



UNIVERSITY OF ICELAND
FACULTY OF MEDICINE

Appendix B

Additional natural products tested on THP-1 cells

This appendix contains results from experiments on plants and compounds that were tested in the beginning of the project but were not chosen for further analysis. Most of the experiments were not repeated as the results were only used to choose which plants or compounds should be chosen for further analysis. The plants and compounds tested were fractionated birch bark extract, Bogbean and Yarrow extracts, the purified compound Annotine, and Ths-2, a beta glucan from the lichen *Thamnolia vermicularis*.

1 Birch bark

Birch (IS: Birki or Ilmbjörk), also known as Downy birch or White birch refers to *Betula pubescens* (Figure B 1), a broad leaf deciduous tree common in Iceland and Northern Europe as well as Greenland and Northern Asia. It is the most common tree-type in Iceland. When found in windy and harsh areas it can grow up to 2-3 meters high, but in areas of a kinder climate it may reach up to 10 meters.



Figure B 1. *Betula pubescens* or Birch (IS: Birki or Ilmbjörk).

As seen in the picture, the leaves are ovate-acute, 2-5 cm long and 1.5-4.5 cm wide with a serrated margin. Image by Hörður Kristinsson taken at Arnarhóll in 1963,

Several different compounds isolated from *Betula pubescens* have been shown to have an effect on cellular responses, the most researched being the triterpenes Betulinic acid and Betulin (1). Dry extracts of birch bark have been shown to have anti-allergenic and anti-inflammatory activities (2) and

therefore it has been suggested that birch bark might influence immune responses. When tested in a dendritic cell model (described in (3)), ethanol extract of birch bark and two fractions had an anti-inflammatory effect by reducing the amount of secreted pro-inflammatory cytokines (4).

The birch bark fraction III was tested on both IFN- γ pre-treated THP-1 monocytes (Figure B 2) and PMA differentiated macrophage-like THP-1 cells (Figure B 3).

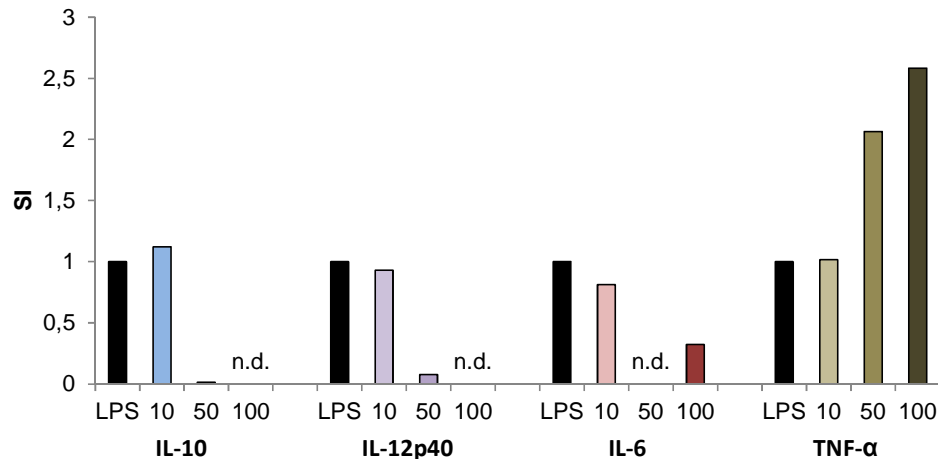


Figure B 2. The effect of fraction III from ethanol birch bark extract on THP-1 monocyte cytokine secretion.

THP-1 monocytes were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 100 U/ml IFN- γ for 3 hours before stimulation with 0.5 μ g/ml LPS with or without fractionated birch bark ethanol extract at concentrations of 10, 50 or 100 μ g/ml for 48 hours. Bars represent a single experiment. Cytokines were measured by ELISA. "N.d."= not detectable.

