UNIVERSITY OF COPENHAGEN
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## Ph.D. Thesis

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# The impact of bark extracts from Norway spruce and Scots pine on gastrointestinal parasites in ruminants

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Cover Photo: Spruce forest: Photographer Morten Gåsvand
*T. circumcincta* and lamb: Berit M. Blomstrand

# Preface

The work presented in this Ph.D. thesis has been conducted from 2017 to 2022. While conducting the work, I was enrolled as a Ph.D. student at the Department of Veterinary and Animal Sciences, University of Copenhagen, in the graduate programme of Molecular Bacteriology and Infection, and I was employed at the Norwegian Centre for Organic Agriculture in Tingvoll, Norway (NORSØK). The work was conducted at NORSØK, The Norwegian Veterinary Institute in Oslo (NVI), and at Scotland's Rural College (SRUC). Stig Milan Thamsborg from the Section of Parasitology and Aquatic Pathobiology has been the main supervisor, with Håvard Steinshamn (Norwegian Institute of Bioeconomy Research) as the principal co-supervisor and Heidi L. Enemark (NVI), and Spiridoula Athanasiadou (SRUC) as co-supervisors. Additional assistance was received from Kristin Marie Sørheim (NORSØK), Ian D. Woolsey (NVI), and Sokratis Ptochos (SRUC).

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This Ph.D. thesis consists of introduction, background, methodology, summary of results and discussion of each publication and manuscript, a general discussion, and conclusions and perspectives. The following publications and manuscripts are included:

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# Acknowledgements

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# Summary

Gastrointestinal parasite infections cause a decrease in animal health and welfare, productivity, and farm profitability worldwide. The development of parasite resistance towards current antiparasitic pharmaceuticals stresses the importance of finding alternative approaches to reducing the impact of parasitism on the hosts. Exploring the antiparasitic traits of bioactive plants, i.e. a plant which has the ability to interact with compounds of the living tissue of animals by presenting a wide range of probable effects, may be a way to contribute to this. Coniferous bark is rich on condensed tannins and other plant secondary metabolites (PSM). Norway has a large forestry industry with bark as an under-exploited by-product. The overall aim of this project was to explore the antiparasitic effects of bark extracts from Norway spruce and Scots pine harvested in Norway as a novel bioactive agent against parasites in ruminants. Bark from Norway spruce and Scots pine was harvested at two seasons (winter and summer) at processing plants using two different debarking methods (ring and drum debarking). The bark was extracted with three different solvents (acetone, methanol, and water), which produced 18 different bark extracts.

In **Paper I**, we investigated the ability of the bark extracts to inhibit the development of *Cryptosporidium parvum* in HCT-8 cell cultures. We found that pine bark extracts extracted with methanol and acetone as solvents inhibited *C. parvum* development in a sigmoid, dose-dependent manner. We did not see any parasite inhibition of the water extracts at the tested concentrations.

To assess the anthelmintic activity of spruce and pine bark, 18 different extracts were tested against two important gastrointestinal nematodes in sheep (**Paper II**). The extracts were tested on the abomasal parasite *Teladorsagia circumcincta* and the intestinal parasite *Trichostrongylus colubriformis* at two life stages (egg hatching and 3$^{rd}$ stage larvae). For the egg hatch assay (EHA), the half maximum inhibitory concentration ($IC_{50}$) was estimated for all extracts. For the larval motility assay, the motility of L3 treated with bark extract was measured with a real time cell analyser and compared to the motility of the dead larvae. The bark extracts were analysed for the content of condensed tannins (CT) as well as for other possible antiparasitic compounds. Putative annotations of identified masses were procured, and the abundance of CT and of the masses were correlated with the EHA $IC_{50}$. We found that several of the tested extracts inhibited the egg hatching up to 100%. The intestinal parasite was more susceptible to treatment compared to the abomasal parasite, pine bark, winter bark, and bark extracted with organic solvents (acetone or methanol) inhibited the parasites to a larger extent compared to spruce bark, summer

bark, and water extracts. CT was negatively correlated to EHA $IC_{50}$ for *T. colubriformis* but not for *T. circumcincta*, and several putative annotations were obtained, suggesting the identity of novel antiparasitic masses.

In **Paper III we** explored the anticoccidial properties of water extracted spruce bark. Young pre-ruminant lambs infected with ovine *Eimeria* spp. were treated with spruce bark extract. The bark extract treated lambs exhibited a lower *Eimeria* oocyst count and lower rate and severity of diarrhoea compared to the untreated lambs. Simultaneously, the treated lambs experienced detrimental effects like reduced milk intake with a subsequent lower growth during the treatment period, which subsided after discontinuing the treatment.

When assessing acetone extracted pine bark harvested during the winter using *Heligmosomoides bakeri* infected mice as a model (**Paper IV**), we found no significant effects in parasitological parameters, though the bark extract treatment increased the body weight and carcass weight and reduced the feed intake-bodyweight ratio in the most susceptible group only: the infected slow responder mice (C57BL/6). Furthermore, the treatment reduced the tolerance to the parasite of the more resistant, fast responder mice (BALB/c). These results indicate that the host responses to PSM may be sensitive to variation in the genetic susceptibility to the parasite.

In conclusion, the results obtained in this project have provided new and essential knowledge to the antiparasitic efficacy of bark extracts from *P. abies* and *P. sylvestris* against a wide range of gastrointestinal parasite infections in various host species. There was a negative correlation between CT content of the extracts and EHA $IC_{50}$ of *T. colubriformis*, and several novel compounds with a significant negative correlation with EHA $IC_{50}$ were putatively annotated. However, investigations of the chemical compounds in the bark extracts responsible for the antiparasitic effects has only begun. Thus, it is of importance to gain further knowledge into the identification of the actual antiparasitic compounds, their concentrations, and their effect against various parasites, alone or synergistically.

# Sammendrag (Norwegian summary)

Infeksjoner med gastrointestinale parasitter forårsaker reduksjon i dyrehelse og dyrevelferd samt redusert produktivitet og lønnsomhet over hele verden. Den pågående utviklingen hos parasitter av resistens mot antiparasittære legemidler understreker viktigheten av å finne alternative tilnærminger for å redusere følgene av parasittisme på vertsdyret. Å utforske de antiparasittære egenskapene til bioaktive planter, en plante som har evne til å samhandle med komponenter i det levende animalske vevet ved å presentere et spekter av sannsynlige effekter, kan være en måte å bidra til dette. Bark fra bartrær er rik på kondenserte tanniner og andre sekundære plantemetabolitter (PSM). Norge har en stor skogindustri hvor furu- og granbark er et underutnyttet biprodukt. Det overordnede målet med dette prosjektet var å utforske de antiparasittære egenskapene til barkekstrakter fra gran (*Picea abies*) og furu (*Pinus sylvestris*) høstet i Norge, som et nytt bioaktivt middel mot parasitter hos drøvtyggere. Bark fra gran og furu ble høstet ved to sesonger (vinter og sommer) ved prosessanlegg som brukte to ulike avbarkingsmetoder (ring- og trommelavbarking). Barken ble ekstrahert med tre forskjellige løsemidler (aceton, metanol og vann), og totalt ga dette oss 18 forskjellige barkekstrakter.

I **Artikkel I** undersøkte vi barkekstraktenes evne til å hemme utviklingen av *Cryptosporidium parvum* i HCT-8 cellekulturer. Vi fant at furubarkekstrakter ekstrahert med metanol og aceton hemmet utviklingen av *C. parvum* på en sigmoid, doseavhengig måte. Vannekstraktene hemmet ikke utviklingen av parasitter i de testede konsentrasjonene.

For å vurdere den anthelmintiske aktiviteten til gran- og furubark, ble 18 forskjellige ekstrakter testet mot to viktige gastrointestinale nematoder hos sau (**Artikkel II**). Ekstraktene ble testet på løpenematoden *Teladorsagia circumcincta* og tynntarmsnematoden *Trichostrongylus colubriformis*, i to livsstadier: egg og 3. stadiums, infektive larver (L3). Under eggklekkingstesten (EHA) ble det for alle ekstraktene fastslått den konsentrasjonen som er nødvendig for å redusere parasittnivået til det halve ($IC_{50}$). I L3 motilitetsanalysen (LMA) ble bevegeligheten til barkekstraktbehandlede larver målt med en sanntidscelleanalysator og sammenlignet med motiliteten til kontrollen (døde larver). Barkekstraktene ble analysert for kondenserte tanniner (CT) samt andre komponenter. Resultatene ble interpretert og de oppdagede massene ble forsøkt identifisert ved automatisk og manuell sammenlikning av massevekt i Metlin PLCL-databasen. Til slutt ble komponenter med høy tilstedeværelse i ekstraktene korrelert med EHA $IC_{50.}$ Vi fant at flere av de testede ekstraktene hemmet eggklekkingen med opptil 100%. Tarmparasitten var mer mottakelig for behandling sammenlignet med løpeparasitten, og furubark, bark høstet i løpet av vinteren, og bark ekstrahert

med organiske løsemidler (aceton eller metanol) hemmet parasittene i større grad sammenlignet med granbark, bark høstet i løpet av sommeren og vannekstrakter. CT kunne korreleres til EHA $IC_{50}$ til *T. colubriformis* men ikke til *T. circumcincta*, og ble identiteten til nye komponenter med antiparasittær effekt ble antydet.

I **Artikkel III** utforsket vi de koksidiostatiske egenskapene til vannekstrahert granbark for å redusere koksidiebelastningen i spedlam de første ukene på beite. Unge melkefôrede lam infisert med et feltisolat av *Eimeria* spp. ble behandlet med granbarkekstrakt daglig i 11 dager. De behandlede lammene hadde lavere oocystetall i avføringen og lavere frekvens og alvorlighetsgrad av diaré sammenliknet med lammene som ikke ble behandlet. Samtidig så vi at de behandlede lammene fikk redusert melkeinntak med påfølgende lavere tilvekst under behandlingsperioden, noe som gikk over da behandlingen ble seponert.

Den anthelmintiske effekten av acetonekstrahert furubark høstet om vinteren ble vurdert ved bruk av *Heligmosomoides bakeri*-infiserte mus som modell (**Artikkel IV**). Vi så ingen signifikant parasittreduserende effekt. Likevel så vi at behandlingen økte kroppsvekten og skrottvekten, og bedret fôrutnyttelsen i den mest utsatte gruppen: infiserte mus som bare langsomt utviklet immunitet mot parasitten (C57BL/6). Videre reduserte behandlingen toleransen mot parasitten til de mer resistente musene (BALB/c). Disse resultatene indikerer at vertens respons på sekundære plantemetabolitter kan være følsom for variasjon i vertens genetiske mottakelighet for parasitten.

Samlet sett har resultatene fra dette prosjektet gitt ny og essensiell kunnskap om den antiparasittære effekten av barkekstrakter fra *P. abies og P. sylvestris* mot et bredt spekter av gastrointestinale parasitter i ulike vertsdyr. Vi kunne demonstrere en negativ korrelasjon mellom CT-innholdet i ekstraktene og EHA $IC_{50}$ til *T. colubriformis*, og flere nye komponenter med en signifikant negativ korrelasjon med EHA IC50 ble identifisert. Imidlertid har undersøkelser av de kjemiske komponentene i barkekstraktene som er ansvarlige for de antiparasittære effektene bare akkurat begynt. Det er derfor viktig å få ytterligere kunnskap om identifisering av de faktiske antiparasittære komponentene, deres konsentrasjoner og deres effekt mot ulike parasitter, alene eller synergistisk.

# Abbreviations

| | |
|---|---|
| Ac | Acetone |
| AH | Anthelmintic drugs |
| ANOVA | Analysis of variance |
| AR | Anthelmintic resistance |
| BALB/c | Fast responding mouse line |
| BW | Body weight |
| C57BL/6 | Slow responding mouse line |
| Cq | Quantification cycle |
| CT | Condensed tannins |
| CW | Carcass weight |
| DAD | Diode array detector |
| dH2O | Distilled water |
| DM | Dry matter |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DP | Degree of polymerisation |
| EHA | Egg hatch assay |
| EIC | Eggs in colon |
| EU | European Union |
| FCS | Faecal consistency score |
| FEC | Faecal egg count |
| FECRT | Faecal egg count reduction test |
| FI | Feed intake |
| FOC | Faecal oocyst count |
| FOCRT | Faecal oocyst count reduction test |
| GIA | Growth inhibition assay |
| GIN | Gastrointestinal nematodes |
| H2O | Water |
| HCT-8 | Human ileocecal colorectal adenocarcinoma cells |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IFAT | Indirect Fluorescent Antibody Technique |
| IF | Immune fluorescence |
| IIA | Invasion inhibition assay |
| L1 | First stage larvae |
| L3 | Third (infectious) stage larvae |
| LC | Liquid chromatography |
| LMA | Larval motility assay |
| MB | Master batch |
| mBW | Metabolic body weight ($BW^{0.75}$) |
| mDP | Mean degree of polymerisation |
| Me | Methanol |
| MS | Mass spectrometry |
| NEP | Non-extractible phenolics |
| PA | *Picea abies* |
| PBS | Phosphate buffered saline |
| PC | Procyanidins |
| PD | Prodelphinidins |
| PS | *Pinus sylvestris* |
| PSM | Plant secondary metabolites |
| PVPP | Polyvinyl polypyrrolidone |
| qPCR | Quantitative real-time polymerase chain reaction |
| qTOF | Quadruple time-of-flight |
| RTCA | Real time cell analyser |
| S | Summer |
| SEM | Standard error of the mean |
| SI | Small intestines |
| SL | Sesquiterpene lactones |
| spp. | Species |
| TWC | Total worm count |
| UV | Ultraviolet |
| W | Winter |
| WST-1 | Water-soluble tetrazolium salt assay |

# 1. Introduction and objectives

## 1.1. Introduction

Gastrointestinal parasites are major pathogens of livestock worldwide, causing reduced animal health and welfare as well as having a negative impact on productivity, and consequently on the farmers' economy. Parasites such as gastrointestinal nematodes (GIN, responsible for parasitic gastroenteritis) and protozoa like *Eimeria* (responsible for coccidiosis) and *Cryptosporidium parvum* (responsible for cryptosporidiosis), have a high prevalence in ruminants (Bangoura et al., 2012, Chartier and Paraud, 2012, Rinaldi et al., 2015). Coccidiosis leads to high economic losses in the livestock industry (Alzieu et al., 1999, Lassen and Østergaard, 2012), and helminth infections, including GIN infections, in ruminants are estimated to cause an annual financial cost of € 1.0-2.7 billion (Charlier et al., 2020). In Norway, both GIN infections and coccidiosis are common diseases of livestock, and Cryptosporidiosis is generally believed to be underdiagnosed (Helle, 1971; Nygård et al., 2003). Cryptosporidiosis is mainly a disease of calves, but it can also cause diarrhoea and reduced growth in sheep and goats (Guo et al., 2021).

Today, the most common method for controlling gastrointestinal parasites in livestock is using antiparasitic pharmaceuticals (Molento, 2009; Domke et al., 2011; Odden et al., 2017). Unfortunately, resistance to such drugs is commonly reported, with examples of resistance reported for GIN and coccidia in particular, around the world (Domke et al., 2012; Odden et al., 2018a; Vineer et al., 2020). Due to this worldwide setback, there is need for alternative options in the control of parasites. Amongst the possibilities currently under consideration are plant secondary metabolites (PSM) that have been thoroughly researched *in vitro* and *in vivo* for their antiparasitic properties (Anthony et al., 2005; Hoste et al., 2015; Spiegler et al., 2017). Leguminous forages rich in condensed tannins (CT) have been shown to reduce the GIN burden in ruminants, but such forages have limited cultivation potential in Scandinavia (Desrues et al., 2016a, Novobilsky et al., 2011). In contrast, bark from conifer tree species is readily available in colder climates and contain high levels of PSM such as CT. Conifers are trees of the division *Pinophyta* (Coniferae) that consist of more than 600 species out of which only four genera are naturally found in Norway, namely Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*), juniper (*Juniperus communis*), and common yew (*Taxus baccata*) (Farjon, 2018). These evergreen gymnosperms have characteristic needle shaped leaves and most of them keep their foliage year-round. Norway has a thriving forestry industry with a production volume of 7.21 million $m^3$ of wood logs from Norway spruce and Scots pine, equivalent to 721.000 $m^3$ of wet

bark (Prosess21, 2020). Today, bark is mainly combusted for energy production or used as garden mulch, and there is a high incentive from the sawmill and paper pulp industry to find new practices to better utilise the hitherto unexploited biproduct.

