinal plants yields crude extracts with a great diversity of natural products, including among others carbohydrates and phenolics. Flavonoids and other polyphenols are known to contribute to anti-inflammatory properties (Yahfoufi et al., 2018) while polysaccharides may contribute to immune activation (Meijerink et al.,

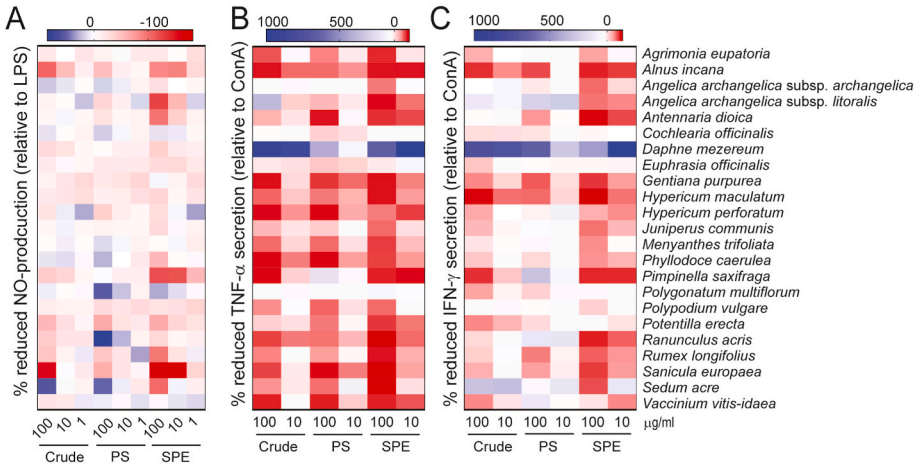

Fig. 5. Immune inhibitory effect of plant extracts and fractions. A) Percentage reduced NO-secretion after culturing J477.1 macrophages over night with plant extracts and fractions, relative to LPS-stimulation or medium alone. Reduction in B) TNF-α and C) and IFN-γ secretion measured by ELISA in supernatants from PBMCs cultured overnight in plant extracts and fractions, relative to PMA/Ionomycin and medium alone. Percentage values are measured relative to LPS stimulation alone and PBMC without stimulation.

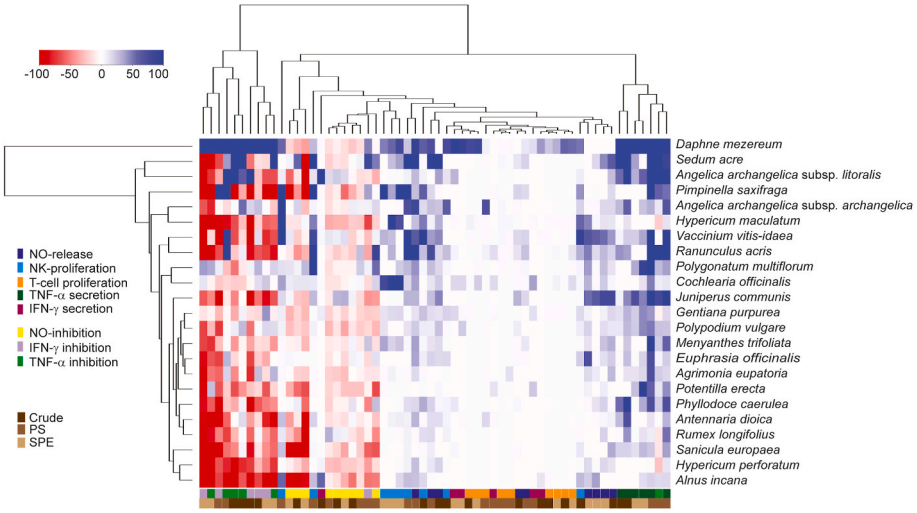

Fig. 6. Hierarchical clustering of all plant extracts and fractions with immunological assays. Red indicates reduced immune activity, while blue color indicate activating effect. Plant extracts and fractions, and immunological assays is colored coded. The immunological assays are given along the x-axis, and plants along the y-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2018). When such crude extracts are screened in *in vitro* systems, these classes of compounds may mask or counterbalance each other's activities. Therefore, the water extract was fractionated into a polysaccharide enriched fraction and a fraction enriched in phenolics. The historical sources gave generally no detailed information about how the decoctions or infusions were prepared. The plants were therefore extracted with boiling water using the same conditions for all plants, and a modern pressurized liquid extraction instrument was chosen as it ensured the same conditions for all samples.

It was interesting to note that plants with high immune inhibitory activity (*A. incana*, *H. perforatum*, *S. europaea*, *R. longifolius*, and *A. dioica*) were all reported in the sources as wound-healing remedies. In contrast, plants with primarily immune activating activities (*D. mezereum*, *S. acre*, *A. archangelica* species, and *P. saxifraga*) shared usage against the cold or upper airway infections that could support a function in promoting viral immune defense. Some plants, such as *A. eupatoria*, *Euphrasia. officinalis* L. [Orobanchaceae] and *G. purpurea*, showed low activities with our screens, and these plants shared a usage against diarrhea and other gastrointestinal-related ailments that could indicate that effects are not mediated through the immune system. Surprisingly, *Menyanthes trifoliata* L. [Menyanthaceae] and *J. communis* showed little activity in the screens despite multiple reported indications and broad use in folk medicine.

Plant-derived polysaccharides have been shown to induce macrophage activation measured as NO-production, release of TNF-α, and/or increase in phagocytic activity (Yin et al., 2019). Especially plants rich in pectic type polysaccharides have shown potent activity (Beukema et al., 2020; Inngjerdingen et al., 2008; Vogt et al., 2016). The observed general activation of macrophages by PS-fractions in our study was thus expected. Interestingly, the observed macrophage stimulatory effects of PS-fractions from different plants was quite diverse, and the observed effects are likely related to differences in structures and monosaccharide composition. Pectic polysaccharides consist of a backbone of GalA (homogalacturonan), but also of branched rhamnogalacturonan regions (RG-I and RG-II). RG-I has a backbone of rhamnose and GalA units, and is decorated by side chains consisting of primarily Ara and Gal (Wusigale et al., 2020). Among strong inducers of NO-release, the polysaccharide fractions of *D. mezereum*, *S. acre*, and *R. acris* are enriched in GalA, Ara and Gal which indicates presence of pectic structures. However, *A. archangelica* subsp. *archangelica* shows low enrichment in these monosaccharides, yet the PS-fraction of this plant has strong macrophage stimulatory activity. *S. acre* and *R. acris* induced TNF-α secretion from PBMCs, with the PS-fraction being the most potent TNF-α inducer. To date, little research has been performed on polysaccharides of these two plants. From the presented screening, *S. acre* appears to contain a considerable amount of mannose (Man) (13%), which could indicate the

presence of hemicelluloses like glucomannan or galactomannan in the plant. Polysaccharides from *S. acre* have not been studied and further detailed analysis is necessary. *R. acris* contained considerable amounts of GalA, Ara and Gal which could indicate the presence of pectic polysaccharides. Likely, the fine structure, in terms of backbone and branching, of the polysaccharides present in plant extracts is determining the functional effects.

While the PS-fractions generally were more potent in terms of NO-production than the SPE-fractions isolated from the same plant material, the SPE-fractions from *H. perforatum* and *V. vitis-ideaea* showed higher NO-release capacity compared to the PS-fractions. In fact, the SPE-fractions from these two plants were the most potent SPE-fractions across all tested plants. The activity is likely reflected in specific compounds, as there were no general association between high phenolic content and macrophage activation by the SPE-fractions. Interestingly, we noted that *H. perforatum* showed more potent immunomodulating activity than the closely related, and much less studied, *H. maculatum*. This could be related to slight differences in chemical structures between the two plants, although the overall phenolic content and monosaccharide composition of the polysaccharides were very similar.