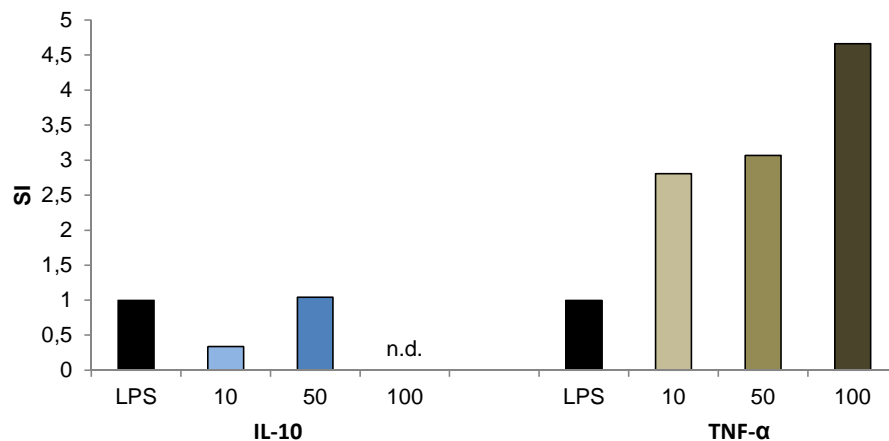


Figure B 3. The effect of fraction III from ethanol birch bark extract on PMA differentiated macrophage-like THP-1 cell cytokine secretion.

THP-1 monocytes were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 0.2 μ g/ml PMA for 48 hours before stimulation with 0.5 μ g/ml LPS with or without fraction III from ethanol birch bark extract for 48 hours. Cytokines were measured by ELISA. Bars represent the secretion index of a single experiment. "N.d."= Not detectable.

Treatment of PMA differentiated THP-1 cells with fraction III of the ethanol extract from birch bark resulted in the complete reduction of IL-10 secretion in the highest concentration tested (Figure B 3, blue bars). TNF- α secretion was however increased in a dose-dependent manner, reaching 4.5 times

the secretion of an untreated control with 100 µg/ml concentrations of the fraction. No IL-12p40 or IL-6 was detected.

The birch bark fraction notably reduced the amount of IL-10 and IL-6 secretion in both the stimulation methods tested (Figure B 2 and Figure B 3) and IL-12p40 secretion when pre-treated with IFN- γ . Most notably it completely abolished the IL-6 secretion from the PMA differentiated macrophage-like THP-1 cells in all the concentrations tested. This effect coincides with the results seen in (4) when the same fraction was tested in the dendritic cell model. Any speculation on cell death (which was not seen in microscopic observations) or reduced cellular function can be eased by the largely increased TNF- α secretion seen in the same samples, indicating that these cells were in fact alive and capable of cytokine secretion.

Although these measurements were not repeated, they are in accordance with results obtained using the dendritic cell model, underlining the need for further testing of this fraction as an immunomodulatory substance.

2 Bogbean and Yarrow

Bogbean (Figure B 4, IS: Horblaðka or Reiðingsgras) also known as buckbean or *Menyanthes trifoliata* (trifoliata or trifoliate meaning to have three leaflets) grows in bogs, marshes and shallow water throughout Europe and most of North America. The medicinal use of bogbean ranges from using it as a remedy for scurvy, constipation or rheumatism (5-7) to a flavoring additive in beer making.



Figure B 4. Bogbean/bucbean, *Menyanthes trifoliata* (IS: Horblaðka or Reiðingsgras).

Image by Hörður Kristinsson taken in Kaupangsmýri in Eyjafjörður in 1991,

The phytochemistry of bogbean and the activity of its components on specific cell types or promised effects are rarely studied. When tested in an *in vitro* dendritic cell model (as previously mentioned) aqueous extracts of bogbean had general anti-inflammatory effects (unpublished results from Guðbjörg Jónsdóttir as part of her masters study) by lowering the dendritic cell IL-12/IL-10 cytokine secretion ratio. Some bogbean extracts have been tested in the past for immunomodulatory effects with contradicting results (6, 7).

Yarrow (IS: Vallhumall) is also known as milfoil or *Achillea millefolium* L. (Figure B 5). Both its flowers and leaves have been used in traditional medicine as a herbal tea or tincture to lower blood pressure and to treat arthritis, as well as externally to facilitate wound healing (5).