Bark powder or extracts from pine and spruce have been tested *in vitro* and found active against various GIN. For instance, purified pine bark extracts inhibited egg hatching, feeding, development, or larval and adult motility in important GIN species in ruminants in various *in vitro* tests (Molan, 2014, Quijada et al., 2015, Desrues et al., 2016b). Athanasiadou et al. (2021) demonstrated that bark extracts from Norway spruce and Scots pine have antiparasitic properties against *Teladorsagia circumcincta in vitro*. From these studies it is evident that the chemical composition of the bark extract depends on the tree species, the age of the tree, and the solvent used in the extraction process and further, that the variation in the chemical composition of bark results in variation in antiparasitic efficacy. The i*n vivo* evidence is scarce; Min et al. (2015) showed that goats infected with *H. contortus* had 30% lower faecal egg counts when fed ground *P. taeda* bark compared to the non-supplemented controls.

Bark powder or extracts are also tested against coccidia. Molan et al. (2009) found that water extracts from *P. radiata* bark suppressed the sporulation of *Eimeria* spp. oocysts *in vitro*. Hur et al. (2005) concluded that the consumption of a feed containing pine (*P. densiflora*) needles, oak (*Quercus acutissima*) leaves, and lucerne (*Medicago sativa*) chaff resulted in lower oocyst output in goats naturally infected with *Eimeria* spp. Kim and Healey (2001) found that mice fed bark extract from *P. pinaster* (Pycnogenol®) had a reduced *C. parvum* oocyst count compared to the untreated control. Mice infected with GIN or protozoa are often used as model hosts to test the antiparasitic properties of plant extracts; they are regularly the hosts of choice due to various resource limitations (e.g., costs, availability of extracts in large amounts, availability of parasites, etc.) (Wahid et al., 1989; Behnke et al., 2008).

The intestinal roundworm of the mouse is phylogenetically closely related to common and important GIN in ruminants, it has a similar life cycle. It is responsible for chronic infections in mice, which greatly imitate helminth infections in livestock (Behnke et al., 2009). Host protective immunity to this nematode is mediated by the Th2 cytokine response, similarly to livestock GIN, and for these reasons *H. bakeri* is extensively studied and frequently used as a model organism for GIN infections in other host species (Behnke et al., 2009). The *H. bakeri* infected mouse is a good model to investigate the antiparasitic properties of plant extracts. Nevertheless, the monogastric digestive system of the mouse is very different from that of the ruminants. Therefore it is important to acknowledge that results derived from mouse experiments

cannot be directly extrapolated to other host-parasite systems and should be validated with targeted studies in livestock.

In the work leading to this thesis we wanted to explore the antiparasitic properties of bark extracts from Nordic coniferous trees. We aimed to assess the bark extracts against coccidia and GIN *in vitro* and *in vivo*, both common parasite types in ruminants. The first description of an *in vitro* culture system for *Eimeria ovinoidalis* macromeront formation was described by Carrau et al. (2016). Due to difficulties establishing this novel method at our laboratory, we were forced to deviate from this plan and decided to use the protozoan parasite *C. parvum* in a cell culture as the method of choice, an *in vitro* method already established at the premises. Furthermore, we planned to assess selected bark extracts against GIN in lambs. Since we failed to find a production facility able to produce large enough amounts of bark extract for animals of that size (approximately 35 kg BW), we had to go for smaller host animals, and a mouse model was chosen.

We hypothesised that bark extracts will be active against gastrointestinal parasites and that variation in the bark extract composition would have an impact on the antiparasitic activity measured. We aimed to do this by first assessing the direct antiparasitic activity of bark extracts *in vitro* to then, based upon results from the *in vitro* trials, test selected bark extracts *in vivo*.

## 1.2. Objectives

Main objective:

The overall objective of this thesis was to assess the antiparasitic activity of bark extracts from Norway spruce and Scots pine against selected gastrointestinal parasites of ruminants and mice, applying the latter as a model organism for ruminants.

The specific objectives were:

-to determine whether bark extracts from Scots pine (*P. sylvestris*) exhibited anti-cryptosporidial properties against *C. parvum* by means of an *in vitro* growth inhibition test (**Paper I**)

- to assess the antiparasitic effect *in vitro* of bark extracts on selected ovine GIN and the variation in biological activity related to the bark extract constituents (**Paper II**)

-to assess the effect of a water extract of bark from Norway spruce (*Picea abies*) against *Eimeria* infections in pre-ruminant, milk-fed lambs (**Paper III**)

-to quantify the impact of pine bark extract administration on the resistance, performance, and tolerance of genetically diverse mice infected with *H. bakeri* (**Paper IV**)

## 1.3. Conflicts of interest

The author of this thesis has no conflicts of interest to declare. This synopsis was written with reference to the attached research articles and manuscripts.

The experimental work was conducted at the Norwegian Veterinary Institute, Oslo, Norway (**Paper I**), Norwegian Centre for Organic Agriculture, Tingvoll, Norway (**Paper III**), and at Scotland's Rural College, Edinburgh, UK (**Paper II** and **IV**).

# 2. Background

## 2.1. Gastrointestinal parasites in animals

Endoparasites are considered as organisms which live a portion of their lives inside another organism, the host. Parasites are dependent on the host and benefit by deriving nutrients at the host's expense (Taylor et al., 2016). They thereby cause harm to their hosts, although this is not always easy to demonstrate. Often parasites cause subclinical infections, but clinical disease may manifest itself in case of high infection pressure (Gunn and Irvine, 2003).
In this thesis we have assessed the biological activity of coniferous bark extracts against important parasites in ruminants.

### 2.1.1. Protozoan parasites in cattle and sheep: *Eimeria* spp.

Coccidiosis is a common intestinal disease of livestock caused by protozoa of the genus *Eimeria* belonging to the phylum *Apicomplexa*. *Eimeria* are monoxenous parasites, i.e. parasites restricted to a single host species, with high potential on causing diarrhoea, reduced growth, increased mortality, and consequently reduced animal welfare, in young animals in particular (Enemark et al., 2013, Chartier and Paraud, 2012).
There are 15 *Eimeria* species known to infect sheep, out of which only two are considered as major pathogens: *E. ovinoidalis* and *E. crandallis* (Joachim et al., 2018). In Norway, sheep are kept on pastures close to the farms the first weeks after release in spring. Most ewes and lambs are moved to mountain or forest pastures during the summer, with low stocking densities and infection pressure (10-80 animals per $km^2$) (Mysterud et al., 2001; Vatn, 2009; Domke et al., 2011). Thus, in Norway clinical coccidiosis in sheep is a disease of major importance in young lambs the first 2-3 weeks after release onto spring pasture (Helle, 1970; Vatn, 2009).
Fourteen *Eimeria* species are known to infect goats, with *E. ninakohlyakimovae* and *E. arloingi* as the most pathogen species. Coccidiosis in Norwegian goats is mainly a problem in housed kids (Gjerde, 2011). In cattle, more than 20 *Eimeria* spp. have been described, with particularly three species, *E. bovis, E. zuernii*, and *E. alabamensis* considered pathogenic (Daugschies and Najdrowski, 2005). Calves may develop eimeriosis (typically *E. alabamensis)* shortly after turn out to pasture (Svensson et al., 1994).
The transmission of *Eimeria* oocysts is via the faecal-oral route. After ingestion and subsequent exposure to mechanical and chemical action, such as trypsin, bile, and $CO_2$ (Jackson, 1962), the oocyst wall is broken down, which results in excystation and the release of sporozoites. The sporozoites invade enterocytes (Figure 1), where they undergo an asexual reproduction phase

(several merogony stages) and a sexual phase (gamogony), resulting in the production of zygotes and finally oocysts. The unsporulated oocysts are passed with the faeces and the oocysts sporulate outside the host within 1-10 days. The exact time needed for sporulation depends on several factors such as the species, temperature, oxygen level, and moisture (Eckert et al., 1995). Following *Eimeria* infection, ruminants (as well as other host species) generally develop a strong protective immune response (Catchpole et al., 1993, Taubert et al., 2008).

Clinical coccidiosis leads to high economic losses, both directly due to increased mortality and treatment costs, but also indirectly manifested as delayed fertility due to reduced weight gain and retarded development (Alzieu et al., 1999, Svensson et al., 1994). A study from Estonia showed that *Eimeria* infections in cattle were estimated to incur an annual loss of 8-9% of gross margin (Lassen and Østergaard, 2012).

A Norwegian survey found that more than 80% of the farmers treated their lambs with anticoccidials, mainly without a laboratory-based diagnosis as only 12.3% of the farmers submitted faeces for a laboratory analysis (Odden et al., 2017). Furthermore, as many as 37.9% of the farmers reported clinical signs continuing after treatment with an anticoccidial drug, indicating a potential treatment failure.

Coccidiosis is mostly diagnosed by clinical signs as well as on coproscopic examination by flotation, e.g., by a modified McMaster technique (Eckert et al., 1995). In some host species, e.g., in cattle, *Eimeria* species can be differentiated by microscopic examination of the oocyst morphology. In other species, e.g., sheep, the microscopic species identification is more cumbersome, and polymerase chain reaction (PCR) may be preferred. The digital droplet PCR and next generation sequencing technologies are robust techniques in identifying and quantifying the *Eimeria* species (Snyder et al., 2021). Histological scoring can be used to confirm a tentative diagnosis, and lastly, serology can be performed, although seroconversion is observed rather late in the course of the infection, i.e. after the onset of patency or in the post patent period (Bangoura and Daugschies, 2018).

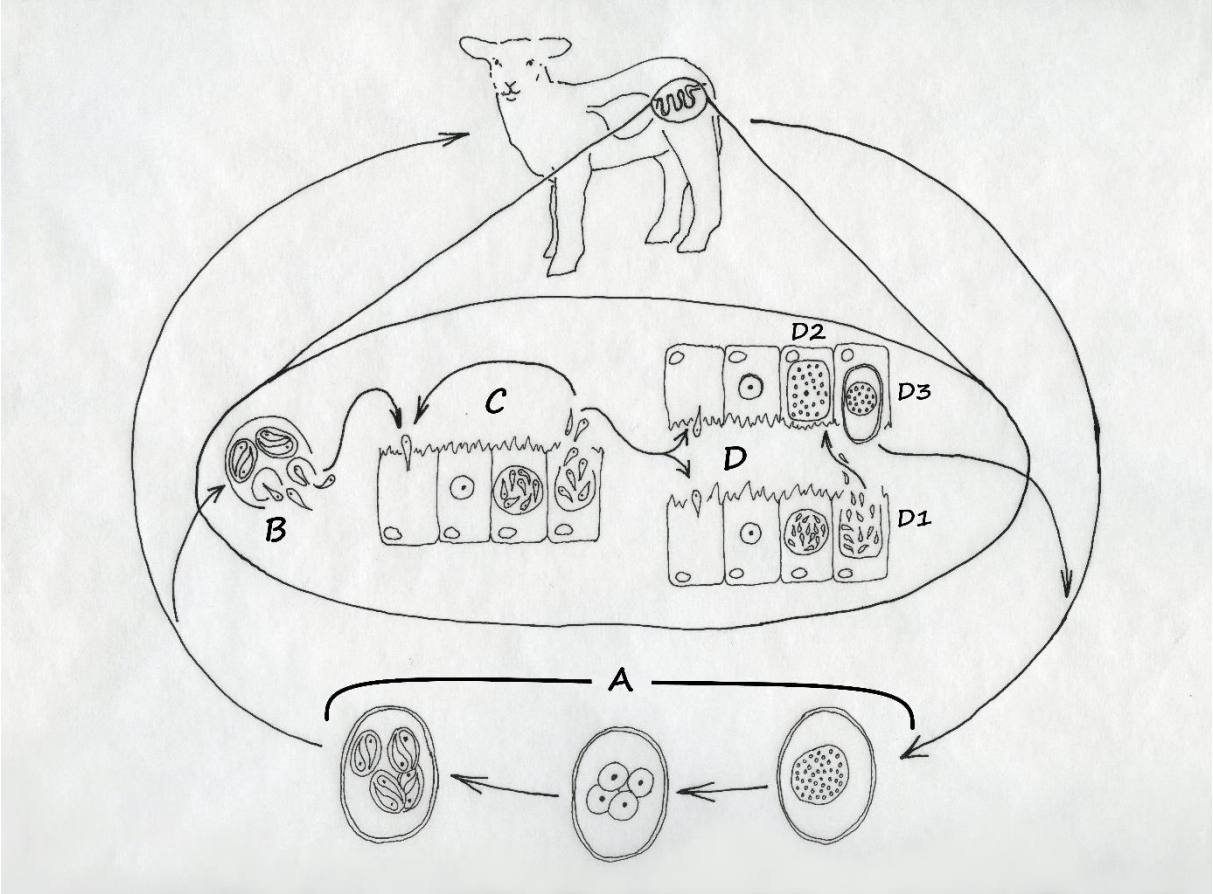


Figure 1: The life cycle of ovine *Eimeria* spp. A: Sporogony, sporulation of the oocysts, B: excystation, C: Merogony, the asexual reproduction with the development of merozoites and infection of new enterocytes, D: Gametogony, with formation of microgametes (D1), macrogametes (D2), zygote (D3), and finally new oocysts. (Anne de Boer)

#### 2.1.2. Protozoan parasites in cattle and sheep: *Cryptosporidium parvum*

*Cryptosporidium parvum* is a zoonotic, apicomplexan parasite with a worldwide distribution. The classification is being debated, but at present it has been designated the class *Gregarinomorphea* (Ryan et al., 2016). The parasite, which is transmitted via the faecal-oral route, has a broad host spectrum and can cause severe intestinal disease in calves at 1-4 weeks of age (Mosier and Oberst, 2006), with symptoms like abdominal pain, profuse, often intermittent diarrhoea, and in severe cases, death. Cryptosporidiosis in sheep and goats is also caused by *C. parvum*, but *C. ubiquitum*, and *C. xiaoi* may also play a role (Guo et al., 2021). There seems to be less marked age-associated distribution of *Cryptosporidium* spp. in sheep and goats, with *C. parvum* demonstrated in lambs and kids up to 12 weeks with the peak at 4-6 weeks of age. Jacobson et al. (2018) reported that *Cryptosporidium* infection in small ruminants was associated with diarrhoea and reduced growth both pre and post weaning.

Cryptosporidiosis in animals has been reported in Norway on a few occasions. Two surveys undertaken to determine the prevalence of *C. parvum* in dairy calves and in free ranging wild cervids in Norway revealed that the parasites are relatively widespread (Hamnes et al., 2006a, 2006b). In 2006, an outbreak of human cryptosporidiosis was attributed to contamination from calves (Robertson et al., 2006), and in 2014, an outbreak of *C. parvum* in school children was associated with *C. parvum* positive lambs and goat kids at a holiday farm in Norway (Lange, 2014).

*C. parvum* has a monoxenous, direct life cycle similar to that of *Eimeria* spp. (Figure 1). The sporulated oocysts containing four naked sporozoites (no sporocysts) are excreted from the host with faeces and are therefore, in contrast to *Eimeria* spp., directly infective. The process of excystation of sporozoites from oocysts normally requires exposure to stomach acids, bile salts, and pancreatic enzymes, although some sporozoites have the ability to excyst in warm aqueous solutions (Dubey et al., 1990). After excystation, the sporozoites attach themselves to the apical membrane of the enterocytes in ileum and jejunum, where they are enveloped in a parasitophorous, epicellular vacuole. The further developmental stages continue through several generations of merogony, an asexual multiplication stage where a vast amount of merozoites is released into the gut lumen and new enterocytes are infected. Merogony is followed by gametogony, which is the sexual stage producing diploid zygotes finally developing into oocysts which are released from the enterocytes. Thin-walled and thick-walled oocysts are produced, where the former release their sporozoites in the intestinal lumen, hence are responsible for autoinfection in the immunocompromised host, and the latter (the majority) are passed in the faeces (Taylor et al., 2016).

The oocysts of *C. parvum* are quite small (approximately 5×4 µm) and are difficult to identify in a coproscopic sample. The primary way of detecting oocysts in the faeces is with immunodetection, e.g., IFAT. Using fluorescein-labelled mouse monoclonal antibodies made to oocyst outer wall epitopes of *C. parvum* is a secure method of identifying *C. parvum* in faeces, but it also requires advanced microscopic equipment of high cost (Waterborne, 2022). Additionally, the fluorescein stain is light sensitive and might fade when exposed to light. Using real time, quantitative PCR (qPCR) to detect and quantify *C. parvum* DNA is a relatively new diagnostic method, providing faster and more reliable results.