A. archangelica subsp. *litoralis* and *A. archangelica* subsp. *archangelica* are very similar with regards to their high content of Glc. However, minor differences in amounts of GalA, Man and 4-*O*-Me GlcA could potentially contribute to the observed differences in biological effects of the PS-fractions obtained from these two plants. A more potent effect on TNF-α secretion was observed with the PS-fraction of *A. archangelica* subsp. *litoralis*. In contrast, the PS-fraction from *A. archangelica* subsp. *archangelica* induced higher proliferation of T cells and NK cells, as well as NO-production from macrophages. Although no studies have been conducted on the polysaccharides of *A. archangelica*, heteropolysaccharides isolated from the Chinese angelica *Angelica sinesis,* an important Chinese medicinal plant, have previously been reported to induce secretion of NO and TNF-α in macrophages (Sun et al., 2005; Yang et al., 2006). While the PS-fractions of both angelica subspecies induced varying degrees of immune activation, we observed that the SPE-fraction of *A. archangelica* subsp. *litoralis* strongly suppressed macrophage NO-release and TNF-α secretion from PBMCs. This was not evident with the SPE-fraction from *A. archangelica* subsp. *archangelica*. To date, no pharmacological studies have been conducted on *A. archangelica* subsp. *litoralis*, but *A. archangelica* subsp. *archangelica* has a longstanding tradition in Norwegian folk medicine as an "angel plant" with miraculous curative power (Teixidor-Toneu et al., 2020). Previous studies have identified active compounds in extracts from the plant, in particular furanocoumarins, coumarins and terpenes (Kaur and Bhatti, 2021). A wide range of effects of these compounds are reported, including neurological related diseases (Dahija et al., 2014; Gorick and Melzig, 2013; Kupchan and Baxter, 1975; Prakash et al., 2015; Ren et al., 2017; Vidal et al., 2012; Zhang et al., 2007), anti-cancer activity, antiviral activity, and gastro-protective activity (Altınyay et al., 2015; Fraternale et al., 2014; Fraternale et al., 2018; Joshi, 2016; Kaur and Bhatti, 2021; Krasilnikova et al., 2018; Li, Webster, Johnson and Gray, 2015; Prakash et al., 2015).

T cells are highly antigen specific through recognition of peptides in complex with MHC-molecules, and not believed to be directly stimulated through plant polysaccharides. However, antigen-independent activation occurs through lectins such as ConA via cross-linkage of the T cell receptor complex. T-cell proliferative activity was only observed in presence of PS-fractions from *A. archangelica* subsp. *archangelica* and *A. dioica*. The monosaccharide composition of the PS-fractions from these two plants are apparently quite different, thus the structural component mediating the observed T-cell proliferative effects is as yet uncertain.

Interestingly, the SPE-fraction, but not the PS-fraction, from *D. mezereum* uniquely induced high T-cell proliferation, as well as high secretion of IFN-γ and TNF-α by PBMCs. IFN-γ is secreted selectively by T and NK cells, while TNF-α is broadly secreted by macrophages, NK cells, and T cells. In light of this dichotomy, it is interesting to note that IFN-γ was induced almost exclusively by the *D. mezereum* SPE-fraction. This indicates a unique mode of action by as yet uncharacterized compounds in the *D. mezereum* SPE-fraction. Several bioactive compounds from *D. mezereum* have been characterized. Aqueous alcohol extracts from *D. mezereum* and the isolated compound mezerein possess anti-leukemic activity, and the diterpenoids daphnetoxin and gniditrin are shown to have cholesterol-lowering activity (Kupchan and Baxter, 1975; Vidal et al., 2012; Zhang et al., 2007). However, no immune modulating activity has been reported. Moreover, *D. mezereum* has been reported to have strong toxic effects due to the presence of diterpen esters, such as gniditrin (Gorick and Melzig, 2013). It should be noted that many of the assumed toxic compounds are highly lipophilic, and are thus not expected to be found in the water extract studied here.

Immune inhibitory activities were also observed in the SPE-fractions from *A. incana*, *S. europaea*, and *P. saxifraga*. Phenolic compounds isolated from *A. incana* have previously been reported to have anti-inflammatory properties (Dahija et al., 2014; Joshi, 2016; Prakash et al., 2015; Ren et al., 2017). Previous studies have attributed the anti-inflammatory effects of *A. incana* to the diarylheptanoid glycoside oregonin, which has been found to reduce inflammation in macrophages and structurally resembles the well-known antioxidant curcumin (Krasilnikova et al., 2018).

5. Conclusion

In summary, a wealth of information on Scandinavian folk medicine was collected from 19th- and 20th-century sources, forming the foundation of this study. These historical descriptions of ailments treated by each plant allowed for the recognition of plants with possible immunomodulatory activity. However, details on the preparations of plants for medicinal usage were sometimes lacking or vague. These missing details made it difficult to predict the nature of the compounds and bioactivity that may have been responsible for a plant's place in a remedy. While the focus on polysaccharides and polyphenols in this investigation may not have captured the most relevant compounds for every plant's recorded immunomodulatory function, overall we were able to detect the predominant immune activating or inhibitory activities with most of the studied plant extracts. This could underscore the feasibility of our selection approach. Our findings thus support an approach of combining historical sources with modern pharmacology in the discovery of plant sources containing potentially new pharmacological compounds.

Author contributions

ESU, HSB, AO, AK, RAB, HW, MI, KTI conceived and designed the study; ESU and HSB performed experiments, ESU, HSB, AO, AK, RAB, HW, MI, KTI analyzed and interpreted data, ESU and HSB did the historical literature review and wrote the manuscript, AO, AK, HW, RAB, MI, KTI edited the manuscript. All authors approved the final version of the manuscript.

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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part in plant collections and discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115402.

Glossary

ASE	accelerated solvent extraction
CFSE	5- (and 6)-carboxyfluorescein succinimidyl ester
ConA	Concavilin A
ELISA	enzyme-linked immunosorbent assay
IL-2	interleukin 2
LPS	lipopolysaccharide;
PBMC	peripheral blood mononuclear cells
PS	polysaccharides
SPE	solid phase extraction

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Phytochemical characterization and anti-inflammatory activity of a water extract of *Gentiana purpurea* roots

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ABSTRACT

Ethnopharmacological relevance: *Gentiana purpurea* was one of the most important medicinal plants in Norway during the 18th and 19th centuries, and the roots were used against different types of gastrointestinal and airway diseases.

Aim of the study: To explore the content of bioactive compounds in a water extract from the roots, a preparation commonly used in traditional medicine in Norway, to assess the anti-inflammatory potential, and furthermore to quantify the major bitter compounds in both roots and leaves.

Materials and methods: *G. purpurea* roots were boiled in water, the water extract applied on a Diaion HP20 column and further fractionated with Sephadex LH20, reverse phase C18 and normal phase silica gel to obtain the low molecular compounds. 1D NMR, 2D NMR, and ESI-MS were used for structure elucidation. HPLC-DAD analysis was used for quantification. The inhibition of TNF-α secretion in ConA stimulated peripheral blood mononuclear cells (PBMCs) was investigated.

