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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

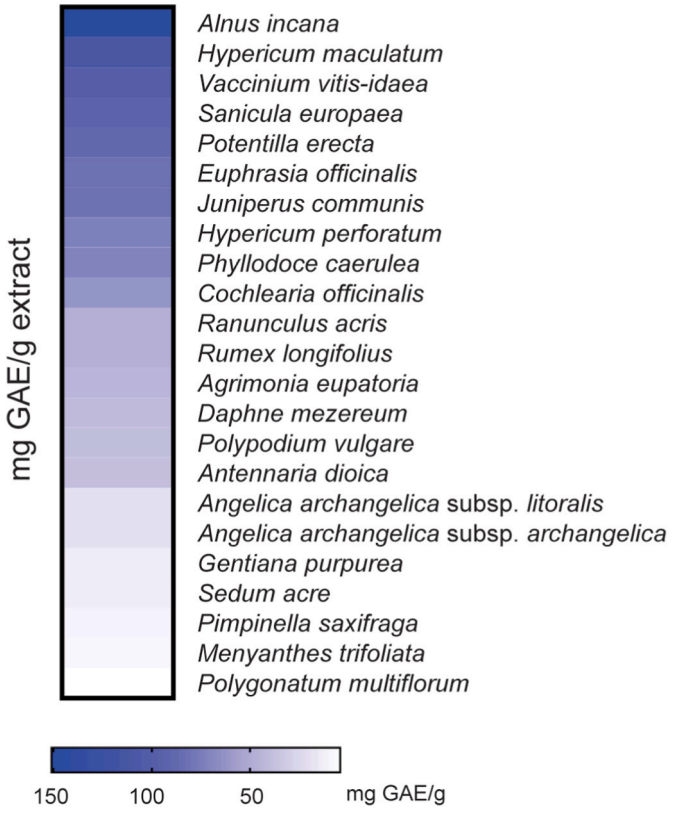

Fig. 1. Total phenolic content in SPE-fractions expressed as gallic acid equivalents (mg of GAE/g of extract).

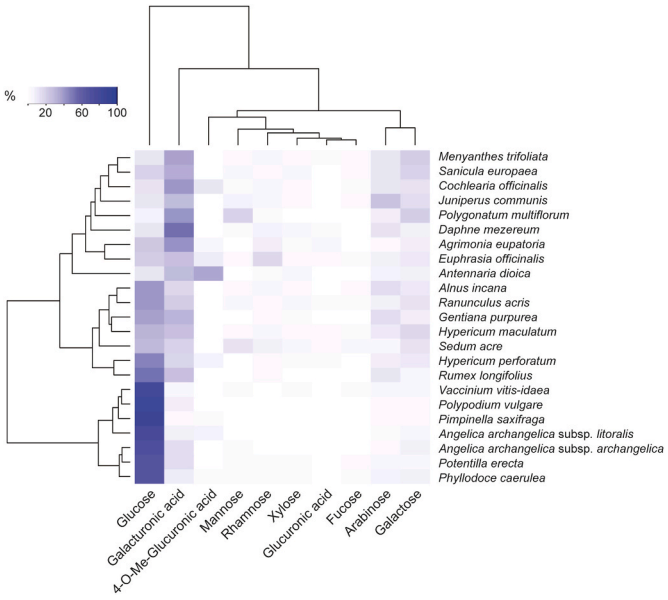

Fig. 2. Illustrative representation of the monosaccharide composition of PS-fractions obtained from each plant. Data are presented as the percentage of each monosaccharide in relation to total amount of carbohydrates in the sample, and hierarchical clustering of monosaccharides versus plant species.

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

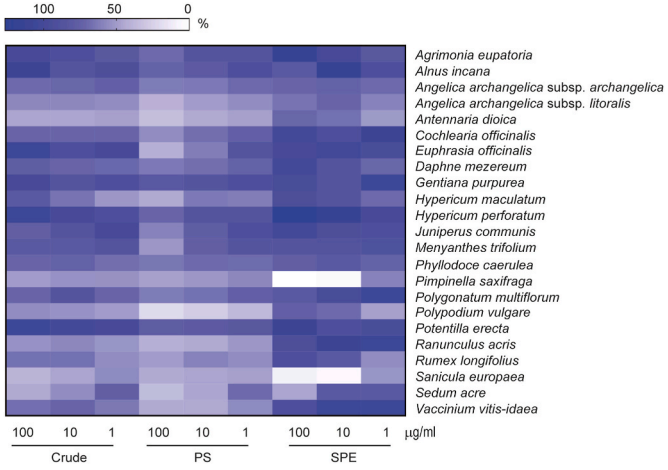

Fig. 3. Assessment of cellular toxicity of extracts. Cell viability of the J774.1 macrophage cell line, measured by the MTT assay for each plant extract and fraction. Data are presented as percentage live cells of treated cells relative to untreated cells.

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).