#### 2.1.3. Important gastrointestinal nematodes in ruminants

The cost of nematode infections is high in all important livestock species. Charlier et al., 2020, calculated an annual cost of helminth infections to be € 3.5 million and 18.2 million in meat

sheep production and dairy cattle in Norway, respectively, including the production loss and the cost of treatment. Trichostrongylidae type parasites are quite small, slender worms, usually <3 cm long, with a direct life cycle (Figure 2). They are important nematodes of ruminants, causing disease and reduced performance (Deplazes et al., 2016). Strongyle eggs are difficult to distinguish from each other, hence speciation is done based on the third larval stage (L3) and adult male morphology. The eggs are passed in the faeces, and develop within the faeces to the first stage larvae (L1), second stage (L2), and to L3 (the infective stage) within one to two weeks under optimal conditions. Under optimal moist conditions, L3 migrate or are dispersed with rain from the faeces to the herbage where they may be ingested during host foraging.

After ingestion of abomasal GIN, like *T. circumcincta* and *O. ostertagi*, L3 exsheath in the rumen and the further development to L4 and L5 occurs within the lumen of abomasal glands. The L5 emerges from the abomasal gland around 18 days post infection and becomes sexually mature. Disregarding the hypobiotic stages, where L3 and early L4 might become arrested in development for as long as six months, the cycle is completed in approximately three weeks, i.e. the prepatent period.

In temperate climate, the majority of the *H. contortus* L3 ingested in late season get arrested as hypobiotic L4 and do not complete development until the following spring (Taylor et al., 2016), when the maturation of these L4 coincide with the periparturient relaxation of immunity and may cause acute haemonchosis. For the intestinal GIN, e.g., *T. colubriformis* and *C. oncophora, the L3* exsheath in the abomasum, travel to the small intestine where they moult twice subepithelial in the crypts before emerging as adult worms. Ruminants may host a range of other GIN.

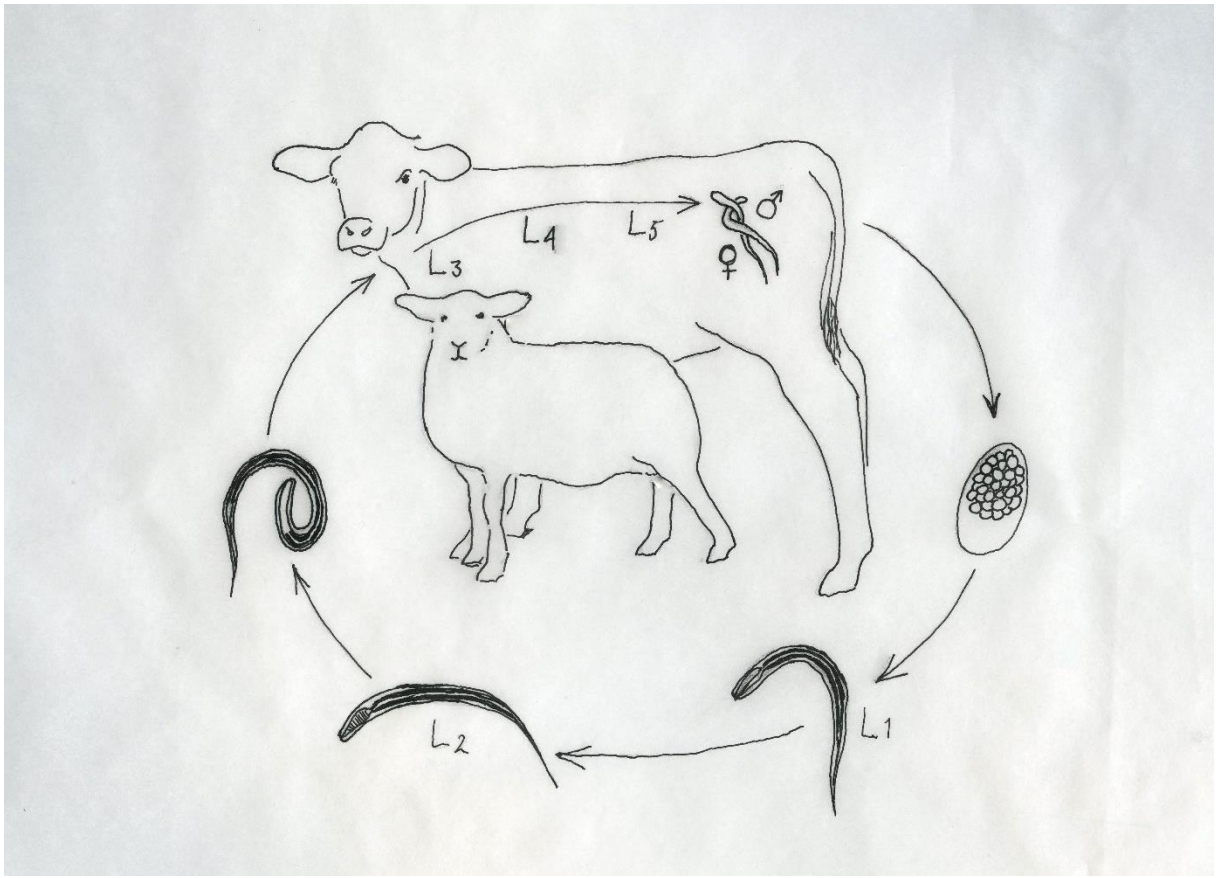


Figure 2 Life cycle of trichostrongyloide type nematode parasites. L1-L5: 1st-5th stage larvae (Anne de Boer).

While *T. vitrinus* is more prevalent in Denmark, *T. colubriformis* is the more prevalent of the two in Norway (Domke et al., 2013; Maingi et al., 1996). Both species are described to have similar pathogenicity and have been reported from various areas in southern and northern parts Norway, although both species have a low ability to survive on the pasture during Nordic winters, as for *H. contortus* (Waller et al., 2004). Examining lambs by necropsy, *T. colubriformis* was found in lambs from the coastal and inland areas of southern Norway (prevalence: 22%) but none in lambs from North Norway, while *T. vitrinus* was found in North Norway only (prevalence: 3%). Similarly, *H. contortus* eggs were found in faeces collected from sheep flocks from all regions included in the survey, as far north as Troms County (Domke et al., 2013). *H. contortus* in goats was only reported from the south-western region of Norway.

*T. circumcincta* was the most prevalent GIN species found in a prevalence study in Norway (Domke et al., 2013). It was found in 75.0% and 81.2% of all necropsied sheep and goats, respectively, and in all the regions included in the study (coastal, inland, and northern regions). The abomasal parasite may survive the Nordic winter both on pasture as well as an inhibited

larvae within the host, which is probably the main reason of success for this parasite (Waller et al., 2004).

The most frequent clinical sign of GIN-infections in sheep is intermittent diarrhoea and a noticeable loss of weight. In temperate areas, *Trichostrongylus* spp. is rarely a primary pathogen but more often a component in parasitic gastroenteritis (Taylor et al., 2016). In the subtropics, on the other hand, *T. colubriformis* is one of the most important causes of gastroenteritis. *T. circumcincta* and *T. colubriformis* are both known to cause a protein deficiency in the host, not due to a reduced protein absorption, but rather as a result of leakage of plasma protein into the intestinal lumen in addition to an increased mucoprotein production and sloughing of epithelial cells into the alimentary tract (Coop et al., 1985; Kyriazakis et al., 1996). This results in reduced growth and immunity of young lambs.

Acute haemonchosis is characterised by anaemia, submandibular oedema and ascites, lethargy, and dark coloured faeces. *H. contortus* can, in severe cases, cause acute haemorrhagic gastritis with sudden deaths (Taylor et al., 2016). History and clinical signs are often sufficient for diagnosis, which can be supported by a faecal egg count (FEC) or necropsy of fatal cases.

#### 2.1.4. *In vitro* and *in vivo* models for assessment of antiparasitic activity

##### 2.1.4.1. *In vitro* models

There are various experimental models and methods also available for assessing the efficacy of plant products against intestinal parasites. *In vitro* models, i.e. experiments performed outside the body, in a laboratory, (lat.: “in glass”), can provide useful information in a cheap, time-saving, and ethical manner. Following Russell and Burch (1959), who proposed a new applied science that would improve the treatment of laboratory animals, we should strive to replace, reduce, and refine the use of animals in research, and the implementation of *in vitro* models may remedy this. Furthermore, *in vitro* models may provide information on the antiparasitic efficacy on different life stages of the parasite, which can provide important information related to the mode of action. The On the other hand, an *in vitro* model lacks an actual anatomy and physiology of the intestine and cannot simulate the complexity of a complete organism such as the host animal. Due to this, there will be no interindividual differences. Furthermore, using *in vitro* models increases the risk of interlaboratory variation. Nevertheless, due to the high throughput of *in vitro* studies, they can be helpful on deciding which bark extracts to use in the targeted *in vivo* study. We selected specific *in vitro* tests for this project based upon the recommendations of Coles et al. (1992) and Smout et al. (2010).

The organoid technology is a simple and reliable *in vitro* multicellular culture system and addresses some of the limitations of *in vitro* methods, as some kind of structure is evident. Organoids are derived from pluripotent stem cells or isolated organ progenitors and grown in the laboratory to resemble the original organ tissue architecture and function, which has been done also for ruminant intestinal structures (Smith et al., 2021). Nevertheless, due to the lack of immune cells, fibroblasts, microbiome, and ingesta, organoids will not display the same gene expression as seen in *ex vivo* and *in vivo* experiments.

*Ex vivo* models are a compromise between *in vitro* and *in vivo* models (Xu et al., 2021). Here, the experiment is performed on tissue extracted from a host animal and kept in a controlled environment resembling the natural conditions. In this way, the integrity of the tissue can be maintained, with the complete intestinal anatomy. This model is complex to establish, and the static system without blood supply is difficult to maintain for long. Therefore, the use of living animals to evaluate the antiparasitic efficacy of bark extracts is still important.

#### 2.1.4.2. *In vivo* models

*In vivo* studies, i.e. “within the living”, allow experiments on intact, living organisms, including the complete anatomy and the complex interplay between different physiological and biochemical processes in a living biological system (Xu et al., 2021). On the downside, *in vivo* studies are time-consuming and expensive, and it is important to consider the ethical issues related to animal experiments, acknowledging the principles of the 3R: Replacement, refinement, and reduction (National Centre for the Replacement, Refinement, and Reduction of Animals in Research, 2022). Performing studies on the relevant parasite in its host species is important prior to any implementation steps, but the use of model organisms is a well-recognised tool that can contribute to understanding the host-pathogen relationship before these are investigated at the target host.

*H. bakeri* (s. *H. polygyrus bakeri*, formerly known as *Nematospiroides dubius*) (Behnke and Harris, 2010; Cable et al., 2006), is a nematode parasite of the subfamily *Trichostrongylina* with a direct life cycle similar to that of other trichostrongylids (Figure 2). The transmission is through ingestion of infective L3. The larvae migrate to the subserosal layer of the duodenum where they encyst, go through two moulting stages before returning to the intestinal lumen as adult worms approximately eight days after infection (Johnston et al., 2015). Eggs are passed in the faeces 9-11 days after infection and infective L3 can be found in faeces after 2-6 days. *H. bakeri* is phylogenetically closely related to *H. contortus*, *T. circumcincta,* and *T. colubriformis* with a similar life cycle, thus, *H. bakeri* is extensively studied and frequently used as a model organism for ruminant GIN infections (Behnke et al., 2009). Nevertheless, it is important to

recognise that mice and ruminants are different species with diverse digestive systems. Results from mice experiments cannot be directly extrapolated to other host-parasite systems and should be confirmed with targeted studies in livestock.
Due to the limited bark extract resources, extracting costs, and the lower cost of hosting smaller animals, *H. bakeri* in a mouse model was chosen.

#### 2.1.5. Antiparasitic pharmaceuticals: current availability and drug resistance

##### 2.1.5.1. Pharmaceuticals against protozoa

Control of parasites in livestock is today largely depending on the use of antiparasitic pharmaceuticals (Molento, 2009). In the EU and the USA, there are only two drugs registered for use against coccidia (e.g., *Eimeria* spp.) in mammals, namely toltrazuril (s. ponazuril) and diclazuril, and there are only two moderately effective drugs registered against cryptosporidiosis in calves: halofuginone lactate and paromomycin (The European Commission, 2020; US food and drug administration, 2020). To achieve the optimal induction of immunity against *Eimeria* spp., a metaphylactic treatment with toltrazuril one week after exposure to coccidia is recommended, which in Norway corresponds to one week after the lambs are let out on spring pasture. Coccidia have a large capacity for multiplication in the intestine, and resistant strains may rapidly become the dominant type. Furthermore, treatment is largely implemented without a definitive diagnosis, and this uncritical use of anticoccidial drugs increases the risk of resistance against the anticoccidial drug toltrazuril, which was reported for the first time in 2018 (Odden et al., 2018a). Odden et al. (2017) concluded that underdosing due to visual appraisal of the body weight, poor timing of the treatment, and uncontrolled and extended use of anticoccidials may be risk factors for the development of ACR in Norwegian sheep farms. Furthermore, by delaying the treatment of the lambs after letting out, untreated oocysts will be shed on the pasture and will increase the refugia. Frequent and underdosed treatment at the wrong time can efficiently increase the resistant population of *Eimeria* spp.
It is challenging to determine treatment failure and anticoccidial resistance for *Eimeria* spp. as there is no practicable *in vitro* technique available. Reduced efficacy of toltrazuril against *Eimeria* spp. can be assessed by a faecal oocyst count reduction test (FOCRT), which requires faecal oocyst counts before and after the treatment with the anticoccidial drug (Odden et al., 2018b).

##### 2.1.5.2. Anthelmintic drugs

As of today, drugs belonging to several chemical groups are registered for the use against GIN in livestock in the European Union (The European Commission, 2020) and include benzimidazoles

(BZ) (e.g., albendazole, fenbendazole), imidazothiazoles/tetrahydropyrimidines (e.g., levamisole, pyrantel), macrocyclic lactones (sub-grouped in avermectins and milbemycins), spiroindoles (e.g., derquantel), and amino acetonitrile derivates (monepantel). Salicylanilides (closantel) is a separate drug group mainly used to treat fasciolosis and haemonchosis. The treatment routines depend largely on local conditions on the farm, and in Norway, a large proportion of the farmers treat without diagnosis (Domke et al, 2011). A major threat against animal health and welfare today is the emerging worldwide resistance against existing antiparasitic pharmaceuticals. A recent meta-analysis of 197 publications representing 22 European countries found that reports on AR were widespread throughout Europe, particularly to BZ (Vineer et al., 2020). The prevalence of anthelmintic resistance (AR) was different in various host species, and in sheep, AR was reported against all AH groups tested (BZ, levamisole, macrocyclic lactones, monepantel, and closantel). They also found that AR prevalence tended to increase over time, This supports previous research communicating that only a few years after introduction of new AH drugs to the market, AR is reported (De Graef et al., 2013).

GIN have a high rate of reproduction, consequently a high genetic diversity, which promotes the development of AR. Resistance against anthelmintics can be triggered by using drugs from a single class only, frequent use, underdosing, and using long-acting drugs with a prolonged elimination curve which maintains the selection pressure with a low dose over time, favouring the selection of resistant parasites. In fact, any factor reducing the size of the refugia is likely to enhance development of AR. Refugia is defined as the proportion of a parasite population that is not exposed to antiparasitic drugs, thus escaping selection for resistance, contributing to maintain susceptible parasites in the population (van Wyk, 2001; Sangster et al., 2018). The higher the proportion of the population in refugia, the slower the selection for resistance. Refugia can be maintained by treating only a proportion of the host population. In contrast, refugia can be lost by combining treatment with a move to clean pasture ("dose and move") or by repeated treatments with short intervals, which will ultimately select for resistant parasites. (van Wyk, 2001; Charlier et al., 2014).