Results: Eleven compounds were isolated and identified from the hot water extract of *G. purpurea* roots. Gentiopicrin, amarogentin, erythrocentaurin and gentiogenal showed dose-dependent inhibition of TNF-α secretion. Gentiopicrin is the major secondary metabolite in the roots, while sweroside dominates in the leaves.

Conclusions: The present work gives a comprehensive overview of the major low-molecular weight compounds in the water extracts of *G. purpurea*, including metabolites produced during the decoction process, and show new anti-inflammatory activities for the native bitter compounds as well as the metabolites produced during preparation of the crude drug.

List of compounds

Compound	CAS RN
Swertiamarin	17388-39-5
Gentiopicroside/gentiopicrin	20831-76-9
Sweroside	14215-86-2
Angelone	904293-35-2
Gentiogenal	87042-24-8
Syringic acid	530-57-4
Erythrocentaurin	50276-98-7
Erythrocentaurin dimethyl acetal	1002101-86-1
Acanthoside B	7374-79-0
Naringenin 4'-O-β-glucopyranoside	81202-36-0
Amarogentin	21018-84-8

1. Introduction

Gentiana purpurea L., family Gentianaceae, is a 20–80 cm high perennial plant with dark purple corollas, and is known for the intensely bitter taste of its roots. It has limited distribution in Europa, growing only in the mountain area in southern parts of Norway and in the Alps (Roskov et al., 2019). *G. purpurea* was regarded as one of the most important medicinal plants in Norway during the 18th and 19th centuries. The roots were used as medicine both for humans and animals and prepared as a water decoction, an alcoholic tincture, boiled in milk, or even in cream or beer (Høeg, 1974). Indications were all kinds of

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List of abbreviations

ConA – Concanavalin A
$CDCl_3$ – deuterated chloroform
CD_3OD – deuterated methanol
DMSO-d_6 – deuterated dimethyl sulfoxide
ESI-MS – electrospray ionization-mass spectrometry
GAE – gallic acid equivalents
HPLC-DAD – high performance liquid chromatography – diode array detector
LOD – limit of detection
LOQ – limit of quantification
PBMC – peripheral blood mononuclear cell
NMR – nuclear magnetic resonance
TNF-α – tumor necrosis factor alpha

stomach diseases, especially diarrhea, but also against colic and to stimulate appetite. The roots were also used against chest diseases such as bronchitis, and to treat coughing and the cold (Ulriksen et al., 2022). The roots were collected during autumn, preferably October, and the dried roots were sold to pharmacies and at local markets, but also exported, e.g. to Sweden (Svanberg, 2002). Due to overextensive and unsustainable harvesting, the plant was eradicated several places in Norway, but is today regarded as a vigorous plant in Norway (Artsdatabanken, 2021).

A few chemical constituents from *G. purpurea* are reported: Secoiridoids identified in the roots are gentiopicrin (also known as gentiopicroside) (Bridel, 1914), desoxyamarogentin (amaropanin) (Wagner and Vasirian, 1974), amarogentin and amaroswerin (Sticher and Meier, 1978, 1980), and gentiolactone (Suhr et al., 1978). An alkaloid, gentianine, was reported to be present in *G. purpurea* (Steinegger and Weibel, 1951). It was later suggested that this is an artefact from treatments of iridoids, such as gentiopicrin and amarogentin, with NH_3 during isolation (Budzikiewicz et al., 1967). The xanthone gentisin is reported from the roots (Verney and Debelmas, 1973). The *C*-glycosylflavones isoorientin, isovitexin and their 4'-glucosides are reported from *G. purpurea* leaves (Hostettmann et al., 1975). The trisaccharide gentianose was first reported in the roots (Meyer, 1882). Other identified carbohydrates are sucrose from the roots (Bridel, 1920), and bornesitol identified in the leaves (Hostettmann and Jacot-Guillarmod, 1974). Also a series of phenolic acids are known from the plant (Dombrowicz and Swiatek, 1987; Hatjimanoli et al., 1988). Reviews of constituents of the genus *Gentiana*, including *G. purpurea*, have been published (Pan et al., 2016; Xu et al., 2017; Yang et al., 2010), however, these reviews lack several of the references mentioned above.

Despite the popularity of this plant in traditional medicine in Norway, no systematic phytochemical characterization of the water extract is reported in the scientific literature. The purpose of the study was to characterize the chemical composition in a root decoction, as this has been a popular preparation in traditional medicine in Norway. Gentiana species are known to have to anti-inflammatory effects (Pan et al., 2016), and we wanted to study anti-inflammatory activities of the decoction and selected isolated constituents to get a deeper insight in how the roots from *G. purpurea* can contribute to medicinal effects. An HPLC-DAD method was developed to quantify the major secoiridoid glucosides in both leaves and roots.

2. Materials and methods

2.1. Plant material

Gentiana purpurea (local name "søterot") (Supplementary Material Fig. S1) was collected in Vang in Valdres, Norway (coordinates 60°59'29.5"N 8°37'41.7"E), 1060 masl, the 13th of August 2020; roots for isolation of constituents, and the 30th of June 2021 (before flowering), the 12th of August 2021 (flowering) and the 8th of October 2021 (after flowering); leaves and roots for quantitative analysis. The identity was verified by botanist Dr. Anneleen Kool, Natural History Museum, University of Oslo, Norway. Voucher specimens, RL-20200813-gp, RL-20210630-gp-r, RL-20210630-gp-l, RL-20210812-gp-r, RL-20210812-gp-l, and RL-20211008-gp-r are deposited at the Department of Pharmacy, University of Oslo, and a herbarium specimen is deposited at the Natural History Museum, University of Oslo. The roots were cleaned and washed, cut in small pieces and air-dried. The leaves were air-dried.

2.2. General methods

1D and 2D NMR spectroscopy was conducted on a Bruker AVIII400 or a Bruker AVII600 instrument (Bruker, Rheinstetten Germany). CD_3OD or $CDCl_3$ was used as solvent with tetramethylsilane as reference (Sigma-Aldrich, St. Louis, MO, USA). HPLC analysis was performed on a LaChrom Elite HPLC system (VWR-Hitachi, Tokyo, Japan) equipped with an L- 2455 diode array detector and a Kinetex C18 100A (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA). Mass spectra were recorded on a Maxis II-ETD instrument (Bruker), positive or negative mode. Preparative chromatography was performed on a Biotage Select Flash instrument equipped with Biotage Sfär C18 or silica columns (Biotage, Uppsala, Sweden) or with laboratory packed Diaion HP-20 gel (Sigma-Aldrich) or Sephadex LH-20 gel (Pharmacia, Uppsala, Sweden) columns. Preparative HPLC was carried out on a ProStar Polaris system (Varian, Palo Alto, CA, USA) equipped with a Kinetex C18 100A (150 × 21.2, 5 μm) column (Phenomenex), flow rate 15 ml/min. Fractions from CC were combined as indicated by UV-absorbance (Biotage Select flash), or by analytical TLC for open column fractions. Silica gel 60 RP-18 F254S, 0.2 mm thickness foils (Merck, Darmstadt, Germany) were used for TLC, and spots were visualized by UV irradiation (254 and 366 nm), and by spraying with $Ce(SO_4)_2$ (1% in 10% aqueous H_2SO_4) followed by heating (105 °C, 5 min).

2.3. Water extraction

The dried roots were ground in an Ultra Centrifugal Mill ZM 100, 1 mm (Retsch GmbH & Co. KG, Haan, Germany), and 570 g powder was boiled with 5 l distilled water for 1 h. The decoction was centrifuged, plant residue extracted once more under the same conditions and the supernatants combined (crude water extract).