Figure B 5. Yarrow or *Achillea millefolium* L. (IS: Vallhumall)

Both the flowers and leaves have been used as herbal remedies in Iceland. Image taken by Hörður Kristinsson on August 18th 2009.

In vivo anti-inflammatory effect of yarrow has been observed in various animal models as well as a diuretic effect (8). When aqueous extract of yarrow was tested in an *in vitro* dendritic cell model it was shown to possess an anti-inflammatory effect (unpublished results by Guðbjörg Jónsdóttir as part of her M.Sc. project) in a similar manner as the aqueous extract of bogbean.

Two types of extracts from both bogbean and yarrow were made by Guðbjörg Jónsdóttir as part of her M.Sc. project, an ethanol extract and an aqueous extract. The bogbean was collected in Mýrarkotsland, Grímsnes, Iceland in June 2008. The yarrow was collected at Hvanneyri in July 2008. An ethanol extract was made by the same method as the birch bark ethanol extract (see above) but only the bogbean ethanol extract was used in this study. Before use the ethanol extract was dissolved in 10% DMSO and 90% RPMI 1640 culture medium. Aqueous extracts were prepared by decoction (85°C, 2 hours). The warm extract was then filtered and lyophilized. The aqueous extracts were dissolved in RPMI 1640 culture medium for one hour at RT before use.

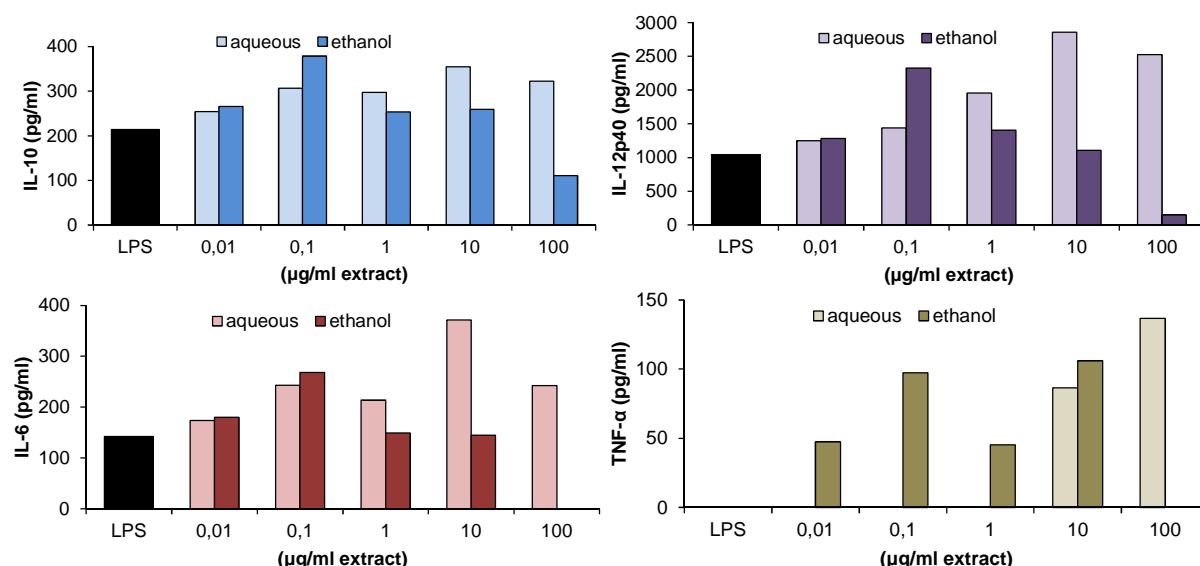


Figure B 6. The effect of bogbean extract on THP-1 monocyte cytokine secretion.

THP-1 monocytes were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 100 U/ml IFN- γ for 3 hours before stimulation with 0.5 μ g/ml LPS with or without aqueous/ethanol bogbean extract in the total concentration of 0.01 to 100 μ g/ml for 48 hours. Bars represent one experiment. Cytokines were measured by ELISA.

Both ethanol and aqueous extracts of bogbean were tested but only the aqueous yarrow extract. The extracts were tested on both IFN- γ pre-treated THP-1 monocytes (Figure B 6) and PMA differentiated macrophage-like THP-1 cells (Figure B 7).