Currently, the gold standard of diagnosing resistance in GIN is by means of faecal egg count reduction tests (FECRT) by calculating the reduction in egg excretion in the animal following treatment, compared to before treatment (Levecke et al., 2018). Like FOCRT, the method requires the use of infected animals and is time consuming. For GIN, there are several *in vitro* methods applied routinely for assessing the anthelmintic sensitivity of helminth eggs or larvae, e.g., the egg hatch inhibition test, the larval development test, or the larval migration inhibition test (Le Jambre, 1976; von Samson-Himmelstjerna, 2009; Demeler et al., 2010). All these have

the advantage of requiring only a faecal sample and no anthelmintic treatment of live animals. However, in some cases there have been deviations between the *in vitro* test result and the test result of the FECRT, showing a FEC reduction >99% while the *in vitro* test results showed resistance (Königová et al., 2021). Moreover, some *in vitro* tests are limited to a single drug class. A novel, sensitive, and fast technique to verify AR is the molecular test to verify resistance-associated alleles, although such a method has been established for BZ resistance only. The lack of more effective diagnostic methods for detecting resistance prevents resistance to be diagnosed at an early stage, causing the resistance alleles to be widely spread in the parasite population (Ahuir-Baraja et al., 2021).

#### 2.1.6. Complementary parasite control strategies

Three principal strategies may be applied for control of gastrointestinal parasites: 1) reducing the parasite population in the host; 2), reducing the contact between the host and the parasite and 3) stimulating the host response on the parasite (Hoste and Torres-Acosta, 2011). Use of antiparasitic drugs, as mentioned above, is the most common method for reducing parasites in the host, but nutritional intervention is an alternative. This will be thoroughly covered in the next section. The most important measure to reduce the contact between the host and the parasite is to employ a good management to keep the infection pressure at a minimum. For protozoa control for instance, this can be done by improving the housing hygiene, particularly in and around feeding areas. Hygiene measures, such as keeping the indoor beddings dry, has the same oocyst reducing effect as treatment with trimethoprim/sulfadoxine (Lopes et al., 2014). Cleaning of the housing area is important, especially before employing disinfectants. If applying disinfectants, it is important to select the correct agent wisely since protozoa and some GIN eggs tend to be resistant to commonly used disinfectants (Fayer, 2004; You, 2014).

Another important way of reducing contact between parasites and ruminants, which is relevant for both protozoa and GIN, is to implement a well-planned grazing strategy. It is important to keep the infection pressure low at pastures for young and naïve animals. This can be done by reducing the stocking rate, which results in lower contamination of the pastures. Furthermore, increasing the time between grazing periods and alternate grazing or co-grazing of different host species will limit the contact between the host and infective stages of parasites on pasture (Ruiz-Huidobro et al., 2019; Charlier et al., 2022). Since several parasite species induce a lasting immunity in the host, it is beneficial to keep naïve animals on the less contaminated pastures for a limited time, move the naïve animals to a new, low-contaminated pasture and let more resistant animals move to the first. While the earlier mentioned “dose-and-move” method should be

avoided, repeated moves to clean pasture without any treatment provide solid control of GIN in cattle (Eysker et al., 2005).

A novel method of limiting the number of infective larvae of GIN on pasture is the use of nematophagous fungi. Bioworma®, a commercially available product containing a nematode-predacious fungi, *Duddingtonia flagrans*, marketed in 2018, has shown promising results in reducing the pasture contamination and ultimately the parasitic load of cattle infected with common pathogenic GIN, subsequently increasing the hosts' weight gain (De Oliveira et al., 2021).

Stimulating the host's response on the parasite, vaccination has a role to play for protozoa and GIN control. In 1996, the first *Eimeria* multi-valent vaccine for poultry was registered for use in the EU (Legemiddelverket, 2021). This enabled the reduction in use of antimicrobial drugs against poultry coccidiosis and lead to Norway abolishing the use of in-feed coccidiostats like ionophores in 2016 (The Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM), 2018). Research has been done on development of vaccines against coccidiosis in mammals, but there is still work to be done to procure an effective vaccine against important pathogenic *Eimeria* sp. (Ruiz et al., 2014).

There are only a few vaccines against parasitic helminths in livestock on the market, and only one vaccine can be obtained for active immunisation against GIN, namely against *H. contortus* in sheep (Claerebout and Geldhof, 2020). Currently, there is research going on to develop further vaccines, e.g., against *Ostertagia ostertagi*, *Cooperia oncophora*, and *T. circumcincta*, amongst others.

As there is some evidence that early exposure to *Eimeria* oocysts may give weaker clinical signs and a better immunity (Gregory and Catchpole, 1989), turnout of lambs at the youngest possible age may help increasing immunity and reducing clinical signs of coccidiosis in young lambs.

Breeding to achieve more resilient and/or resistant host animals is an available method to combat parasitic infections (Mugambi et al., 1997; Behnke et al., 2006; Greer et al., 2018; Yan et al., 2021). Host animals with a higher resistance and resilience to GIN may have less need for antiparasitic treatment and breeding more resistant and resilient host animals can help preventing AR. This can be achieved by selecting rams for resistance against the worms, exemplified by breeding values recommended by e.g., the Australian Sheep Industry Cooperative Research Centre, 2005. Breeding for resistance and resilience against parasites is a time-consuming process. Still, for breeding organisations it is important to focus on the resistance and resilience traits of sires used in breeding programs.

Overall, when comparing control strategies for protozoa with GIN, one may say that, due to the lower efficacy of the drugs, good hygiene management may be more important for prevention of protozoa. In the control of protozoa, it is important to prevent indoor infections by good hygiene measures and dry bedding. Since host animals are exposed to GIN on pasture, such measures are less effective for controlling GIN. Furthermore, low lamb age at turnout will reduce the implications of *Eimeria* spp., this has not been seen for GIN. Well managed grazing strategies are important for both parasite types. Nevertheless, co-grazing strategies may have less effect for preventing protozoa since host animals are mainly infected by soil contaminated with oocysts, although oocyst may leave the soil with growing vegetation (Lassen et al., 2014). Pasture management strategies depend on the parasites' ability to survive on winter pasture and on the prepatent time of the respective parasite. By alternating which pastures to use for livestock when let out, not using the same pasture two adjacent years, both GIN and coccidiosis can be managed efficiently.

## 2.2. Plant bioactive compounds and health

### 2.2.1. Plant secondary metabolites and their biological activity

Medicinal plants represent the oldest source of pharmacotherapy used by mankind (Fürst and Zündorf, 2015) and have been used to treat various ailments in humans and animals, e.g., as treatment against alimentary, urogenital, or pulmonary diseases. In medieval times, plants used against parasites were often mixed with arsenic or copper mineral salts, or more questionable materials like blood or faeces from reptiles or wild animals (Waller et al., 2001). With the arrival of the safer and more effective synthetic pharmaceuticals from the 1940-ies, e.g., phenothiazine (1940), piperazine (1954), and thiabendazole (1961) against GIN (De Graef et al., 2013), and sulphonamides (1940-ies) and toltrazuril (1987) against *Eimeria* spp. (Gjerde et al., 2009; Noack et al., 2019), the use of plants as antiparasitic agents diminished.

The development of new techniques within fields of biochemistry and molecular biology has, together with the rapid emergence of AR against existing antiparasitic pharmaceuticals, given incentive to bring forth old knowledge of plants as antiparasitic agents. The process of defining the compounds, e.g., PSM, responsible for the biological activity and developing new knowledge within modes of action has been accelerated (Mueller-Harvey, 2006; Haslam, 2007).

PSM are compounds produced in the plants involved in the adaptation to the plants' environment, but not directly involved in their growth, development, or reproduction (Makkar et al., 2007). The functions of PSM are diverse and range from defence from herbivores, microbes and competing plants, UV-protection, over to attraction of pollinating insects, seed dispersing

animals, root nodule bacteria, etc. (Wink, 2010). There is a multitude of PSM with pronounced biological activity, and various plants with traits against various ailments can be seen in Table 1. The use of PSM against GIN in the livestock industry has been extensively reviewed (Anthony et al., 2005; Hoste et al., 2006, 2015, 2016, 2022; ; Spiegler et al., 2017; Ali et al., 2021), while less attention has been dedicated to protozoa (Anthony et al., 2005; Wunderlich et al., 2014; Muthamilselvan et al., 2016). Moreover, with the development of the concept of nutraceuticals, meaning any substance that may be considered a food or part of a food which provides health benefits (Andlauer and Fürst, 2002), the focus has shifted from searching for new antiparasitic remedies to a more holistic approach to parasite control.

It has long been a challenge to determine the chemical compound in the plant which is responsible for the biological activity. In recent years, much research has had focus on rather narrow groups of compounds, e.g., sesquiterpene lactones (SL), essential oils, and CT. SL have been identified as the most likely group of anthelmintic compounds in chicory (*Cichorium intybus*) (Valente et al., 2021). Woolsey et al. (2019), on the other hand, concluded that the biological activity of chicory extracts against *C. parvum* was not solely related to SL content, with the chicory extract with lower SL content exhibiting higher anti-cryptosporidial inhibition.

#### 2.2.2. Phytochemicals in bark of conifers and other trees

Different phytochemicals have been identified in bark of Scandinavian coniferous trees, and there are several reports documenting relatively high concentrations of phenolic compounds, e.g. CT (Table 2). CT, also referred to as proanthocyanidins, is a class of plant polyphenolic compounds common in some forages (sainfoin (*Onobrychis viciifolia*), big trefoil (*Lotus pedunculatus*), birdsfoot trefoil (*Lotus corniculatus*)) and in bark from both tropical and temperate trees (wattle (*Acacia* spp.), quebracho (*Schinopsis* spp.), spruce (*Picea* spp.), pine (*Pinus* spp.)) (Hoste et al., 2022). They have a bitter taste and act as a deterrent for the protection from herbivores. CT have traditionally been used for tanning of leather, hence the name. Polyphenols are organic compounds constituted by two or more phenol units (benzene rings) with more than one hydroxyl group. Tannins are one of the more abundant classes of PSM among the phenolics (Haslam, 2007), broadly categorised into two major groups: hydrolysable tannins, consisting of a central core of carbohydrate with ester-bound phenolic carboxylic acids, and CT, or proanthocyanidins/non-hydrolysable tannins. CT are composed of flavan-3-ol monomers linked by carbon-carbon bonds, with catechin and its stereoisomer epicatechin, and gallocatechin and its stereoisomer epigallocatechin as the most common monomers (Hagerman and Butler, 1981; Makkar et al., 2007).

**Table 1** Examples of plants and their chemical compounds with assumed responsibility for the biological activity

| Common name | Latin name | Chemical component | Effect | Reference |
|---|---|---|---|---|
| Black currant | *Ribes nigrum* | CT, quercetin, myricetin | Antioxidant, anticancerogenic, vasculoprotective, anti-inflamatory | Karjalainen et al., 2009 |
| Garlic | *Allium sativum* | organosulfur components, i.e. thiosulfonates | antibacterial, antifungal, anti-inflamatory, antioxidant | Lee et al., 2012; Palaksha et al., 2010; Ried and Fakler, 2014; Zhu et al., 2022 |
| Pomegranate | *Punica granatum* | CT, HT, and others | Vasculoprotective, antimicrobial, antioxidative, anticarcinogenic | Wang et al., 2018; Wong et al., 2021 |
| Chicory | *Cichorium intybus* | SL++? | Antiparasitic | Woolsey et al., 2019 |
| Macroalgae | | Fatty acids | Antiparasitic | Bonde et al., 2021 |
| Chicory | *Cichorium intybus* | SL | Antiparasitic | Valente et al, 2021 |

CT: Condensed tannins; HT: hydrolysable tannins; SL: sesquiterpene lactones.

**Table 2** Phytochemicals in bark of some Scandinavian trees

| Common name | Latin name | Chemical components | Reference |
|---|---|---|---|
| Norway spruce | *Picea abies* | CT, monoaryl components, stilbene glucosides, lignans, flavonoids | Pan and Lundgren, 1995 |
| Conifer and broad-leaved tree species | *Coniferae, Betula sp., Castanea sp., Quercus sp*. | CT | Matthews et al., 1997 |
| Scots pine | *Pinus sylvestris* | lipophilic resin acids, steryl esters, triglycerides, diterpenoids, stilbene glycosides, flavonoids, mono-and disaccharides, suberin, and lignin | Kylliäinen and Holmbom, 2004 |
| Conifers | *Coniferae* | CT | Bianchi et al., 2014, 2015, 2019 |
| Nordic trees | *Coniferae, Betula pubescens* | CT | Athanasiadou et al., 2021 |

CT: Condensed tannins

CT are known to complex strongly with carbohydrates and proteins. Procyanidins (PC) are oligomeric catechin and epicatechin molecules while prodelphinidins (PD) are oligomeric molecules composed of gallocatechin and epigallocatechin units, both with a degree of polymerisation of 2-50 or more. Gallocatechin has, compared to catechin, one extra hydroxyl group, which means that PD have a higher number of hydroxyl groups compared to PC. PD is therefore recognised to have a higher binding affinity, an affinity which is pH dependant.
The affinity (of proteins) for tannins is proportional to the size of the polymer; the larger the CT molecule, the higher is the affinity to precipitating proteins. Proteins are precipitated by CT most efficiently at pH values near their isoelectric points (Hagerman and Butler, 1981). Consequently, CT will have different potential to bind proteins depending on the type of proteins and the pH, e.g., in the various compartments of the digestive system.
Many CT are water soluble (as are the hydrolysable tannins), but some, particularly large CT, are not (Haslam, 2007). This difference in solubility is likely to affect the biological functions. Apart from the factual compounds of the bark and tree species, the extractability (i.e. the extract output per unit of bark) depends on e.g., the particle size and the wood content of the bark, sampling season, sampling location, the age of the tree, bark storage duration and temperature, and potential pre-treatment of the bark (e.g., drying) (Athanasiadou et al., 2021; Matthews et al., 1997; Miranda et al., 2012). Furthermore, the type of solvent, the temperature used during the extraction process, and the isoelectric point (pI) of the extractives are factors affecting the amount of extract retrieved (Zeller, 2019).
A commonly used technique to assess whether CT are indeed responsible for the antiparasitic activity is to test purified plant extracts rich in CT with dose response curves Williams et al. (2014a, 2014b), Quijada et al. (2015), and Desrues et al. (2016b). Furthermore, inactivating CT with compounds binding to and precipitating CT has been a frequently used method, as described by Doner et al. (1993). Polyvinyl polypyrrolidone (PVPP) precipitates CT from plant extracts, making it possible to remove CT from the extract e.g., by centrifugation. In that way it is possible to determine whether CT are likely responsible for antiparasitic effects. Mengistu et al. (2017) used this method and found that for some plants, CT were responsible for inhibiting *Haemonchus contortus* larval exsheathment, while for other plants there was no difference between samples with or without PVPP. Furthermore, molecular networking with correlation analyses has been used with success to determine probable PSM compounds responsible for the biological activity (Bonde et al., 2021; Valente et al., 2021).

### 2.2.3. Bark extracts against parasites

Traditionally, helminth infections of humans and livestock living in temperate climate zones were treated with bark or other products from various tree species (Waller et al., 2001). Bark derived from tree species like pine (*Pinus* spp.), spruce (*Picea* spp.), willow (*Salix alba*), and quebracho (*Schinopsis* spp.) is known to possess anthelmintic properties and may provide an easily accessible resource (Table 3). Furthermore, bark from *Berberis lycium*, *Pinus* spp., and *Salix* spp. has been tested and found effective against protozoa infections *in vitro* and *in vivo*. Factors influencing the anthelmintic efficacy include tree species, age of the tree at processing, CT type, and relative CT content. Some *in vitro* trials reported that a high concentration of PD with a high mean degree of polymerisation (mDP) was associated with high levels of antiparasitic effects, and immune-modulating activity was stronger in CT with an mDP higher than 6 compared to CT with lower mDP (Desrues et al., 2016b; Quijada et al., 2015; Williams et al., 2017). However, extracts with high PC levels have also been found to have marked antiparasitic effects (Desrues et al., 2016b). Athanasiadou et al. (2021) reported that the total CT decreased with the age of the tree and that mDP was higher for spruce (8.2) compared to pine and birch (6.6 and 5.7, respectively). They compared water extracted spruce and birch bark, both low in CT, and found that the spruce extract, with 100% PC and a high mDP, had higher inhibiting effect on *T. circumcincta* egg hatching. These findings were consistent with findings of Dhakal et al., 2015. Other studies have shown that PD-rich extracts were more efficient in reducing larval exsheathment, larval feeding, and adult motility *in vitro* (Brunet and Hoste, 2006; Desrues et al., 2016b). Such observations may suggest that the developmental stage of the parasite is also of importance when it comes to the parasite's susceptibility to PSM. Furthermore, when it comes to the antiparasitic efficacy of PSM against GIN residing in various compartments of the gastrointestinal tract, Paolini et al. (2003) and Cenci et al. (2007) concluded that when assessing the effect of the extract on the worm burden, tannin enriched extracts were more effective against intestinal (*T. colubriformis*, *Cooperia* sp.) than against abomasal species (*T. circumcincta*, *H. contortus*). Contrastive, Max et al. (2005) and Minho et al. (2008) assessed CT rich drenches of Quebracho and Acacia, respectively, and found a reduction in FEC and total worm count (TWC) for the abomasal parasite (*H. contortus*) but not for the intestinal parasite (*T. colubriformis*). It may be challenging to see whether *in vivo* activity of PSM is related to parasite species or the location. If PSM have the same effect on all GIN located in the same gastrointestinal compartment, it is likely a location effect. If the effect varies between species located at the same compartment, it is likely a species effect.