2.4. Isolation of low-molecular compounds

The crude water extract (72% of total amount, 3.6 L) was filtered and applied to a Diaion HP-20 column (42 × 5 cm) eluting with a stepwise gradient of H_2O and methanol to yield fractions D1 (H_2O), D2 (20% methanol), D3 (50% methanol) and D4 (100% methanol). Fraction D2 (500 mg) was applied to a Biotage Sfär C18 column (60 g) and fractioned with a gradient of H_2O and acetonitrile (5–25%), detection by UV absorbance at 250 nm. UV-absorbent fractions were rechromatographed on a Sfär C18 column (12 g) using the same conditions to obtain swertiamarin (**1**) (9 mg), gentiopicrin (**2**) (147 mg) and sweroside (**3**) (14 mg). Fraction D4 (4.5 g) was chromatographed on a Biotage Sfär C18 column (60 g) with a gradient of H_2O and methanol (5–95%), to yield fractions D4F1-D4F9. Fraction D4F1 (47 mg) was chromatographed on a Biotage Sfär C18 column (12 g), UV-detection 270 nm, using a gradient of H_2O and methanol (5–90%) to yield angelone (**4**) (2.3 mg). Fractions D4F2 (217 mg) was applied to a Sephadex LH-20 column (30 × 2 cm) and eluted with a stepwise gradient of H_2O and methanol (25–100%) to give five subfractions. D4F2S3 and S4 were purified on a Biotage Sfär C18 column (12 g), UV detection 270 nm, (gradient H_2O and methanol, 5–90%) to give gentiogenal (**5**) (3.6 mg) and syringic acid (**6**) (1 mg). D4F4 (220 mg) was fractionated on a Biotage Sfär silica column (50 g)

with a stepwise gradient of dichloromethane and ethyl acetate (1:0, 1:9, 1:3, 1:1, 0:1, 2 CV each), 270 nm, to obtain erythrocentaurin (**7**) (44 mg). D4F5 (319 mg) was chromatographed on a Biotage Sfär C18 column (60 g), UV-detection 270 nm, with a gradient of H_2O and methanol (5–95%), to give erythrocentaurin (**7**) (23 mg) and erythrocentaurin dimethylacetal (**8**) (8 mg). The fraction between the peaks representing compounds **7** and **8** (D4F5F2, 117 mg) was further purified on a Biotage Sfär silica column (10 g), UV detection 260 nm, with a gradient of dichloromethane and methanol (5–95%), to give acanthoside B (**9**) (26 mg). D4F8 (989 mg) was chromatographed on a Biotage Sfär C18 column (60 g), UV detection 270 nm, with a gradient of H_2O and methanol (20–95%) to yield four subfractions. Fraction D4F8F2 (31 mg) was rechromatographed on a Biotage Sfär C18 column (60 g) followed by a Biotage Sfär C18 column (12 g) using a H_2O-methanol gradient (20–95%), UV detection 270 nm, to give naringenin 4'-*O*-β-glucopyranoside (**10**) (2.1 mg). D4F8F4 (204 mg) was applied on a Sephadex LH-20 column (30 × 2 cm) and eluted with a step wise gradient of H_2O and methanol (25–100%) and yielded amarogentin (**11**) (69 mg). NMR spectra of compounds **1**–**11** are shown in supplementary material, Figs. S3–S13.

Compound **1** (swertiamarin, $C_{16}H_{22}O_{10}$)

ESI-MS (+) m/z 397.11 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 7.63 (s, 1H, H-3), 5.72 (d, J = 1.4 Hz, 1H, H-1), 5.44 (m, 1H, H-8), 5.36 (dd, J = 17.0, 2.5 Hz, 1H, H-10), 5.29 (dd, J = 9.5, 2.5 Hz, 1H, H-10), 4.75 (ddd, J = 12.8, 10.9, 2.7 Hz, 1H, H-7), 4.64 (d, J = 7.9 Hz, 1H, H-1'), 4.34 (ddd, J = 10.9, 5.1, 1.7 Hz, 1H, H-7), 3.89 (dd, J = 12.0, 2.1 Hz, 1H, H-6'), 3.67 (dd, J = 11.9, 5.6 Hz, 1H, H-6'), 3.26–3.40 (overlapping signals, H-3', H-4' and H-5'), 3.21 (dd, J = 9.1, 7.9 Hz, 1H, H-2'), 2.92 (dd, J = 9.2, 1.4 Hz, 1H, H-9), 1.91 (ddd, J = 14.1, 12.8, 5.1 Hz, 1H, H-6), 1.75 (brd, J = 14.3, 1H, H-6), in accordance with (Li et al., 2015); ^{13}C NMR (100 MHz, CD_3OD) δ 168.03 (C-11), 154.82 (C-3), 133.85 (C-8), 121.24 (C-10), 108.89 (C-4), 100.26 (C-1'), 99.13 (C-1), 78.54 (C-5'), 77.82 (C-3'), 74.44 (C-2'), 71.43 (C-4'), 65.99 (C-7), 64.31 (C-5), 62.60 (C-6'), 51.94 (C-9), 33.75 (C-6), in accordance with (Boros and Stermitz, 1991).

Compound **2** (gentiopicrin, $C_{16}H_{20}O_9$)

ESI-MS (+) m/z 379.10 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (d, J = 1.5 Hz, 1H, H-3), 5.78 (ddd, J = 17.2, 10.3, 6.9 Hz, 1H, H-8), 5.68 (d, J = 2.9 Hz, 1H, H-1), 5.64 (m, 1H, H-6), 5.26 (m, 1H, H-10), 5.22 (dt, J = 10.3, 1.2 Hz, 1H, H-10), 5.09 (m, 1H, H-7), 5.01 (m, 1H, H-7), 4.67 (d, J = 7.9 Hz, 1H, H-1'), 3.92 (dd, J = 11.9, 2.2 Hz, 1H, H-6'), 3.67 (dd, J = 11.9, 6.1 Hz, 1H, H-6'), 3.27–3.41 (overlapping signals, H-9, H-3', H-4' and H-5'), 3.18 (dd, J = 9.2, 7.9 Hz, 1H, H-2'), in accordance with (Li et al., 2015); ^{13}C NMR (100 MHz, CD_3OD) δ 166.30 (C-11), 150.65 (C-3), 135.01 (C-8), 127.01 (C-5), 118.54 (C-10), 117.20 (C-6), 104.94 (C-4), 100.19 (C-1'), 98.52 (C-1), 78.40 (C-5'), 77.96 (C-3'), 74.54 (C-2'), 71.52 (C-4'), 70.91 (C-7), 62.77 (C-6'), 46.60 (C-9), in accordance with (Boros and Stermitz, 1991).

Compound **3** (sweroside, $C_{16}H_{22}O_9$)

ESI-MS (+) m/z 381.12 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 7.59 (d, J = 2.5 Hz, 1H, H-3), 5.55 (m, 1H, H-8), 5.55 (d, J = 1.7 Hz, 1H, H-1), 5.31 (m, 2H, H-10), 5.27 (m, 1H, H-10), 4.68 (d, J = 7.9 Hz, 1H, H-1'), 4.45 (ddd, J = 11.1, 4.3, 2.2 Hz, 1H, H-7), 4.37 (td, J = 11.5, 2.8 Hz, 1H, H-7), 3.89 (dd, J = 11.9, 2.1 Hz, 1H, H-6), 3.66 (dd, J = 11.9, 5.7 Hz, 1H, H-6), 3.19 (dd, J = 9.2, 7.9 Hz, 1H, H-2'), 3.28–3.96 (overlapping signals, H-5, H-3', H-4' and H-5'), 2.70 (ddd, J = 9.7, 5.5, 1.8 Hz, 1H, H-9), 1.77 (m, 1H, H-6), 1.70 (m, 1H, H-6), in accordance with (Li et al., 2015).