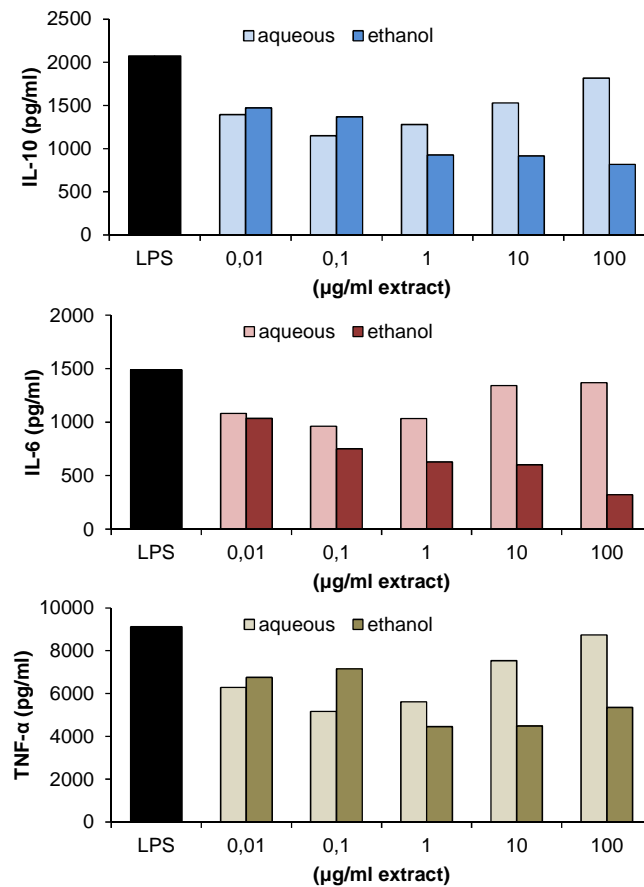


Figure B 7. The effect of bogbean extracts on PMA differentiated macrophage-like THP-1 cell cytokine secretion.

THP-1 monocytes were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 0.2 µg/ml PMA for 3 hours before stimulation with 0.5 µg/ml lipopolysaccharide with or without aqueous/ethanol bogbean extract in concentrations 0.01 to 100 µg/ml. Bars represent a single experiment. Cytokines were measured by ELISA.

In both of the stimulation methods tested (PMA differentiation and IFN- γ pre-treatment) the ethanol bogbean extract had a notably more prominent effect on the cytokine secretion (Figure B 6 and Figure B 7), completely abolishing the IL-6 secretion in the highest concentration tested (100 µg/ml) on the IFN- γ treated cells.

The IFN- γ treated, LPS stimulated monocytes secreted more IL-10, IL-12p40 and IL-6 when treated with the aqueous extract than monocytes stimulated without extracts. This effect was reversed when monocytes stimulated the same way were treated with the ethanol extract. This difference may be reflecting the difference in the compounds extracted by the different extraction methods. Although only preliminary results, it is interesting that this is the opposite of the effect seen in the dendritic cell model previously mentioned, where the ethanol extract had little effect on cytokine secretion but a clear anti-inflammatory effect was seen with the aqueous extract.

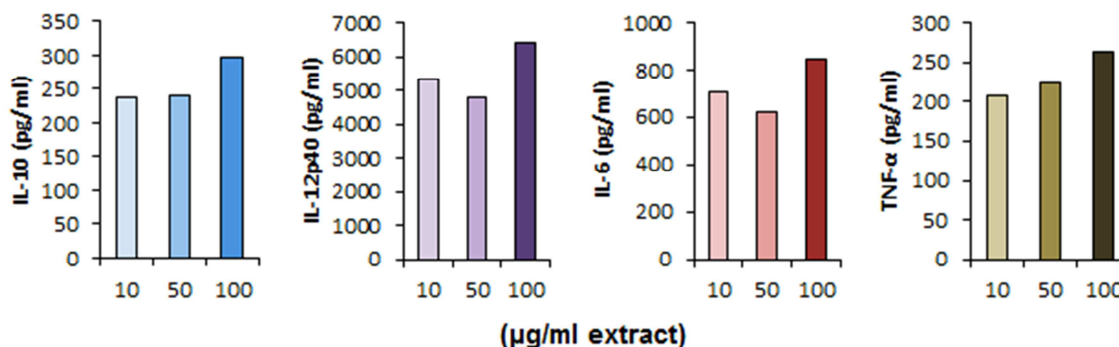


Figure B 8. The effect of an aqueous yarrow extract on THP-1 monocyte cytokine secretion.