Bark extracts have been assessed against parasites other than nematodes. A commercially available bark extract from *Pinus pinaster* was found to reduce FOC in *C. parvum* infected mice (Kim and Healey, 2001), and water extracted bark from *Pinus radiata* inhibited the sporulation of *Eimeria* spp. by up to 84% (Molan et al., 2009).

Norway has a large forestry industry with an annual production volume of more than 700 000 $m^3$ wet bark from coniferous tree species (Prosess21, 2020). There are various methods how to harvest bark from trees (Chahal and Ciolkosz, 2019). The most common methods within the Norwegian forestry industry are drum and ring debarking. Both methods can include treating the logs with hot or cold water to ease the dislodging of the bark from the wood, particularly during winter when the wood-bark shear strength is particularly high (the connection between wood and bark). Furthermore, steaming or water sprinkling is sometimes incorporated with drum debarking.

The treatment of the bark after debarking may vary depending on the further use of the bark, and bark for combustion is often shredded. The bark from the Norwegian forestry industry is mainly used for bioenergy and as garden mulch and is considered an unexploited by-product. The forestry industry is highly interested in developing bark into a more valuable by-product. Bark meets many criteria set for sustainable biorefining feedstocks as it does not compete with food production and is a by-product with low economic value.

**Table 3** Bark from various tree species, with antiparasitic properties against common ruminant GIN and protozoa *in vitro* and *in vivo*

| Plant species | Study | Parasite | Reference |
|---|---|---|---|
| *Schinopsis* spp. | *in vivo* | *T. colubriformis* | Athanasiadou et al., 2000a |
| *Schinopsis* spp. | *in vivo* | *T. colubriformis* | Athanasiadou et al., 2000b |
| *Schinopsis* spp. | *In vitro, in vivo* | *H. contortus, T. circumcincta, T. vitrinus, N. battus* | Athanasiadou et al., 2001a |
| *Schinopsis* spp. | *In vivo* | *T. colubriformis* | Athanasiadou et al., 2001b |
| *Albizia anthelmintica* | *In vivo* | *H. bakeri, H. contortus* | Githiori et al., 2003a |
| *Acacia mearnsii* | *in vivo* | Ovine GIN | Cenci et al., 2007 |
| *Acacia molissima* | *in vivo* | H. contortus, T. colubriformis | Minho et al., 2008 |
| *Azadirachta indica, Melia azedarach* | *in vitro* | Bovine GIN | Amin et al., 2009 |
| *Acacia mearnsii* | *In vitro, in vivo* | *Haemochus* spp., *Oesophagostomum* spp., *Trichostrongylus* spp., *Cooperia* spp. | Max, 2010 |
| *Pinus radiata* | *in vitro* | *T. colubriformis, T. circumc.* | Molan et al., 2014 |
| *Pinus* spp. | *in vivo* | *H. contortus* | Min et al., 2015 |
| *Pinus sylvestris, Salix spp.* | *In vitro* | *H. contortus, T. colubriformis* | Quijada et al, 2015 |
| *Pinus sylvestris* | *In vitro* | *O. ostertagi, C. oncophora* | Desrues et al., 2016b |
| *Pinus sylvestris, Picea abies, Betula pubescens* | *in vitro* | *T. circumcincta* | Athanasiadou et al., 2021 |
| *Pinus pinaster* | *in vivo* | *C. parvum* | Kim et al., 2001 |
| *Berberis lycium* | *In vivo* | *Eimeria spp* | Malik et al., 2009 |
| *Pinus radiata* | *In vitro* | *Eimeria spp* | Molan et al, 2009 |
| *Salix spp.* | *in vitro* | *C. parvum* | Teichmann et al., 2016 |

There is a multitude of bark extracts commercially available on the market for human use, with various indications. For animals, on the other hand, there are only a few products. P-FENOL® is a hydrolysed, lignocellulose product from pine pulp, high on polyphenols (lignin). According to the specifications (METHODO S.r.l., Italy), P-FENOL® has documented effects against coccidia, fungi, and bacteria, with immune-stimulating properties, though the references are hard to come by. Pycnogenol® is a bark extract obtained from the maritime pine (*Pinus pinaster*, previously named *Pinus maritima*), primarily composed of PC, bioflavonoids, and phenolic acids (Horphag Research, 2018). It is commercially available as a supplement for humans, and is marketed with indications like asthma and allergy, cardiovascular diseases, diabetes, joint health issues, etc. Pycnogenol® has been tested against *C. parvum* in a mouse trial, with antiparasitic

attributes (Kim and Healey, 2001). Flavangenol® is extracted from maritime pine bark using hot water, containing mainly oligomeric proanthocyanidins, with a preventive effect against skin cancer, hypertension, and arteriosclerosis (Mármol et al., 2019).

## 2.3. Summary of background

Based on the outlined challenges with livestock parasites and their resistance against frequently used pharmaceuticals, there is a dire need to investigate less conventional approaches to control. These should be biodegradable, environmentally friendly, and mitigate the problem of resistance against antiparasitic drugs. PSM from coniferous tree species have potential as agents against livestock parasites, both with regard to the potential of limiting the consequences of parasites and to the amounts of bark needed for a future large-scale production of an antiparasitic agent. Still, only little has been done to throw light on the antiparasitic effect of bark extracts from Norwegian spruce and pine. The current study aimed to further elucidate the antiparasitic properties of bark extracts against selected parasites *in vitro* and *in vivo*. We wanted to do a large-scale screening to assess the antiprotozoal and anthelmintic effect of 18 different spruce and pine bark extracts *in vitro* (**Paper I** and **II**, respectively). Based upon these results, we selected two extracts to assess in live animals infected with *Eimeria* spp. (**Paper III**) and GIN (**Paper IV**). We also aimed to associate chemical compounds in the bark extracts with the anthelmintic efficacy of the bark extract (**Paper II**).

# 3. Methodology

This chapter shows a brief overview of the methods and experimental setups applied in the current work. Further details on specific materials and methods used can be found in each of the relevant **Papers I-IV** that form the basis of this thesis.

## 3.1. Bark extracts

### 3.1.1. Bark collection and processing

For the laboratory scale production of bark extract, bark from Scots pine (*P. sylvestris*) and Norway spruce (*P. abies*) was sampled at three different sawmills or wood processing plants in Norway during two collection periods (Summer: July-August 2017, and Winter: February-March 2018). These bark samples constituted the three master batches (MB I, II, and III) (Table 4). MB I and III were collected at sawmills using a ring debarking method, while MB II was from a pulp mill using a drum debarking method. At the pulp mill the logs were sprayed with cold water prior to debarking. Subsequently, the water was removed by pressing. Each bark MB consisted of three equivalent amounts of sub-batches collected weekly over a three-week period. Each MB was mixed thoroughly, milled to chips of 0.5-2 cm in a hammer mill, freeze-dried and ground to particle sizes of approximately 2 mm in a coffee grinder.

The moisture content of each MB (in %) was determined according to ISO/TS 18134-2 (2017) by drying in a laboratory oven at 105 °C until the change of mass did not exceed 0.2% during a heating period of 60 minutes. The wood percentage of the bark was determined according to SCAN-CM 53:94 (1994) by separating bark and wood manually with subsequent drying of each division at 105 °C for 24 h.

For production of a larger amount of extract for the lamb trial (MB S, **Paper III**), fresh spruce bark was collected from a sawmill in Mid-Norway (Bøfjorden sag AS, Surnadal) in March 2019 (Table 4). The spruce logs were debarked using a ring debarking device (without the use of water). The collected bark was dried to approximately 40% dry matter before milling to a particle size of 1-3 cm with an apple crusher.

The bark was stored at −20 °C until further processing.

### 3.1.2. Bark extract production and analyses

For the laboratory scale extraction process (**Paper I**, **II**, and **IV**), we used aqueous acetone (70%), aqueous methanol (80%), and water as solvents, producing three different extracts of

each MB, from two seasons: summer and winter (Table 4 and 5). Acetone and methanol were added to ground bark (20-30 °C for 20 min), centrifuged, and filtered, as described in Paper I. The organic solvents were removed by evaporation, followed by freezing and freeze-drying. To produce laboratory scale water extracts, 100 ml water (65 °C) was added to 10 g fine-ground bark. After 1 h, the extract was separated by centrifugation, and the procedure repeated with the sediment. After centrifugation and filtration, the extract was concentrated in a vacuum centrifuge at 65°C to approximately 50% of the volume, before freezing and freeze-drying. Due to the need of a higher amount of bark extract for the animal trial with lambs (MB S; **Paper III**), the extraction was performed with water as a solvent and slightly different from the lab scale production. Briefly, the dry bark (66 kg) was divided into two batches and each batch was extracted twice for 1-1.5 h in a stirred tank at 80 °C. After each extraction step, stirring was turned off to let the bark sink, and the liquid phase was collected from the top by pumping and transferred to a holding tank. The combined extract was evaporated in a mechanical vapour recompression evaporator with forced recirculation before freeze-drying.

The content of CT was quantified by the butanol-hydrochloric acid assay as described by Grabber et al., 2013. The relative monomer composition, mDP, and the cis-trans ratio was analysed for the methanol bark extracts (**Paper I and II**). Briefly, the extracts were thiolysed with cysteamine hydrochloride and analysed by HPLC using an Ascentis Express C18 column and a flow rate of 0.3 mL/min (Bianchi et al., 2015).

As described in **paper II**, all 18 extracts were analysed using an Agilent 1200 series Liquid Chromatography-Mass Spectrometry (LC-MS) system equipped with an Ascentis express C18 column connected to a Diode Array Detector (DAD) and an Agilent 6520 Quadruple Time-of-Flight (QTOF) mass spectrometer. Putative annotations of identified masses were procured by automatic recording or manual search using the Metlin PLCL database (Metlin.scripps.edu). For all putative annotations, the observed isotopic distributions were compared with the theoretical distribution.

To assess the extracts' solubility in common solvents, a solubility test on the Pine-S extract (summer collected pine bark) was performed. Lyophilised extract (1 mg) was put in a round bottomed glass test tube, 10 µL of methanol, dimethyl sulfoxide (DMSO, 100%), ethanol, acetone, or water was added, the mixture was vortexed for a few seconds, and the solubility assessed visually. More solvent was added in increasing amounts and the process was repeated with 10 µL, 20 µL, 40 µL, 100 µL, 200 µL solvent (Table 6).

**Table 4** Bark master batch (MB) name, tree species, master batch number, collection period, processing plant, and debarking method of four master batches of bark used to produce 20 bark extracts.

| MB name | Tree species | MB | [1]Collection period | Processing plant | Debarking method |
|---|---|---|---|---|---|
| Spruce1 | *Picea abies* | I | S, W | Bergene Holm, Brandval | Ring |
| Spruce2 | *Picea abies* | II | S, W | Norske Skog, Saugbrugs | Drum |
| Pine | *Pinus sylvestris* | III | S, W | Bergene Holm, Kirkenær | Ring |
| SpruceS | *Picea abies* | S | W | Bøfjorden sag, Surnadal | Ring |

[1]S: Summer; W: Winter

**Table 5** CT yield from dry bark, and CT concentration in DM bark extract of 18 bark extracts used in the *in vitro* trials (**Paper I** and **II**), and the two bark extracts used in *the in vivo* trials (**Paper III** and **IV**).

| **Extract name**[1] | **CT yield per unit dry bark [mg CT/g bark]** | **CT content [mg/g DM]** | **Paper** |
|---|---|---|---|
| Spruce1-S-H2O | 2.7 | 70 | I and II |
| Spruce1-S-Me | 5.2 | 98 | I and II |
| Spruce1-S-Ac | 11 | 107 | I and II |
| Spruce2-S-H2O | 2.5 | 17 | I and II |
| Spruce2-S-Me | 3.6 | 68 | I and II |
| Spruce2-S-Ac | 7.8 | 91 | I and II |
| Pine-S-H2O | 1.5 | 65 | I and II |
| Pine-S-Me | 3.8 | 142 | I and II |
| Pine-S-Ac | 5.4 | 153 | I and II |
| Spruce1-W-H2O | 8.7 | 80 | II |
| Spruce1-W-Me | 10.3 | 106 | II |
| Spruce1-Ac | 15.7 | 122 | II |
| Spruce2-W-H2O | 3.5 | 48 | II |
| Spruce2-W-Me | 3.8 | 68 | II |
| Spruce2-W-Ac | 8.7 | 101 | II |
| Pine-W-H2O | 1.8 | 33 | I and II |
| Pine-W-Me | 4.2 | 91 | I and II |
| Pine-W-Ac | 5.3 | 95 | I and II |
| SpruceS-H2O | 4.5 | 49 | III |
| Pine-W-Ac 2019 | 5.3 | 80 | IV |

[1]Deciphering of the extract name: see Table 4. H2O=water, Me=methanol, Ac=acetone. Pine-W-Ac 2019: Acetone extracted bark from *Pinus sylvestris* collected in winter, extracted anew in 2019. CT: Condensed tannins; DM: dry matter bark extract.

**Table 6** Extract solubility in increasing amounts of solvents added to 1 mg dried summer pine extract (Pine-S).

| Extract[1] | Solvent | 10 µL/g DM | 20 µL/g DM | 40 µL/g DM | 100 µL/g DM |
|---|---|---|---|---|---|
| Pine-S-H2O | Methanol | - | - | - | - |
| Pine-S-Me | Methanol | + | + | + | |
| Pine-S-Ac | Methanol | + | + | + | |
| Pine-S-H2O | **DMSO** | **+** | **+** | **+** | |
| Pine-S-Me | **DMSO** | **+** | **+** | **+** | |
| Pine-S-Ac | **DMSO** | **+** | **+** | **+** | |
| Pine-S-H2O | Ethanol | - | - | - | - |
| Pine-S-Me | Ethanol | - | - | - | + |
| Pine-S-Ac | Ethanol | - | - | + | + |
| Pine-S-H2O | Acetone | - | - | - | - |
| Pine-S-Me | Acetone | - | - | - | - |
| Pine-S-Ac | Acetone | - | - | - | - |
| Pine-S-H2O | H2O | + | + | + | + |
| Pine-S-Me | H2O | - | - | - | - |
| Pine-S-Ac | H2O | - | - | - | - |

[1]Deciphering of the extract name: see Table 4 and 5. DM: dry matter bark extract, DMSO: dimethyl sulfoxide. +: extracts dissolved, -: extracts not dissolved.

## 3.2. Antiparasitic effects *in vitro*

### *3.2.1. Cryptosporidium parvum*

In **Paper I**, we investigated the anti-cryptosporidial properties of bark extracts. In previous studies, plant extracts have been assessed against separate parasitic stages of *C. parvum*: the oocyst excystation and invasion of sporozoites into the cells (Invasion Inhibition Assay; IIA) and the intracellular development of *C. parvum* sporozoites; the merogony and the gametogony (Growth Inhibition Assay; GIA) (Gaur et al., 2018; Woolsey et al., 2019). Due to lack of *C. parvum* inhibitory efficacy of plant extracts in previously performed IIA, we decided to perform GIA only (Gaur et al., 2018; Woolsey et al, 2019).