Compound **4** (angelone, $C_9H_8O_4$)

ESI-MS (+) m/z 203.03 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 8.40 (s, 1H, H-7), 4.54 (t, J = 6.0 Hz, 2H, H-3), 3.19 (t, J = 6.0 Hz, 2H, H-4), 2.50 (s, 3H, H-11), in accordance with (Mulholland et al., 2006).

Compound **5** (gentiogenal, $C_{10}H_{10}O_4$)

ESI-MS (+) m/z 217.05 $[M+Na]^+$

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.88 (s, 1H, H-11), 7.94 (s, 1H, H-8), 5.64 (q, J = 6.5 Hz, 1H, H-6), 4.41 (m, 2H, H-3), 3.08 (m, 2H, H-4), 1.40 (d, J = 6.5 Hz, 3H, H-12);

^{13}C NMR (100 MHz, $CDCl_3$) δ 185.43 (C-11), 163.2 (C-1), 163.18 (C-8), 142.50 (C-10), 120.1 (C-5), 104.3 (C-9), 73.28 (C-6), 65.04 (C-3), 22.81 (C-4), 19.84 (C-12), in accordance with (Boros and Stermitz, 1991).

Compound **6** (syringic acid, $C_9H_{10}O_5$)

ESI-MS (+) m/z 221.04 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 7.33 (s, 2H, H-2 and H-6), 3.88 (s, 6H, 3-OCH_3 and 5-OCH_3), compared with standard compound (Fluka, Buchs, Switzerland).

Compound **7** (erythrocentaurin, $C_{10}H_8O_3$)

ESI-MS (+) m/z 199.04 $[M+Na]^+$

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H, H-11), 8.38 (dd, J = 7.8, 1.5 Hz, 1H, H-8), 8.07 (dd, J = 7.6, 1.5 Hz, 1H, H-6), 7.64 (t, J = 7.7 Hz, 1H, H-7), 4.57 (t, J = 6.1 Hz, 2H, H-3), 3.59 (t, J = 6.1 Hz, 2H, H-4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.86 (C-11), 164.16 (C-1), 141.14 (C-10), 138.38 (C-8), 135.61 (C-5), 132.61 (C-6), 127.86 (C-9), 126.92 (C-7), 66.74 (C-3), 24.60 (C-4), in accordance with (Wang et al., 2009).

Compound **8** (erythrocentaurin dimethylacetal, $C_{12}H_{14}O_4$)

ESI-MS (+) m/z 245.08 $[M+Na]^+$

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (dd, J = 7.9, 1.4 Hz, 1H, H-8), 7.77 (dd, J = 7.7, 1.4 Hz, 1H, H-6), 7.41 (t, J = 7.7 Hz, 1H, H-7), 5.43 (s, 1H, H-11), 4.51 (t, J = 6.1 Hz, 2H, H-3), 3.32 (s, 6H, H-12 and H-13), 3.16 (t, J = 6.1 Hz, 2H, H-4), in accordance with (Ando et al., 2007).

Compound **9** (acanthoside B, $C_{28}H_{36}O_{13}$)

ESI-MS (+) m/z 603.21 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 6.71 (s, 2H, H-2 and H-6), 6.65 (s, 2H, H-2' and H-6'), 4.85 (overlapped), 4.76 (d, J = 4.0 Hz, 1H, H-7), 4.76 (d, J = 4.2 Hz, 1H, H-7'), 4.28 (m, 2H, H-9$_b$ and H-9'$_b$), 3.90 (m, 2H, H-9$_a$ and H-9'$_a$), 3.86 (s, 6H, 3-OCH_3 and 5-OCH_3), 3.84 (s, 6H, 3'-OCH_3 and 5'-OCH_3), 3.77 (*dd*, J = 12.1, 2.6 Hz, 1H, H-6''$_b$), 3.66 (*dd*, J = 12.1, 5.2 Hz, 1H, H-6''$_a$), 3.47 (m, 1H, H-2''), 3.41 (m, 2H, H-3'' and H-4''), 3.20 (*m* , 1H, H-5''), 3.13 (m, 2H, H-8 and H-8');

^{13}C NMR (100 MHz, CD_3OD) δ 154.44 (C-3 and C-5), 149.37 (C-3' and C-5'), 139.57 (C-1), 136.23 (C-4'), 135.60 (C-4), 133.10 (C-1'), 105.36 (C-1''), 104.85 (C-2 and C-6), 104.53 (C-2' and C-6'), 87.62 (C-7'), 87.21 (C-7), 78.36 (C-5''), 77.85 C-3''), 75.73 (C-2''), 72.92 (C-9), 72.95 (C-9'), 71.35 (C-4''), 62.60 (C-6''), 57.10 (3-OCH_3 and 5-OCH_3), 56.84 (3'-OCH_3 and 5'-OCH_3), 55.75 (C-8'), 55.54 (C-8), in accordance with (Shahat et al., 2004).

Compound **10** (naringenin 4'-*O*-β-glucopyranoside, $C_{21}H_{22}O_{10}$)

ESI-MS (+) m/z 457.11 $[M+Na]^+$

^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (d, J = 8.6 Hz, 1H, H-2' and H-6'), 7.14 (d, J = 8.6 Hz, 1H, H-3' and H-5'), 5.90 (d, J = 2.1 Hz, 1H, H-6), 5.88 (d, J = 2.1 Hz, 1H, H-8), 5.40 (dd, J = 12.6, 3.1 Hz, 1H, H-2), 4.94 (d, J = 7.2 Hz, 1H, H-1''), 3.90 (dd, J = 12.0, 2.1 Hz, 1H, H-6''), 3.70 (dd, J = 12.0, 5.4 Hz, 1H, H-6''), 3.40–3.50 (overlapping signals, H-2'', H-3'', H-4'' and H-5''), 3.09 (dd, J = 17.1, 12.6 Hz, 1H, H-3$_a$), 2.73 (dd, J = 17.1, 3.1 Hz, 1H, H-3$_b$);

^{13}C NMR (100 MHz, CD_3OD) δ 197.41 (C-4), 168.61 (C-9), 165.47 (C-7), 164.69 (C-5), 159.27 (C-4'), 134.19 (C-1'), 128.79 (C-2' and C-6'), 117.80 (C-3' and C-5'), 103.32 (C-10), 102.18 (C-1''), 97.18 (C-8), 96.27 (C-6), 80.12 (C-2), 78.07 (C-5''), 77.97 (C-3''), 74.89 (C-2''), 71.36 (C-4''), 62.50 (C-6''), 44.03 (C-3), in accordance with (da Silva et al., 2013).