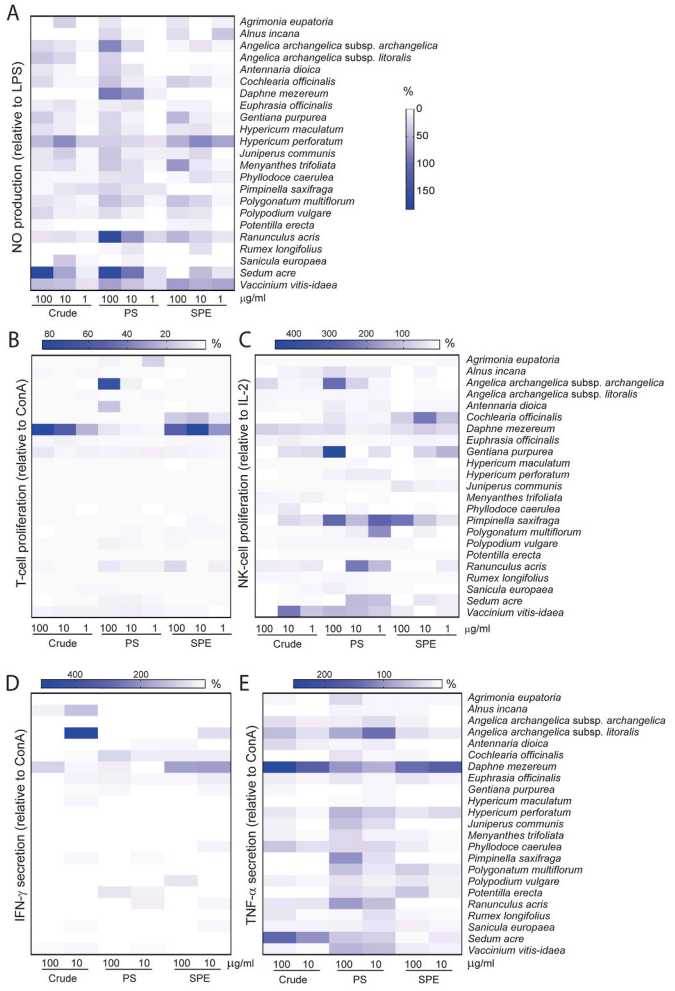

Fig. 4. Immune activating effects of plant extracts and fractions. A) NO-secretion from J477.1 macrophages for each plant extract or fraction, presented as percentage values relative to LPS stimulation. B) T-cell and C) NK cell proliferation after 6-day cultures of CFSE-labelled PBMCs with indicated 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.,

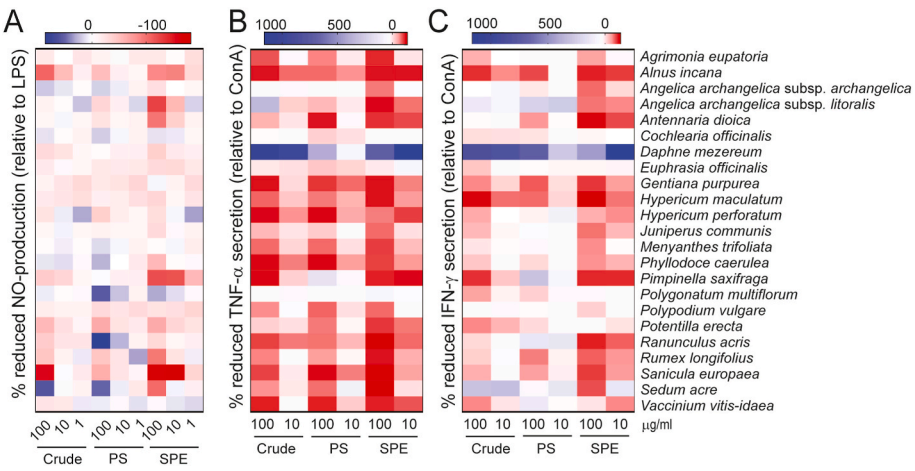

Fig. 5. Immune inhibitory effect of plant extracts and fractions. A) Percentage reduced NO-secretion after culturing J477.1 macrophages over night with plant extracts and fractions, relative to LPS-stimulation or medium alone. Reduction in B) TNF-α and C) and IFN-γ secretion measured by ELISA in supernatants 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.

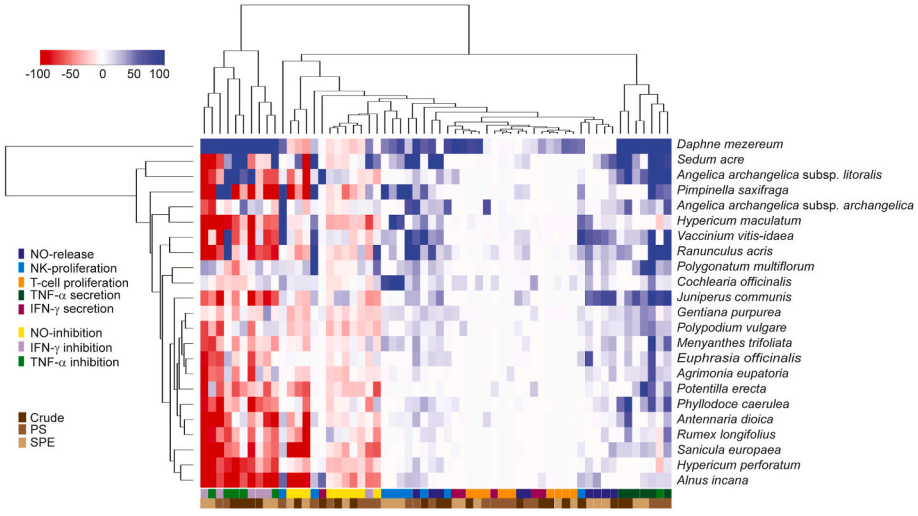

Fig. 6. Hierarchical clustering of all plant extracts and fractions with immunological assays. Red indicates reduced immune activity, while blue color indicate activating effect. Plant extracts and fractions, and immunological assays is colored coded. The immunological assays are given along the x-axis, 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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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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