Initially, we performed a small pilot study where we screened the nine summer extracts for anti-cryptosporidial efficacy using a GIA with fluorescein-labelled rat anti-*C. parvum* sporozoites polyclonal antibody (Sporo-glo™, Waterborne Inc, LA, USA) technique. Human ileocecal colorectal adenocarcinoma (HCT-8) cells were seeded onto Nunclon® 96 well plates and, after 24 h incubation, inoculated with *C. parvum* sporulated oocysts, as described in **Paper I**. After an infection period of 4 h, the cell monolayer was washed, and bark extracts were added to the live oocyst wells at the concentration of 500 µg DM/ml, 250 µg DM/ml, and 125 µg DM/ml with

negative (infected, untreated), positive (infected, treated with 500 µg/mL of the anti-cryptosporidial drug Paromomycin), and uninfected controls (n=3, no replicates). Quantification of *C. parvum* in the cell culture was performed with a parasite immunodetection method as described by Woolsey et al. (2019): After assessing the cell monolayer viability with a water-soluble tetrazolium salt (WST-1) test, the monolayers were washed with phosphate buffered saline (PBS), then fixed with ice cold methanol, and subsequently remaining *C. parvum* was identified with Sporo-glo™ as per manufacturer's instructions. The plates were incubated in a humid chamber in the dark at 22 °C for 45 min before Sporo-glo™ was aspirated from each well, washed with PBS and counterstain added. After removing the counterstain and washing the wells with PBS, the wells were washed and one drop of No-fade mounting medium was added to each well. Ten fields of each well were then viewed under a fluorescence microscope (AX10, Zeiss, Germany), with the FITC/AF 488 filter at ×200 magnification (480-nm excitation and 550-nm emission). Fluorescent particles (Figure 3) were counted to determine the amount of *C. parvum*. The results from the pilot GIA (Table 7) corresponded with the results from EHA (**Paper II**), and we proceeded with the pine bark extracts collected during the winter 2018 (Pine-W-H2O, Pine-W-Me, Pine-W-Ac) in a *C. parvum* GIA described in detail in **Paper I**. Briefly, HCT-8 cells were seeded in 96 well plates and grown until confluence (24 h). The cell monolayers were inoculated with sporulated, pre-treated *C. parvum* oocysts, left for 4 h and then washed. Acetone, methanol, and water extracts of pine bark were added in triplicates to the wells in 300 µg/mL, 250 µg/mL, 200 µg/mL, 150 µg/mL, 100 µg/mL, and 50 µg/mL dry matter extract in 1% DMSO and left to incubate for 48 h, together with negative, positive, and uninfected controls as mentioned above. The wells were washed, and the cell viability was assessed using a WST-1 assay. The extracts were assessed independently on separate plates, replicated once. Subsequently, *C. parvum* DNA was extracted and the amount of DNA was assessed by performing qPCR with 18S primers and hydrolysis probe (Shahiduzzaman et al., 2009). From each biological repeat, extracted DNA was run in duplicate on the qPCR plate, and DNA template from a separate oocyst titration study was added to serve as DNA standards. BioRad CFX manager v3.1 was used to analyse the amplification curves and the Cq values from the standard templates were used to estimate the DNA level of the test templates.

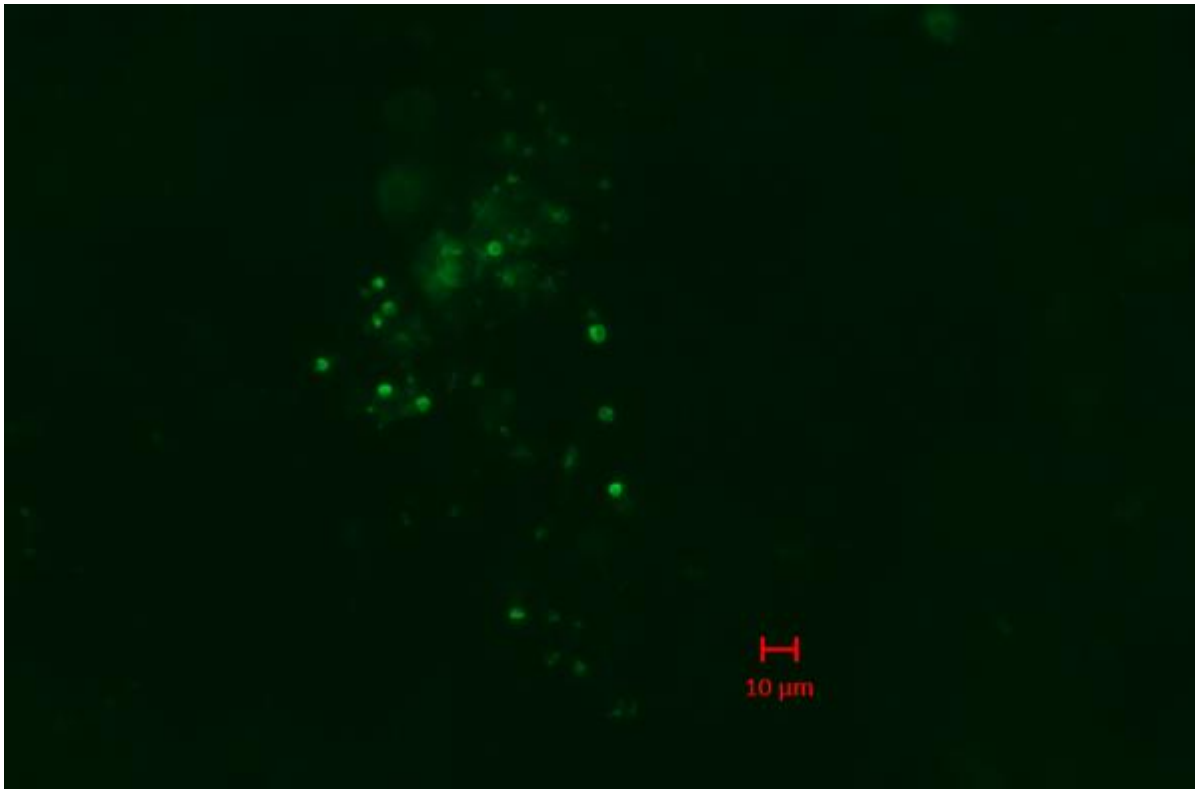


Figure 3: *C. parvum* dyed with fluorescein-labelled rat anti-*C. parvum* sporozoite polyclonal antibody (Sporo-glo™) technique, viewed under a fluorescence microscope with the FITC/AF 488 filter. The green dots present intracellular stages of *C. parvum*.

**Table 7** The mean immune fluorescent (IF) *C. parvum* particle count (n=3) for each extract and extract concentration (µg DM/mL).

| Extract[1] | Concentration | Mean IF count | SEM |
|---|---|---|---|
| Spruce1-S-H2O | 500 | 259 | 8.4 |
| Spruce1-S-H2O | 250 | 86 | 6.7 |
| Spruce1-S-H2O | 125 | 452 | 15.7 |
| Spruce1-S-Me | 500 | **68** | 3.1 |
| Spruce1-S-Me | 250 | 231 | 7.7 |
| Spruce1-S-Me | 125 | 619 | 10.5 |
| Spruce1-S-Ac | 500 | **38** | 4.0 |
| Spruce1-S-Ac | 250 | 250 | 8.6 |
| Spruce1-S-Ac | 125 | 404 | 3.0 |
| Spruce2-S-H2O | 500 | 571 | 10.0 |
| Spruce2-S-H2O | 250 | 122 | 18.0 |
| Spruce2-S-H2O | 125 | 267 | 24.1 |
| Spruce2-S-Me | 500 | **24** | 11.2 |
| Spruce2-S-Me | 250 | 281 | 11.2 |
| Spruce2-S-Me | 125 | 214 | 6.4 |
| Spruce2-S-Ac | 500 | **42** | 1.5 |
| Spruce2-S-Ac | 250 | 332 | 9.9 |
| Spruce2-S-Ac | 125 | 239 | 1.6 |
| Pine-S-H2O | 500 | 126 | 6.4 |
| Pine-S-H2O | 250 | 251 | 17.6 |
| Pine-S-H2O | 125 | 337 | 4.5 |
| Pine-S-Me | 500 | **35** | 2.1 |
| Pine-S-Me | 250 | **19** | 2.7 |
| Pine-S-Me | 125 | 163 | 14.7 |
| Pine-S-Ac | 500 | 198 | 65.0 |
| Pine-S-Ac | 250 | 357 | 10.7 |
| Pine-S-Ac | 125 | 114 | 3.3 |
| NC | 0 | 639 | 4.9 |
| IO | 0 | 39 | 2.2 |
| PC | Paromomycin | 78 | 3.6 |

[1]Deciphering of the extract name: see Table 4 and 5. DM: extract dry matter; SEM: standard error of the mean. NC: negative control (monolayer infected with *C. parvum* oocysts); IO: monolayer with inactivated oocysts: PC: positive control (infected monolayer with paromomycin 500 µg/ml). Figures in bold emphasize low IF particle counts, showing bark extracts of particular interest.

The quantification of *C. parvum* in cell cultures treated or not treated with bark extracts was initially done with the IF method. This method is time consuming and strenuous to the eyes. Additionally it demands advanced equipment (fluorescence microscope with appropriate filters), and the immunofluorescent staining fades easily when exposed to light. On the positive side, the IF method allows a differentiation of the various cycle stages of *C. parvum*, which might be advantageous when looking at the mode of action of potential anti-cryptosporidial PSM or drugs. Using qPCR is a faster method to assess the relative DNA quantity and makes it easier to quantify the amount of *C. parvum* unbiased. The procedure requires advanced equipment, and the procedure itself demands DNA standards in addition to the templates to be analysed. All in all, in this case the qPCR method was decided to be the faster, easier, and more reliable method. In this trial, each extract was tested on separate plates and caution was needed when comparing the efficacy of different extracts. This was done to save time and proved to be necessary to avoid desiccation of the monolayers and increase the cell viability.

#### 3.2.2. Ovine gastrointestinal nematodes

In **Paper II**, we wanted to assess the antiparasitic efficacy of 18 bark extracts on several nematode stages *in vitro*. The methods were selected based upon Coles et al. (1992) and Smout et al. (2010) and are methods that are recommended by WAAVP for the diagnosis of anthelmintic resistance. Guided by the results from these tests, selected bark extracts were tested against GIN *in vivo*.

We utilised the egg hatch assay (EHA) and a high-throughput larval motility assay (LMA) with a real time cell analyser (RTCA) to investigate the *in vitro* antiparasitic effect of all 18 bark extracts against two important GIN in sheep, *T. circumcincta* and *T. colubriformis* (Smout et al., 2010; von Samson-Himmelstjerna et al., 2009). Fresh faeces were collected from sheep monospecifically infected with either parasite and eggs were extracted from the faeces using a flotation technique (Christie and Jackson, 1982). Eggs (100-150) were added to wells in 24 well plates, and bark extract was added to the final concentrations of 1000, 500, 250, 125, and 62.5 µg dry matter (DM)/mL (n=3) and left at 20 °C for 48h. Then a drop of Lugol solution was added to each well to stop the hatching process, and the number of eggs and L1 were counted under 400 x magnification using an inverted microscope.

For LMA, infective L3 from both species were recovered from faecal cultures of monospecifically infected donor sheep, which had been incubated at 23 °C for 10 days and extracted using the Baermann technique. The DP xCELLigence Real Time Cell Analyser measures the electrical impedance on the bottom of tissue culture e-plates. L3 (n=1000) were

added into e-plate wells and incubated in the dark at 20 °C for 24h. The bark extracts were added (n=3) to a final concentration of 1000 µg DM/mL and incubated for another 24 h. The impedance was measured every 15 seconds for the whole experimental period (48h). The impedance data was converted to a motility index before statistical analysis.
Additionally, in **Paper II** we aimed to analyse the extracts to define the chemical compounds possibly responsible for the biological activity. The bark extracts were analysed as described in **Paper II** and chapter 8.1.2. using a butanol-HCl assay for quantification and characterisation of CT. Furthermore, the bark extracts were analysed with an LC-MS and LC-UV system (HPLC-DAD) connected to a QTOF mass spectrometer. A Pearson's correlation analysis was performed between the CT content and $IC_{50}$ (i.e. the extract concentration required to inhibit 50% egg hatching) values obtained using the EHA, and between the abundance of each of the LC-MS determined masses in the extracts and the $IC_{50}$ (EHA). For the correlation, we used a) the whole dataset, b) data from methanol and acetone extracts only, and c) data from the methanol extracts, re-analysed using a more sensitive "Jet Stream" ion source. The aim was to define an association between CT and biological activity, and between other compounds present in the extracts and the biological activity.

## 3.3. Animal studies

### 3.3.1. Lamb study: *Eimeria* spp.

In **Paper III**, CT containing water-extracted spruce bark was used to investigate the effect of bark extract on coccidiosis on young, pre-weaned lambs. The choice of using a water extract was based upon the previous *in vitro* studies (Paper I and II, Table 8). The *in vitro* results encouraged us to use bark extracted with an organic solvent. Practical challenges in finding a production plant with the necessary permissions to use organic solvents in the extraction process made us settle for a water extract. This could be produced in large scale at the same plant where the laboratory scale extracts were produced. Lambs (n=24) of the Norwegian White Sheep breed ("Norsk kvit sau") were removed from their mothers at birth within a three days' timeframe and raised in a parasite free, indoor housing area.
The group size was estimated by the resource equation described by Roger Mead (1990): N-1=T + B + E, where N is the number of animals, T is the degree of freedom (the number of test groups minus 1), B is the number of blocks, and E an error estimation. This confirmed that n=8 would be an appropriate group size when using three treatment groups. We assessed this

group size with a T-test and with various requirements for results, e.g., 10% reduction and 75% reduction in FOC, which confirmed the calculated group size as sufficient.

The lambs were blocked according to BW and sex and randomly allocated into three treatment groups (infected, treated animals; infected, untreated animals; sham-infected, untreated animals). All lambs were fed parasite free hay, concentrate, and water *ad libitum* and a volume of milk replacement depending on age. After an adaptation period of three weeks, the lambs were infected on three consecutive days (day 0-2) with a purified field isolate of 100.000 sporulated *Eimeria* oocysts (5 mL) per day (Eckert et al., 1995) containing 62% highly pathogenic *Eimeria* spp. From day 0 and for 11 consecutive days, the lambs were dosed orally with bark extract using a stomach tube. Based upon previous research (Fraquelli et al., 2015; Saratsis et al., 2012), we aimed to dose the lambs with bark extract with a CT content corresponding to 0.1% of the individual metabolic body weight (mBW). Due to adverse reactions on the extract explained in **Paper III**, the dose had to be adjusted and reduced (Table 9). We aimed for a dose of 0.05% of mBW, which is equivalent to 4.8 g DM extract for a lamb of 20 kg BW.

The animals were regularly weighed, faeces were assessed with regards to consistency, and faeces were sampled for parasitic output. On day 22, the animals were euthanised using a captive bolt gun followed by exsanguination.

**Table 8** A cross tabulation highlighting which extracts were used in the various *in vitro* and *in vivo* studies.

| **Bark**[1] | **Season**[1] | **Solvent**[1] | **Paper I** *In vitro* GIA | **Paper II** *In vitro* EHA | **Paper II** *In vitro* LMA | **Paper III** *In vivo* *Eimeria* lamb | **Paper IV** *In vivo* *H. bakeri* mouse |
|---|---|---|---|---|---|---|---|
| Pine | S | H2O | X | X | X | | |
| Pine | S | Me | X | X | X | | |
| Pine | S | Ac | X | X | X | | |
| Pine | W | H2O | X | X | X | | |
| Pine | W | Me | X | X | X | | |
| Pine | W | Ac | X | X | X | | X |
| Spruce1 | S | H2O | X | X | X | | |
| Spruce1 | S | Me | X | X | X | | |
| Spruce1 | S | Ac | X | X | X | | |
| Spruce1 | W | H2O | | X | X | | |
| Spruce1 | W | Me | | X | X | | |
| Spruce1 | W | Ac | | X | X | | |
| Spruce2 | S | H2O | X | X | X | | |
| Spruce2 | S | Me | X | X | X | | |
| Spruce2 | S | Ac | X | X | X | | |
| Spruce2 | W | H2O | | X | X | | |
| Spruce2 | W | Me | | X | X | | |
| Spruce2 | W | Ac | | X | X | | |
| SpruceS | W | H2O | | | | X | |

[1]Deciphering of the extract name: see Table 4 and 5. GIA: *C. parvum* growth inhibition assay, EHA: Egg hatch test; LMA: Larval motility assay.