Compound **11** (amarogentin, $C_{29}H_{30}O_{13}$)

ESI-MS (+) m/z 609.16 $[M+Na]^+$

^{1}H NMR (600 MHz, CD_3OD) δ 7.43 (d, J = 2.6 Hz, 1H, H-3), 7.17 (t, J = 8.0 Hz, 1H, H-5'''), 6.78 (ddd, J = 8.0, 2.4, 1.1 Hz, 1H, H-4'''), 6.70–6.74 (overlapping signals, 2H, H-2''' and H-6'''), 6.30 (d, J = 2.4 Hz, 1H, H-4''), 6.17 (d, J = 2.4 Hz, 1H, H-6''), 5.44 (dt, J = 17.1, 9.7 Hz, 1H, H-8), 5.39 (d J = 1.8, 1H, H-1), 5.24 (m, 2H, H-10) 4.73 (dd, J = 9.4, 8.0 Hz, 1H, H-2'), 4.37 (m, 1H, H-7), 4.29 (d, J = 8.0 Hz, 1H, H-1'), 4.25 (m, 1H, H-7), 3.84 (dd, J = 12.1, 2.2 Hz, 1H, H-6'), 3.61 (dd, J = 12.1, 6.2

Hz, 1H, H-6'), 3.23 (t, $J = 9.3$ Hz, 1H, H-4'), 3.09 (m, 1H, H-5'), 2.82 (t, $J = 9.3$ Hz, 1H, H-3'), 2.74 (m, 1H, H-5), 2.58 (ddd, $J = 9.6$, 5.5, 1.8 Hz, 1H, H-9), 1.69 (m, 1H, H-6), 1.58 (qd, $J = 12.8$, 4.2 Hz, 1H, H-6); ^{13}C NMR (150 MHz, CD_3OD) δ 171.44 (CO), 167.58 (C-11), 165.97 (C-3''), 163.86 (C-5''), 157.41 (C-3‴), 153.69 (C-3), 148.58 (C-1''), 146.48 (C-1‴), 132.80 (C-8), 129.31 (C-5‴), 121.15 (C-6‴), 121.00 (C-10), 116.48 (C-2‴), 114.53 (C-4‴), 112.85 (C-6''), 105.57 (C-4), 104.04 (C-2''), 103.14 (C-4''), 97.16 (C-1), 96.76 (C-1'), 78.31 (C-5'), 74.82 (C-3'), 74.60 (C-2'), 71.63 (C-4'), 69.51 (C-7), 62.42 (C-6'), 43.38 (C-9), 28.67 (C-5), 25.78 (C-6), in accordance with (Wang et al., 2001).

2.5. Quantitative analysis of secoiridoid glucosides with HPLC-DAD

The dried roots were ground in a knife mill (Brabender, Duisburg, Germany; 4 mm sieve). Leaves were pulverized using mortar and pistil. Analytical standards of gentiopicrin and sweroside were obtained from Sigma-Aldrich (≥99% purity), the purity of isolated amarogentin was determined by HPLC (250 nm, > 99% purity) (supplementary material, Fig. S2).

Methanol extracts were obtained by extracting 25.0 mg powdered roots or leaves in 25 mL methanol in a Falcon tube on ultrasonic bath for 40 min. The extract was centrifuged at 4000 RPM for 10 min, the supernatant was decanted, and the extraction step repeated. The supernatants were filtered through an PTFE syringe filter (0.45 μm) into an accurately weighed round-bottomed flask and dried on a rotavapor. Methanol was added to give a final concentration of 2.0 mg/mL, and the solution was diluted 1:1 with distilled water before HPLC analysis. Elution was performed using a gradient of mobile phase A (H_2O) and B (acetonitrile) with the following time schedule: 5% B, 0–3 min; 5–25% B, 3–20 min; 25–90% B, 20–45 min; 90% B, 45–50 min. The flow rate was 1 ml/min, injection volume 10 μL, temperature 25 °C, and absorbance was recorded at 225, 246 and 275 nm. Quantification was based on individual standard curves for each analyte. Gentiopicrin, sweroside and amarogentin were accurately weighed, dissolved in methanol and diluted to appropriate concentrations. Standard curves were based on three parallels with eight different concentrations, and UV maximum for each standard employed; gentiopicrin 275 nm, sweroside 246 nm, and amarogentin 225 nm (Table 1). The sample solutions were filtered through a PTFE syringe filter (0.45 μm) and analyzed in triplicate. The results are expressed as mg substance per gram dry weight.

2.6. Total phenolic content

The total phenolic content of roots and leaves collected at different dates was measured by use of the Folin-Ciocalteu method as previously described (Ulriksen et al., 2022) using a SpectraMax 190 Microplate Reader (Molecular Devices, San Jose, CA, USA). The results are expressed as mg gallic acid equivalents (GAE) per gram dry weight.

Table 1
Calibration curve, LOD and LOQ for standard compounds.

Standard	Calibration curve	R^2	Concentration range (μg/mL)[a]	LOD (μg/mL)[b]	LOQ (μg/mL)[c]
Gentiopicrin (**2**)	y = 62377.7x + 10806.7	0.9999	1–200	0.31	0.94
Sweroside (**3**)	y = 75695.3x + 79627.7	0.9996	1–200	2.3	6.9
Amarogentin (**11**)	y = 92161.8x + 28187.3	0.9999	0.5–100	0.29	0.88

[a] Based on 8 different concentrations.
[b] LOD (limit of detection), 3.3 × standard deviation of the y-intercepts of regression line/slope of the regression line (σ/S).
[c] LOQ (limit of quantification), 10 × standard deviation of the y-intercepts of regression line/slope of the regression line (σ/S).

2.7. Anti-inflammatory assay

Release of human TNF-α ELISA was tested from human peripheral blood mononuclear cells (PBMCs), using an ELISA kit from Mabtech (Sweden) as described previously (Ulriksen et al., 2022). PBMCs were isolated via Lymphoprep (StemCell Technologies, Vancouver, Canada) from buffy coats obtained from healthy volunteers at the Blood Bank at Oslo University Hospital (the use of PBMCs are approved by the Regional Ethical Committee). In brief, PBMCs were incubated with indicated concentration of substances overnight at 37 °C in a 5% CO_2 cell incubator. In each well, 0.5% DMSO were spiked in to ensure equal DMSO concentration in all wells. 10 ng/mL concanavalin A (ConA) were used as an inducer of TNF-α release. Cell-free culture supernatants (100 μL) and standards were added to 96-well plates pre-coated with capture antibodies and blocked in PBS with 0.05% Tween-20 and 0.1% BSA. After 2 h incubation, the plates were washed and 100 μL/well of human TNF-α monoclonal detection antibody (Mabtech) diluted in incubation buffer (1 μg/mL) were added, plates were incubated at 1 h in room temperature (RT). After washing, streptavidin-HRP (Mabtech) were added, and plates were incubated for 1 h at RT. Plates were developed with TMB substrate for 15 min followed by 1 M HCl. Absorbance at 450 nm was measured using a Molecular Devices FlexStation 3 Reader within 15 min of adding HCl. Data are calculated based on standard curve and presented as percent inhibition based on the ConA alone and DMSO alone. All samples were run in duplicates on the same plate and all runs were repeated with three different donors. All washes were done using a BioTek ELx405 plate washer with 0.05% Tween-20 in PBS.

2.8. Statistics

Statistical analysis was conducted by using the GraphPad Prism 9 software (GraphPad). Analysis was done by ordinary one-way ANOVA test, Tukey's multiple comparison test was used for comparison of secoiridoid and total phenolic content among the samples, while Dunnett's test for comparison of TNF-α secretion against the untreated control. Values are expressed as mean ± SD.