THP-1 cells were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 100 U/ml IFN- γ for 3 hours before stimulation with 0.75 μ g/ml LPS with or without the aqueous yarrow extract in three concentrations (10, 50 and 100 μ g/ml) for 48 hours. The supernatants were then collected and frozen until measured by ELISA. The bars represent data from a single experiment. There was no usable control for this experiment.

The three concentrations tested of the aqueous yarrow extract (Figure B 8) had a similar effect on the cytokine secretion, but comparison with a control was not possible as the control sample got ruined. Given that this was only tested once, and rather unsuccessfully, further speculations must wait for further testing.

3 Lycopodium annotinum

Lycopodium annotinum (IS: Lyngjafni, Figure B 9) is also known as stiff clubmoss and it forms spores which has been used as a laxative, to assist wound healing and as a diuretic (5).



Figure B 9. *Lycopodium annotinum* (IS: Lyngjafni).

Image captured in Fljótavík in Hornstrandir in 1982 by Hörður Kristinnsson.

Lycopodium annotinum is a source of lycopodium alkaloids, a group of compounds which have been a popular research subject because some of these alkaloids are acetylcholinesterase inhibitors and might therefore become valuable substances to delay the symptoms of Alzheimer's disease (9). Annotine, the alkaloid tested in this project, has been tested for acetylcholinesterase inhibitor activity

and found to be a poor inhibitor of the enzyme (10). Annotine has also been tested in the previously mentioned *in vitro* dendritic cell model as part of a masters project in the Faculty of Pharmaceutical Sciences in 2009 (Ingibjörg Sigurðardóttir, unpublished) and found to influence the dendritic cell cytokine secretion, favoring the pro-inflammatory cytokine IL-12p40 at the expense of IL-10 and thus possibly steering a subsequent T cell activation towards a T_H1 response.

The *Lycopodium annotinum* L. was collected and isolated as described in (10). Annotine was only tested on IFN- γ pre-treated THP-1 monocytes.

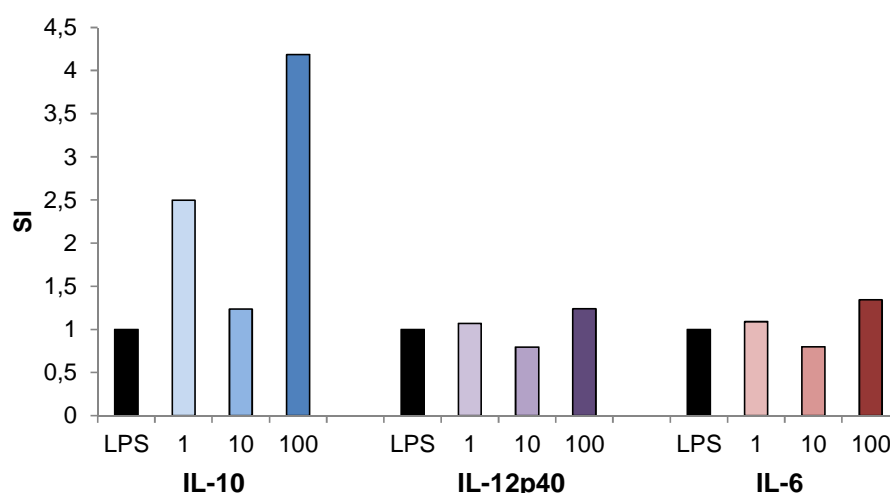


Figure B 10. The effect of Annotine from *Lycopodium annotinum* on THP-1 monocyte cytokine secretion.