**Table 9** Oral dosing with spruce bark extract of lambs infected with ovine *Eimeria* spp. oocysts. The animals were dosed on day 0-11.

| Experiment day | Number of daily doses | Daily dose in % CT of mBW | CT concentration [mg/mL] |
|---|---|---|---|
| 0 | 1 | 0.050 | 15.4 |
| 1 | 2 | 0.075 | 13.0 |
| 2-4 | 1 | 0.025 | 6.7 |
| 5-6 | 2 | 0.050 | 6.7 |
| 7-10 | 2 | 0.050 | 7.3 |
| 11 | 1 | 0.025 | 10.3 |

CT: condensed tannins, mBW: metabolic bodyweight ($BW^{0.75}$)

#### 3.3.2. Mouse study: *Heligmosomoides bakeri*

An *in vivo* model of helminth infection in mice was used to investigate the effect of pine bark extract on the resistance, performance, and tolerance of genetically diverse mouse lines with expected different rate of development of immunity to the parasite *H. bakeri* as a model. Female, five weeks old fast responder mice (BALB/c, expected to clear the *H. bakeri* infection fast) and slow responder mice (C57BL/6 mice, not able to clear the infection fast, developing a chronic infection) where infected or sham infected with *H. bakeri* L3 and treated or not treated with a high or low dose of acetone extracted bark from *P. sylvestris* (**Paper IV**). The choice of using acetone extracted pine winter bark in the mouse trial was based upon the preliminary results from the *in vitro* trial on ovine gastrointestinal nematodes (**Paper II**).

Upon arrival, the mice were weighed and placed in pairs in individually ventilated cages with sawdust, nesting and enrichment material, and *ad libitum* water and feed. The best indicator to evaluate the effect of a plant on the parasites is the worm count. Previous studies have indicated that the coefficient of variation for worm counts is 20%, and for a relative reduction of 40% we need 10 mice per treatment. A reduction of 40% in the worm counts will make a significant contribution in the parasite load and thus we have included 10 mice per treatment in the current study. Mice are individually treated with the parasite and plant extract, and all data are collected from the individual mouse. Therefore, the mouse is the individual unit, though cage number will be included in the statistical model. Group feed intake, i.e. of the two mice combined, will give an indication on anorexic effects, and through measuring this also in sham-infected mice we get

an indication of potential side effects. Within line, the mice were randomly allocated into groups with 10 animals in the infected groups and six animals in the non-infected control groups. On day 0, after an adaptation period of 6-9 days, the mice were infected by oral gavage with 200 infective larvae of *H. bakeri* (200 µL). The non-infected mice were given 200 µL of tap water. Two levels of bark were tested: 150 mg/mL and 75 mg/mL (1.5 and 0.75 g DM/kg BW, respectively). The animals received 200 µL of the bark extract daily for three consecutive days, and the negative control animals were given 200 µL tap water (Table 10).
The animals were regularly sampled over a two-week period (faecal sampling, measuring of body weight and feed intake). At day 28, the animals were euthanised by increasing $CO_2$ inhalation until asphyxiation. The animals were dissected and carcass weight, weight of small intestines and spleen were recorded. Small intestines were opened longitudinally and stored in 70% ethanol for later total worm count and estimation of per capita fecundity of the female nematodes. The colon content was sampled for total colon egg count. Per capita fecundity was calculated as total eggs in colon divided by total female worm count.

### 3.4. Ethical statements

All experimentation on the lambs (**Paper III**) was approved by FOTS Norwegian Food Safety Authority, license number 18555, and performed according to The Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations. The *H. bakeri* mouse experiment (**Paper IV**) was approved by the SRUC's ethical review committee (ROD062020) and carried out under Home Office authorisation PP6868991.

**Table 10** Experimental setup (**Paper IV**). Two mouse lines with different susceptibility to the parasite were infected with a single dose of 200 infective L3 of *H. bakeri* (200 µL, day 0) and treated with acetone extracted pine bark for 3 days (200 µL, day 19-21). The mice were treated with bark extract at 0, 15 and 30 mg DM/kg BW.

| Balb/c, n=48 | | | | | | C57BL/6, n=48 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Infected, n=30 | | | Non-infected, n= 18 | | | Infected, n=30 | | | Non-infected, n= 18 | | |
| 0 mg/kg n=10 | 15 mg/kg n=10 | 30 mg/kg n=10 | 0 mg/kg n=6 | 15 mg/kg n=6 | 30 mg/kg n=6 | 0 mg/kg n=10 | 15 mg/kg n=10 | 30 mg/kg n=10 | 0 mg/kg n=6 | 15 mg/kg n=6 | 30 mg/kg n=6 |

BALB/c: fast responder line. C57BL/6: slow responder line. DM: dry matter extract. BW: live bodyweight

# 4. Summary of results

## 4.1. Bark extract production and characterisation

The bark extract used in **Paper I**, **II**, and **IV** were produced in a laboratory scale manner as described in **Paper II** (chapter 8.1). For the experiment described in **Paper III**, a larger extract quantity was needed, and a modified method was used to produce the extract (**Paper III**).

### 4.1.1. Wood and moisture content in the bark

The moisture content of all MB was significantly different between summer and winter (Table 11): the spruce bark harvested in winter was moister compared to the spruce bark harvested in summer, unlike the pine bark, where the summer bark contained more water than the winter bark. The wood content was higher in the winter bark from both species compared to the summer bark. The extract DM yields were in the range of 20-120 mg DM extract per g dry bark, with the lowest value for water extracts from pine and the highest for acetone extracts from spruce. Using acetone in the extraction process yielded more extract compared to methanol, which again yielded more extract compared to water.

### 4.1.2. The impact of season, tree species, and solvent on the CT yield and concentration

We found that bark harvesting season, tree species, and solvent used in the extraction process contributed to the variation observed in the CT yield (mg CT/g bark) and the CT concentration in the extract. The CT yield, ranging from 2-16 mg CT/g bark, was higher in spruce winter bark (Spruce1-W) compared to the summer bark (Table 5). For Spruce2 and Pine, there was no obvious difference in CT yield between the summer and winter extracts. Furthermore, both spruce barks (Spruce1 and Spruce2) gave a higher CT yield per unit bark compared to pine, and Spruce1 had a higher yield compared to Spruce2 (Table 5).

Overall, acetone produced extracts with the greatest CT concentration of all solvents used. Only the pine extracts had higher CT concentration in summer compared to winter, regardless of solvent used. In this regard it might be worth mentioning that the pine bark also had the lowest (summer) and the highest (winter) wood content (Table 11). The methanol and acetone pine summer bark extracts (Pine-S-Ac and Pine-S-Me) had the highest CT concentration per DM extract, meaning that these extracts were purer with less other compounds extracted. Spruce2 extracts, originating from the pulp mill, using water in the debarking process, had the lowest CT concentration per unit extract DM.

**Table 11** Moisture and wood percentage in each bark master batch (MB)

| MB name[1] | Moisture content [% of wet mass] | | Wood percentage [% of dry mass] | |
|---|---|---|---|---|
| | Summer | Winter | Summer | Winter |
| Spruce1 | 54 | 59 | 19 | 19 |
| Spruce2 | 50 | 67 | 17 | 20 |
| Pine | 63 | 56 | 6 | 25 |

[1]Deciphering of the extract name: see Table 4.

#### 4.1.3. Chemical characterisation and solubility of the bark extracts

The CT yield per unit dry bark and the CT concentration in the bark extract dry matter can be seen in Table 5.

PD were not detected in the pine bark extracts, which contained 100% PC. The PD content in the spruce bark extracts were only 2-3%, with 97-98% PC. The cis isomer of the PC (epicatechin) accounted for 87% for spruce and 79-83% for pine. mDP was similar for all extracts within the range of 5.7-7.8. (**Paper II**).

When assessing the solubility of the summer pine extracts in methanol, DMSO, ethanol, acetone, and water, we found that DMSO was the only solvent able to dissolve all three extracts (Table 6). We also found that water was the only other solvent able to dissolve the water extracts, but water did not dissolve the acetone or methanol extracts. In **Paper IV**, we aimed for an end concentration of 150 and 75 mg extract DM/mL. The extract was dissolved in 5% DMSO and the reconstituted bark extract was analysed for CT content (Table 12). Both the high and the low concentrations contained approximately ⅓ of the calculated concentration.

#### 4.1.4. Liquid chromatography-UV-mass spectrometry characterisation of the bark extracts

A significant variation in the compound profiles of the bark extracts could be seen when characterising the bark extracts using liquid chromatography with UV-detection followed by mass spectrometry (LC-DAD-MS-analyses). It was revealed that Spruce1 and Spruce2 contained the same UV absorbing classes but that Spruce2 had a lower concentration of the compounds (**Paper II)**. Furthermore, Spruce1 collected during winter had a higher concentration of UV absorbing compounds compared to the batch collected during summer, which was not evident for Spruce2.In the spruce extracts, there was a group of compounds that eluted between 8 and 11 min, of which the concentration was higher in the winter extracts compared to the summer extracts. This characteristic group was not present in the pine extracts which contained UV absorbing compounds with a different UV profile. These eluted over a wider time range and

therefor most likely belonged to different chemical groups compared to those of spruce. Similar to spruce extracts, the concentrations in pine extracts were higher in the winter bark compared to the summer bark. It was also evident that the less polar compounds in pine, i.e. compounds eluting later than 10 min in the chromatogram, were not extracted with water (**Paper II**). There was no prominent difference in compounds with high UV absorbance with regards to the three solvents.

Using mass spectrometry to analyse all 18 extracts, 2299 different compounds were detected. We found 878 compounds unique to pine when compared to spruce1, and 595 compounds were unique to spruce1 compared to pine. The extracts from bark collected during winter contained a higher number of different compounds compared to bark harvested during summer, which agreed with the UV data. Pine extracts contained a higher number of compounds compared to extracts from spruce. For both Spruce1 and Spruce2, the methanol extracts contained the highest number of compounds, and for pine, the acetone extracts had the highest number of compounds. By comparing the UV and the MS data, some of the compounds in the extracts could be identified, e.g., coumarin and coumarin derivatives in the pine bark.

The only CT molecules with three or less monomers, detected in the MS analysis at retention times between 6 and 8 minutes, were procyanidin C1 (trimer), procyanidin B1/B2 (dimer), and catechin/epicatechin (monomer), with a higher abundance in pine than in the spruce extracts, not detected in Spruce2.

**Table 12** The high and low concentration of the extract dry matter (DM conc), and the calculated (calc) and analysed (an) concentration of condensed tannins (CT) in the acetone pine bark extract given *H. bakeri* infected mice (**Paper IV**).

| DM conc [mg/mL] | CT calc [mg/mL] | CT an [mg/mL] | SEM |
|---|---|---|---|
| 150 | 12 | 4.2 | 0.45 |
| 75 | 6 | 2.0 | 0.04 |

SEM: standard error of the mean

## 4.2. Paper I

In this study we investigated the anti-cryptosporidial properties of acetone, methanol, and water extracted bark from Scots pine collected during the winter 2018, by means of an *in vitro* growth inhibition test (Slifko et al., 1997). Prior to the main study we performed a pilot study. We found that the methanol extracted pine summer extract, Pine-S-Me, showed promising anti-cryptosporidial activity by inhibiting *C. parvum* both at 500 µg/ml as well as at 250 µg/ml (Table

7). Based upon these results and the results from the analyses of the CT concentrations of the bark extracts (Table 5) we selected which extracts to assess in the *C. parvum* growth inhibition main trial.

In the main trial, we found that Pine-S-Ac and Pine-S-Me had a dose-dependent anti-cryptosporidial effect, which could not be seen for Pine-S-H2O. Furthermore, we showed that Pine-S-Ac and Pine-S-Me were no different from their respective positive controls, paromomycin. When merging the results of Pine-S-Ac and Pine-S-Me, we were able to describe a dose-dependent relationship between the CT concentration and the relative DNA content of the treated wells. Additionally, the extracts with the highest CT concentration demonstrated the highest antiprotozoal effect.

### 4.3. Paper II

In **Paper II**, we investigated the biological activity of 18 different bark extracts against *T. circumcincta* and *T. colubriformis in vitro* by using EHA and LMA-RTCA. We found that certain bark extracts exhibited a high inhibition of egg hatching and larval motility.

Overall, using EHT we found that *T. colubriformis* was more susceptible to the treatment compared to *T. circumcincta*, exhibiting a lower $IC_{50}$ for most of the extracts (Table 13). Pine bark, bark collected during the winter, and bark extracted with organic solvents generally showed higher inhibiting properties in EHA compared to the spruce bark, bark collected during the summer, and bark extracted with water as a solvent (**Paper II**). There was no significant difference in egg hatch inhibition between spruce1 and spruce2. For most of the acetone and methanol extracts we could see a dose response, whereas there was little or no evidence of a dose response for the water extracts. The pine winter acetone extract exhibited the highest efficacy at the lowest concentrations, with the lowest $IC_{50}$ in both GIN species: 48 and 221 µg DM/mL for *T. colubriformis* and *T. circumcincta*, respectively (Table 13).

In the LMA, we found that a higher number of different bark extracts reduced the motility of *T. circumcincta* L3 as compared to the *T. colubriformis* L3 (11 and 6 bark extracts, respectively) (Table 13). Remarkably, a higher number of the water extracts showed biological activity against L3 compared to the acetone and methanol extracts; for *T. colubriformis* and *T. circumcincta*, 4/6 water extracts reduced the L3 motility to the level of the dead control larvae. For the acetone and methanol extracts, the extracts that reduced the motility to the level of the dead control were 0/6 and 2/6, and 4/6 and 3/6, for *T. colubriformis* and *T. circumcincta*, respectively.

In **Paper II**, we aimed to identify the compounds possibly responsible for the observed bioactivity in the EHA. A Pearson's correlation analysis between 2299 detected masses and the EHA $IC_{50}$ of *T. colubriformis* and *T. circumcincta* was performed. When using the whole dataset in the correlation analysis, CT (r=-0.54, $P<0.021$) and one other compound of unknown ID (mass: 662.533 Da, retention time: 18.37) was significantly negatively associated with $IC_{50}$ of *T. colubriformis* egg hatching (**Paper II** Supplementary Table S3). Four compounds were associated with *T. circumcincta* egg hatching, and CT were not among these (r=−0.14, $P=0.579$). When performing the correlation analysis using the methanol and acetone datasets only, 69 compounds were negatively correlated with *T. colubriformis*, out of which most masses could be found in the pine extracts only. Five compounds could be corelated with *T. circumcincta* egg hatching.

Since the acetone and methanol extracts showed relatively similar antiparasitic efficacy, consistently higher than the water extracts, the methanol extracts were analysed anew with a more sensitive Jetstream MS ion source. Using this dataset, a total of 1316 compounds were detected, out of which 89 were new compounds not previously identified. The Pearson's correlation analysis revealed that a total of 46 of these compounds were significantly negatively associated with *T. colubriformis* $IC_{50}$, with seven particularly interesting masses out of which none were correlated to the inhibition of *T. circumcincta* (**Paper II** Supplementary Figure S2). Here, we plotted the relative abundance of candidate compounds against the anthelmintic activity, given as $1/IC_{50}$, to better visualise the possible relationship between these compounds and the antiparasitic effect. These masses were putatively annotated, as can be seen in the figure (A). There were only two masses found in both pine and spruce with $r < -0.90$ and $P<0.01$. The masses of these were 164.0832 Da and 200.1557 Da, and the molecular formulas of these are likely to be $C_{10}H_{12}O_2$ and $C_{15}H_{20,}$ respectively. Possible annotations for these molecules are the monoterpenoid eugenol and a sesquiterpenoid, e.g., calacorene. Furthermore, the mass M=226.1004 Da corresponds to the basic unit of the flavanols ($C_{15}H_{14}O_2$), the group where the tannins belong, and the mass 300.2089 Da ($C_{20}H_{28}O_2$) could potentially be a naphthalene derivative (**Paper II** supplementary Figure S2).