3. Results and discussion

3.1. Phytochemical composition

Eleven low molecular weight compounds were isolated from the hot water extract of *G. purpurea* roots. Their chemical structures are shown in Fig. 1. The structures were identified by 1D and 2D NMR spectroscopy, comparison of their spectroscopic data with literature values, and the structures confirmed with mass spectrometry (ESI-MS). The isolated compounds include the secoiridoid glucosides swertiamarin (**1**) (Boros and Stermitz, 1991; Li et al., 2015), gentiopicrin (**2**) (Boros and Stermitz, 1991; Li et al., 2015), sweroside (**3**) (Li et al., 2015) and amarogentin (**11**) (Wang et al., 2001), the secoiridoids angelone (**4**) (Mulholland et al., 2006), gentiogenal (**5**) (Boros and Stermitz, 1991), erythrocentaurin (**7**) and erythrocentaurin dimethylacetal (**8**) (Ando et al., 2007), the lignan glucoside acanthoside B (**9**) (Shahat et al., 2004), and the flavanone naringenin 4'-*O*-β-glucopyranoside (**10**) (da Silva et al., 2013). Isolated syringic acid (**6**) was identified by comparison with NMR spectra of reference compound obtained from Fluka. Only gentiopicrin, amarogentin, and syringic acid were previously known in this species (Bridel, 1920; Dombrowicz and Swiatek, 1987; Nyiredy et al., 1986; Sticher and Meier, 1978, 1980). Swertiamarin and sweroside are well known from the *Gentiana* genus (Pan et al., 2016), while acanthoside B and naringenin 4'-*O*-β-glucoside have not been reported from the genus before, and the finding in this taxon is therefore of chemotaxonomic interest. Interestingly, flavanones are rare compounds among *Gentiana* species, and only a few flavanones seems to be reported from this taxon

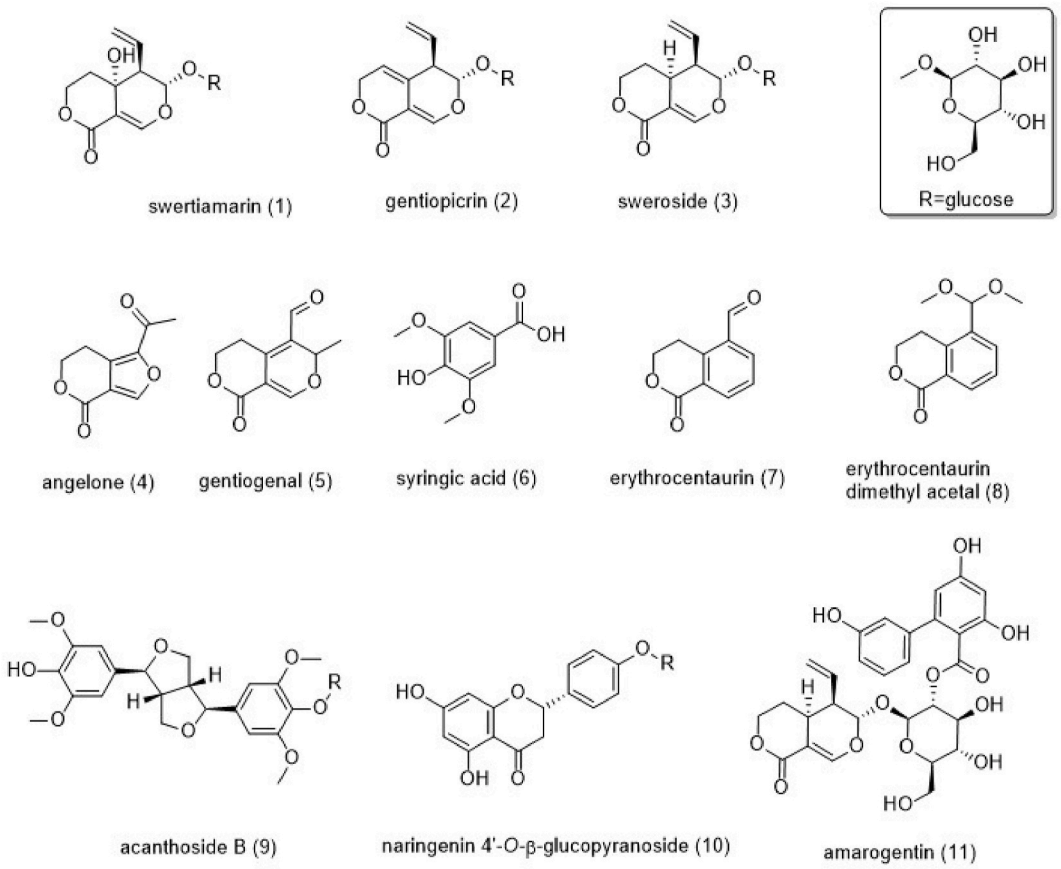

Fig. 1. Chemical structures of compounds **1**–**11** isolated from a water extract of *Gentiana purpurea* roots.

(Pan et al., 2016; Xu et al., 2017; Yang et al., 2010). Gentiogenal and erythrocentaurin were probably formed during hot water extraction. Gentiogenal and erythrocentaurin contain an aldehyde group giving rise to characteristic ^{1}H NMR signals at δ 9.83 ppm (s) and 10.24 ppm (s), respectively, in the water extract (^{1}H NMR, DMSO-d6). These signals were not present in a methanol extract of the gentian roots, and the difference in gentiogenal and erythrocentaurin content in the water and methanol extracts were also verified by HPLC-DAD analysis (Fig. 2). During purification of erythrocentaurin by RP-C18 flash chromatography with a water-methanol gradient, erythrocentaurin dimethylacetal appeared as a new compound and was not present in the crude extract. Erythrocentaurin and gentiogenal are previously reported as degradation products from gentiopicrin, which supports the findings in this study that these molecules are degradation products formed during boiling with water (El-Sedawy et al., 1989; Ishiguro et al., 1983; Wang et al., 2009). The pH of the water was 4.1, which may contribute to an acidic hydrolysis of the glycosidic bond of the iridoid glucosides. However, enzymatic degradation during heating with water cannot be excluded and will be a subject for a follow up study.

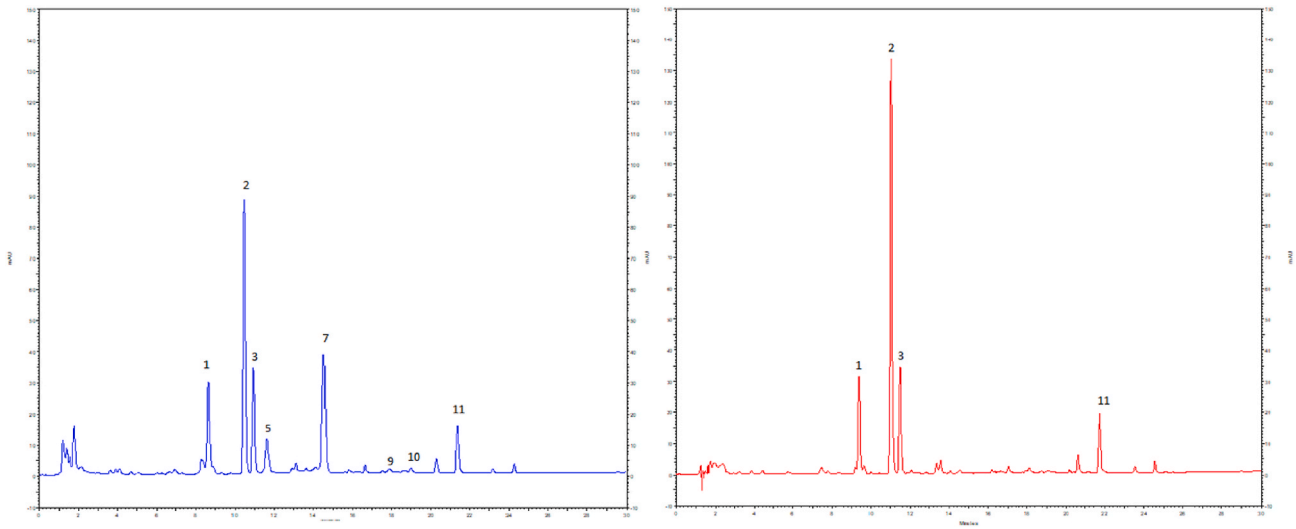

Fig. 2. HPLC chromatograms at 225 nm of *Gentiana purpurea* root water extract (left) and methanol extract (right).