THP-1 cells were seeded in a 48 well culture plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 100 U/ml IFN- γ for 3 hours before stimulation with 0.75 μ g/ml LPS with or without Annotine in three concentrations (1 to 100 μ g/ml) for 48 hours. The supernatants were then collected and frozen until measured via ELISA. The bars represent data from two different experiments (n=2) presented as secretion index (SI).

The effect of Annotine on IFN- γ and LPS treated THP-1 monocytes was tested in two different experiments. It had little effect on IL-12p40 and IL-6 secretion (Figure B 10), however a notable increase was seen in IL-10 secretion in one of the two experiments. Since no further testing of this compound was done, it was impossible to exclude either measurement, thus this difference in IL-10 secretion remains under question. However as previously mentioned, Annotine has been tested in the dendritic cell model where it was found to have the opposite effect, increasing the IL-12p40 secretion while reducing the IL-10 secretion.

4 *Thamnia vermicularis*

Thamnia vermicularis (Sw.) Schaer. var. *subuliformis* (Ehrh.) Schaer. (IS: Ormagrös, Figure B 11) or Whiteworm lichen has traditionally been used in teas in China. Polysaccharides from *Thamnia vermicularis* have been shown to affect immune functions by affecting cytokine secretion from different cell types (11). The most abundant polysaccharide of this lichen is the β -glucan Ths-2 used in this study.



Figure B 11. *Thamnolia vermicularis* (IS: Ormagrös).

Image taken by Hörður Kristinsson in Skaftafell in August 1990.

Thamnolia vermicularis was collected and the polysaccharide Ths-2 was isolated and structurally determined as described in (12).

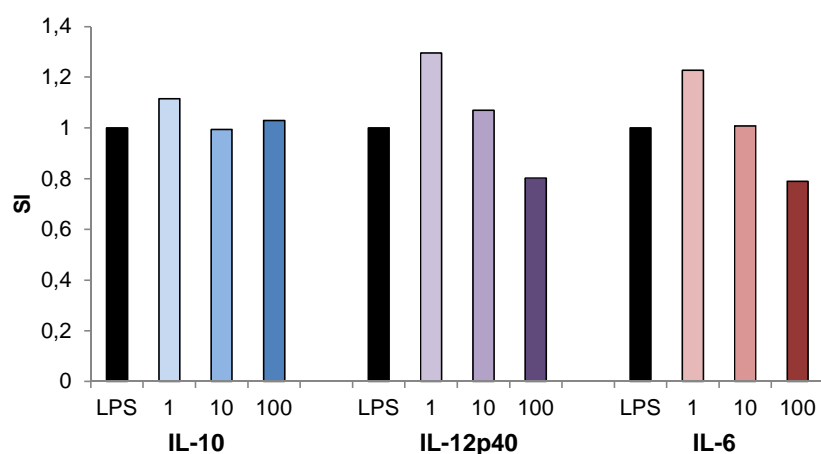


Figure B 12. The effect of Ths-2, a polysaccharide purified from *Thamnolia vermicularis* on THP-1 monocyte cytokine secretion.

THP-1 cells were seeded in a 48 well plate (5×10^5 cells/ml, 1 ml/well) and pre-treated with 100 U/ml IFN- γ for 3 hours before stimulation with 0.75 μ g/ml LPS with or without Ths-2 in three concentrations (1 to 100 μ g/ml) for 48 hours. The supernatants were then frozen until measured via ELISA. The bars represent data from two different experiments ($n=2$) presented as secretion index (SI). Black bars represent the control (IFN- γ for 3 hours, LPS for 48 hours without the polysaccharide).

Ths-2 had little effect on the IL-10 cytokine secretion of the IFN- γ and LPS stimulated monocytes compared with control (Figure B 12). There was, however, a slight dose-dependent decrease in the secretion of the pro-inflammatory cytokines IL-6 and IL-12p40, but further testing will be necessary for confirmation of that.

The preparation of the natural compounds or extracts was not part of this project. The compounds and extracts used were kindly provided by Elín S. Ólafsdóttir and Sesselja Ómarsdóttir and their students at the Faculty of Pharmaceutical Sciences and by Jóna Freysdóttir and Ingibjörg Harðardóttir and their students at the Faculty of Medicine.

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