3.2. Quantitative analysis

Significant differences between the content of gentiopicrin, sweroside, and amarogentin in leaves and roots were observed (Table 2). Gentiopicrin is the major metabolite in the roots (49.7–67.6 mg/g), while sweroside is the major metabolite in the leaves (26.4–26.6 mg/g). Amarogentin was not detected in the leaves. Highest yield of the three analyzed secoiridoids was obtained with roots collected in October, while no significant differences were observed for the secoiridoid content in the leaves collected at different time points. The quantitative content of gentiopicrin and amarogentin is comparable with the values reported in the roots of *G. purpurea* (Sticher and Meier, 1980), 33–100 mg/g for gentiopicrin and 1.86–5.10 mg/g for amarogentin. However, the analysis from 1980 did not distinguish between gentiopicrin and sweroside, which elute quite close to each other on the RP18 column. This is the first report of the identification and quantification of gentiopicrin, sweroside, and amarogentin in *G. purpurea* leaves.

The phenolic content, measured as GAE, was higher in the leaves than in the roots. This is in accordance with other *Gentiana* studies, e.g. (Stefanović et al., 2018). There were only minor differences between the samples collected at different dates. In Norway, *G. purpurea* roots were commonly harvested from August to October, preferably in late autumn. This study supports the local harvesting tradition with a preference for harvesting in October, since a high content of bitter substances is wanted.

3.3. Anti-inflammatory effects

Gentiopicrin (**2**), gentiogenal (**5**), erythrocentaurin (**7**), amarogentin (**11**), and the crude water extract were evaluated for immune inhibitory effects by measuring their capacity to reduce TNF-α secretion in ConA stimulated PBMCs. The tested compounds showed a significant dose-dependent anti-inflammatory effect, with small differences between the four compounds (Fig. 3). TNF-α inhibition was slightly stronger for erythrocentaurin (39.5%) compared to gentiopicrin (27.9%), gentiogenal (27.1%) and amarogentin (27.9%) at the lowest concentration (12.5 μM). The same trend was observed at 25 μM, with 58.3% inhibition for erythrocentaurin compared to 49.0% (gentiopicrin), 46.4% (gentiogenal) and 53.0% (amarogentin). The crude water extract (12.5–100 μg/mL) showed significant inhibitory effects (15.8–26.0% inhibition), but no clear dose dependency was observed. This is probably caused by the high amounts of carbohydrates in the *G. purpurea* water extract, since a polysaccharide enriched fraction from *G. purpurea* has shown an opposite effect with stimulation of TNFα production (Ulriksen et al., 2022).

Previous studies have reported anti-inflammatory effects of gentiopicrin (Jia et al., 2022; Kondo et al., 1994; Wang et al., 2013) and amarogentin (Huang et al., 2020b; Potunuru et al., 2019; Wölfle et al., 2015). However, this is the first study demonstrating a reduction of TNF-α secretion in PBMCs of these two compounds. Very few biological studies have previously been performed on gentiogenal and erythrocentaurin. This is the first study showing anti-inflammatory activity of gentiogenal, while erythrocentaurin was found to inhibit NO formation in macrophages (Huang et al., 2020a).

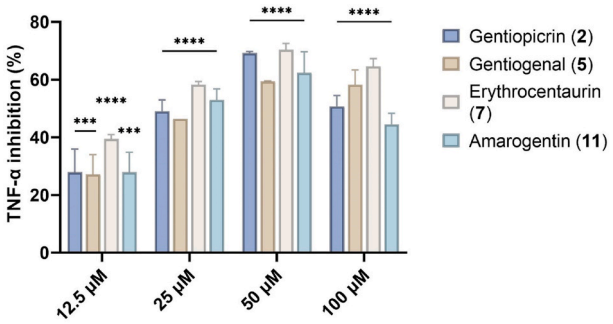

Fig. 3. Anti-inflammatory effects of isolated compounds presented as % inhibition of TNF-α in peripheral blood mononuclear cells (PBMCs) compared to ConA stimulated cells (untreated). Each bar express the average of two individual experiments ±SD. **** = $p < 0.0001$, *** = $p < 0.001$ compared to control cells (ConA + DMSO).

4. Conclusions

This study has explored the chemical composition of a hot water extract from *G. purpurea*, one of the most important and popular medicinal plants in Norway during the 18th and 19th centuries. It includes the finding of low molecular compounds new to this plant in the water extract and the quantification of the major secoiridoid glycosides in roots and leaves collected at different dates. For the first time we report the formation of degradation products in the decoction of *G. purpurea*, with erythrocentaurin (**7**) as the major of these products. Extraction with boiling water can result in chemical modifications of the plant metabolites resulting in new metabolites. There is therefore a potential in the area of traditional medicine research to explore new bioactive compounds developed during different preparation processes. Anti-inflammatory activities, observed as reduction in TNF-α secretion in PBMCs, were observed for the major bitter compounds; gentiopicrin (**2**) and amarogentin (**11**), as well as the degradation products; gentiogenal (**5**) and erythrocentaurin (**7**), with erythrocentaurin showing the strongest effect. Thus, the study has contributed to a better understanding of the traditional use, phytochemical profile and biological properties of a hot water extract obtained from *G. purpurea*.

CRediT authorship contribution statement

Lin Zhang: Formal analysis, Data curation, Writing – review & editing. **Emilie Steinbakk Ulriksen:** Formal analysis, Data curation,

Table 2
Content of gentiopicrin, sweroside, and amarogentin in *Gentiana purpurea* roots and leaves (dry weight) collected at three different dates.

Sample, collection date	Voucher specimen	Gentiopicrin (mg/g ± SD)	Sweroside (mg/g ± SD)	Amarogentin (mg/g ± SD)	Total phenolic content (mg GAE/g ± SD) [a]
Roots, 30.06.2021	RL-20210630-gp-r	57.4 ± 0.85^{a}	17.8 ± 0.34^{a}	2.9 ± 0.040^{a}	10.5 ± 1.3^{a}
Roots, 12.08.2021	RL-20210812-gp-r	49.7 ± 0.68^{b}	11.8 ± 0.15^{b}	2.7 ± 0.027^{b}	8.4 ± 1.3^{ab}
Roots, 08.10.2021[2]	RL-20211008-gp-r	67.7 ± 0.98^{c}	18.2 ± 0.33^{a}	3.6 ± 0.047^{c}	7.1 ± 0.9^{b}
Leaves, 30.06.2021	RL-20210630-gp-l	4.2 ± 0.0083^{d}	26.4 ± 0.59^{c}	< LOD	17.9 ± 3.1^{c}
Leaves, 12.08.2021	RL-20210812-gp-l	3.9 ± 0.049^{d}	26.6 ± 0.36^{c}	< LOD	19.1 ± 2.9^{c}

[2]Leaves were withered and not possible to obtain for analyses at this date. Different letters shown in superscript indicate statistical significant differences between groups according to the Tukey's multiple comparison test, $p < 0.05$; $n = 3$ for gentiopicrin, sweroside and amarogentin, $n = 6$ for total phenolic content.
[a] GAE; gallic acid equivalents.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115818.

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