**Table 13** Biological activity of 18 bark extracts The table shows the extracts' ability to inhibit egg hatching shown as $IC_{50}$, and to inhibit the motility of third stage larvae, shown as rejection of the null hypothesis ($H_0$ rejected), saying that the motility of the larvae in the bark extract treatment differed significantly from the dead controls. Rejected null hypothesis (Y) was indicative of strong antiparasitic activity, whereas not-rejected null hypothesis (N) was indicative of variable and/or no strong antiparasitic activity.

| ***T. colubriformis*** | | | **EHA** | **LMA** | ***T. circumcincta*** | | | **EHA** | **LMA** |
|---|---|---|---|---|---|---|---|---|---|
| Bark | Season | Solvent | $IC_{50}$ [µg DM/mL] | $H_0$ rejected Yes/No | Bark | Season | Solvent | $IC_{50}$ [µg DM/mL] | $H_0$ rejected Yes/No |
| Pine | W | Ac | 48 | N | Pine | W | Ac | 221 | N |
| Pine | W | Me | 63 | N | Spruce2 | W | Ac | 221 | Y |
| Pine | S | Ac | 89 | N | Spruce1 | W | Ac | 353 | Y |
| Pine | S | Me | 93 | N | Spruce2 | W | Me | 407 | N |
| Spruce2 | W | Ac | 95 | N | Pine | W | Me | 407 | N |
| Spruce2 | W | Me | 145 | N | Pine | W | H2O | 459 | N |
| Spruce2 | S | Ac | 159 | N | Spruce1 | W | Me | 475 | Y |
| Spruce1 | W | Ac | 162 | N | Pine | S | Ac | 492 | Y |
| Spruce2 | S | Me | 193 | N | Pine | S | Me | 596 | Y |
| Spruce1 | S | Me | 196 | Y | Spruce1 | S | Ac | 1050 | Y |
| Spruce1 | W | Me | 208 | Y | Spruce2 | W | H2O | 1282 | N |
| Spruce1 | S | Ac | 400 | N | Pine | S | H2O | 3587 | Y |
| Pine | W | H2O | 495 | Y | Spruce2 | S | Ac | 5908 | N |
| Spruce2 | W | H2O | 856 | N | Spruce1 | W | H2O | 7093 | Y |
| Pine | S | H2O | 3498 | Y | Spruce2 | S | Me | 20733 | N |
| Spruce1 | W | H2O | 16203 | Y | Spruce1 | S | Me | 83393 | Y |
| Spruce1 | S | H2O | 212179 | Y | Spruce1 | S | H2O | 4236058 | Y |
| Spruce2 | S | H2O | $1.8\times10^{14}$ | N | Spruce2 | S | H2O | $1.2\times10^{11}$ | Y |

[1]Deciphering of the extract name: see Table 4 and 5. Abbreviations: EHA: egg hatch assay; LMA: larval motility assay; $IC_{50}$: Bark extract dose required to inhibit 50% of the GIN eggs from hatching. DM: dry matter bark extract.

For *T. colubriformis* the correlation between the CT content of the bark extracts and the $IC_{50}$ was significant when using the data from all extracts in the analysis. There was no correlation between the CT content and the $IC_{50}$ of *T. circumcincta*. Nevertheless, numerous other compounds were detected in the bark extracts.. A principal compound analysis of the complete MS data set showed that the extracts of pine collected in winter were different from extracts of the other master batches. However, none of these mostly hydrophilic compounds were more abundant in the extracts with higher bioactivity (Pine-Ac and Pine-Me) compared to the less active Pine-H2O, hence these compounds are most likely not responsible for the antiparasitic activity.

In **Paper II**, we found that a) several of the tested bark extracts demonstrated up to 100% inhibition of the egg hatching, b) tree species, season and method of bark harvesting, solvent used in the extraction process, GIN species, and parasite life stage have a significant impact on the antiparasitic activity of the bark extracts, and c) CT and numerous other compounds detected in the bark extracts may have contributed to the observed antiparasitic effects, alone or synergistically. Several putative annotations were obtained suggesting the identity of some of the masses detected.

### 4.4. Paper III

This study investigated the anticoccidial effect of a water-based extract of bark from Norway spruce (*P. abies*) in pre-weaned, milk-fed lambs (SpruceS, Table 4). The null hypothesis, saying there was no difference in FOC and consequently no reduction in severity of diarrhoea in lambs infected with mixed *Eimeria* spp., had to be rejected as we found that the oocyst output in lambs treated with bark extract was 98% lower than that of the untreated lambs. During the post-treatment period, the treated lambs had more solid faeces (i.e. a lower faecal consistency score, FCS) compared to the untreated infected control animals. The lower FCS coincided with the lower FOC.

During the treatment period, the lambs treated with bark extract had reduced milk intake and showed signs of constipation and diarrhoea. Additionally, the treated lambs had significantly lower growth compared to both infected untreated lambs and the sham-infected, untreated control group, which was probably due to the reduced milk intake.).

### 4.5. Paper IV

Based upon the results from Paper I (bioactivity of acetone pine extracts) and II (activity against ovine GIN *in vitro*), **Paper IV** addressed the impact of acetone pine bark extract on GIN using *H. bakeri* infected house mice (*Mus musculus*) of two strains with expected different rate of development of immunity to the parasite as a model. The aim was to quantify the impact of bark extract administration on the resistance, performance, and tolerance of mice with different susceptibility to *H. bakeri*. We hypothesised that the slow responder line would benefit more from the bark extract treatment compared to the fast responder line and more specifically, slow responder mice would experience a higher reduction in the parasitic burden, a higher increase in performance, and better tolerance to the parasitic infection compared to the fast responder mice. The bark extract exhibited no benefits on the resistance of parasitised mice when using FEC, total count of parasite eggs in colon (EIC), TWC in the intestines, and female worm fecundity as parameters, indicating that the bark extract has no antiparasitic effect against *H. bakeri* at the given doses.

Although the bark extract had no impact on the parasitological parameters, the treatment had a positive impact on the infected, slow responder line on performance parameters expressed in increased body weight and carcass weight, and reduced FI-BW ratio, compared to the untreated infected control group. In contrast, we found that bark extracts reduced the tolerance in fast responder mice, expressed as a negative correlation between parasitological and performance parameters. The bark extracts exerted no influence on the tolerance of the slow responder line. Two of the three predictions stated were confirmed: the infected slow responder mice experienced a higher increase in performance and a better tolerance to the parasitic infection, compared to the fast responder mice. There was no difference in parasitological parameters between the treatment groups. Consequently, the overall hypothesis saying that the infected slow responder mice would benefit more from the bark extract treatment compared to the fast responder mice, had to be rejected.

# 5. Discussion

Bark from coniferous tree species, like *Pinus sylvestris* and *Picea abies*, is an under-exploited by-product from the Norwegian forestry industry and is readily available. The overall objective of the current work was to assess the antiparasitic properties of coniferous bark extracts against parasitic nematodes and apicomplexans.

## 5.1. Bark extract production and characterisation

### 5.1.1. The impact of bark harvesting season and tree species on CT yield, CT concentration, and the content of novel compounds

It is well known that there is large variations in the content of different PSM in plant extracts, with associated variable outcome of tests for antiparasitic activity (Hoste et al., 2005; 2022). The reason for the variations in the chemical composition in bark extracts could be attributed to several factors: the use of different tree species and growth conditions in different trials, the use of different methods to analyse the chemical composition of the material, and analysing crude bark or bark extracted with different types of solvents all have a high influence on the results. Furthermore, the storage conditions of the bark are highly important since the tannin content is known to decrease rapidly in bark stored before extraction (Jylhä et al., 2021).
Overall, the CT yield (mg CT/g bark) and the CT concentration (mg CT/g DM extract) in the pine and spruce bark extracts observed here were at similar levels to those found in other studies using bark from the same tree species (Bianchi et al., 2016; Athanasiadou et al., 2021). Due to water spraying of the tree logs during summer, the moisture was expected to be higher in the spruce summer bark, as seen for the pine bark. The lower moisture in the spruce bark collected in the summer compared to winter is likely due to local storage conditions at the processing plant. The use of water during storage in summer and in the drum debarking process (Spruce2) has been shown to wash out the water-soluble PSM, e.g., CT, from the bark (Chahal and Ciolkosz, 2019). This can also explain the lower concentration of UV absorbing and LC-MS detected compounds in the summer bark compared to the winter bark. The higher CT yield and CT concentration in the summer pine extracts compared to all spruce extracts may indicate that bark from spruce has a higher pre-extraction CT loss compared to bark from pine. This harmonises with the literature saying that CT from spruce is easier extracted than CT from pine (Matthews et al., 1997). Pine is known to contain less soluble polyphenols compared to spruce, and higher extraction temperature and the use of organic solvents rather than water is needed to extract PSM

(Zeller, 2019). Pine had a higher wood content in the winter bark compared to the bark collected during the summer, which may explain the lower CT concentration in the pine winter bark since more other compounds were extracted from the wood. Bianchi et al. (2019) found that increasing the bark particle size would reduce the CT yield. Larger particle size may have contributed to the low CT concentration in the bark extract used in the lamb trial (**Paper III**) where the bark particle size was 1-3 cm, while it was 2 mm for the laboratory scale extracts (Table 5).
Spruce and pine did not only differ in CT yield and concentrations. Spruce and pine extracts had markedly different UV-profiles: the MS-analysis detected 878 compounds that were unique to pine when compared to spruce1, while 595 compounds were unique to spruce1 compared with pine (**Paper II** Supplementary Table S3). Clear differences in the MS-profiles of bark extracts from different conifer tree species, including Norway spruce, Scots pine, and Silver fir, (*Abies alba*) were also observed by Bianchi et al. (2015) and Brennan et al. (2020).

#### 5.1.2. The impact of solvent on CT characteristics

Different solvents and extraction procedures may extract various types of CT with higher or lower DP and various types of compounds other than CT. In a study on *P. nigra*, Bianchi et al. (2019) found that the water extract had lower CT concentration but CT with higher mDP compared to acetone extracts. Such a difference in mDP due to solvents used was not evident in the current study. Bianchi and colleagues (2019) concluded that the higher CT yields with organic solvents, as seen in our study, were due to the disruption of bonds between CT and the stroma of the plant cells.
High mDP and a low ratio between procyanidins and prodelphinidins (PC:PD-ratio) are thought to positively impact the antiparasitic effects of CT (Williams et al., 2014a). The PC:PD-ratio and mDP in the extracts used in this project, containing almost solely PC, agreed with previous findings (Bianchi et al., 2015; Athanasiadou et al., 2021). The analyses done in this work only captured the mean degree of polymerisation (mDP) of the molecules, and the exact size distribution remains unknown. However, since CT accounted for less than 15% of the extract DM, it is unlikely that variation in the antiparasitic efficacy of the extracts is because of difference in molecule size but rather due to contribution from other compounds.

#### 5.1.3. Solubility and effective dose of CT in reconstituted extracts

Bark extracted with organic solvents is not soluble in water. We used DMSO for reconstituting the dry extracts used in the *in vitro* trials and the mouse *in vivo* trial (**Paper IV**). DMSO is considered cytotoxic in concentrations higher than 10% and may display low-dose cytotoxicity

at concentrations above 1% in mice and rats (Willson et al., 1965; Galvao et al., 2014). This puts a maximum concentration limit on the bark extract used in **Paper I** and **II**. The Pine-W-Ac extract used in the mouse trial (**Paper IV**) was extracted with acetone and was reconstituted in DMSO. To achieve the desired extract concentration of 150 mg DM/mL, we could not use 100% DMSO and later dilute it to 1%, as we did for the *in vitro* trials, since this would give us an end concentration $1/10^{th}$ of the desired concentration. Instead, we dissolved Pine-W-Ac in 5% DMSO directly. This likely had an impact on the degree of reconstitution of the extract and ultimately the dose given the animals. The subsequent analysis of the CT content in the extract solution given the mice revealed a discrepancy between the initially calculated CT concentration and the analysed CT concentration in the reconstituted bark extracts. We concluded that 5% DMSO did likely not dissolve the acetone extracts fully and that the actual CT concentration in the product administered to the mice was ⅓ of the initially calculated dose. The extract used in the lamb trial (**Paper III**) was extracted with water and could be reconstituted with water, which gave us the ability to reconstitute the extract at a higher concentration. This might have contributed to the stronger antiparasitic effect of the extract in the lamb trial than in the mouse trial.

### 5.2. *In vitro* experiments

In the *in vitro* trials (**Paper I** and **II**) we found that pine bark extracted with organic solvents (acetone or methanol) resulted in the highest levels of antiparasitic activity. $IC_{50}$ was in a similar range for both GIN and *C. parvum*, which may indicate that bark extract can be efficient against a broad spectrum of parasites. $IC_{50}$ from other studies varies depending on plant species, extraction method, degree of purification of the extract, parasite species, and parasite stage, but on average, $IC_{50}$ lies in the range of 0.5-390 µg CT/mL (Table 14), which corresponds well with the levels of $IC_{50}$ found in our experiments (4.9, 22.6, and 25.4 µg CT/mL for *T. colubriformis*, *T. circumcincta*, and *C. parvum*, respectively). That the pine extracts had comparatively lower $IC_{50}$ than the spruce extracts may indicate that different compounds or different relative concentrations of compounds may contribute to the bioactivity of spruce and pine.
The GIA described in **Paper I** was preceded by a pilot study where we assessed the summer extracts at three different concentrations. The study provided us with the information needed to decide which bark extracts to assess in the main GIA trial with six concentrations and two replicates. The results of the pilot study indicated that bark extracted with organic solvents have a higher potential to inhibit *C. parvum* growth, with methanol extracted pine inhibiting parasite

growth at the two out of three levels tested. These results correspond with the findings in the EHA (**Paper II**).

The IFAT method used in the pilot is time consuming and strenuous to the eyes. It demands advanced equipment, and the immunofluorescent staining fades easily when exposed to light. We decided to rather use qPCR to detect and quantify *C. parvum* DNA, a method providing more reliable results faster (Shahiduzzaman et al., 2009).

In **Paper II**, the potential of antiparasitic activity of novel PSM from bark extract was additionally explored. A compound was identified as the monoterpenoid eugenol, previously reported to significantly reduce worm burdens in humans (El-kady et al., 2019) and another as sesquiterpenoid, e.g., calacorene, which has previously been detected in plant extracts active against the filaroid nematode *Onchocerca* (Metuge et al., 2014) (**Paper II** Supplementary figure S2). Putative annotation of the most abundant masses strongly correlating with the bioactivity of the extracts was done by database searches. However, since there is a high number of bioactive compounds in the bark and many of these compounds are often similar in structure, a reliable identification was not possible (Metsämuuronen and Sirén, 2019). Further identification of these masses would require MS/MS-analysis or nuclear magnetic resonance spectroscopy.

Based upon the results described in **Paper I** and **II**, we concluded that CT from spruce and pine bark were partly but not solely responsible compounds for the antiparasitic effects. It is also possible that CT may have synergistic effects together with other compounds in the bark extracts. Since the tested extracts were not pure CT, and since we did not use CT precipitating compounds like PVPP (Doner et al., 1993), we cannot conclude with certainty if CT were solely responsible for the dose dependent antiparasitic action. Previous studies on CT in plant extracts have left matters open, some concluding that CT is the compound responsible for the antiparasitic activity (Molan et al, 2014; Shepherd et al, 2022), while others have concluded with the opposite (Woolsey et al., 2022). To compare research outcomes in future studies, it is important to report the chemical compounds possibly responsible for the antiparasitic effect of the plant.

The reason for the lack of inhibition of *C. parvum* growth (**Paper I**) and GIN egg hatching (**Paper II**) of the water extracts might have been i) the compounds responsible for the inhibition of this specific parasite or parasite stage were not extracted with water, or ii) the extract levels were too low for these particular assays. Compared to other reports where organic solvents have been used in the extraction process, the $IC_{50}$ values of Barone et al. (2019) are relatively high

(Table 14). The findings of Barone and colleagues support the theory that the dose of the water extracts might have been too low in the GIA and EHA reported in **Paper I** and **II**, respectively. The difference in efficacy of the extracts depending on parasite life stage, may be due to characteristics of the superficial structures of the eggs and larvae (**Paper II)**. It has been suggested that the GIN eggshell is comparatively more permeable to some antiparasitic compounds than the L3 larvae cuticle, and differences in cuticle structure, permeability of the cuticle, and enzymes specific to parasite species or stages may influence the inhibition (Page et al., 2014; Rocha et al. 2020).

We observed that the intestinal nematode *T. colubriformis* egg hatching was more affected by the bark extract than that of the abomasal nematode *T. circumcincta*, while the opposite was the case for larval motility. Previous studies are inconsistent, with some reporting a higher *in vitro* efficacy against intestinal nematodes (Paolini et al., 2003; Cenci et al., 2007), whilst other find that the abomasal nematodes are more susceptible (Max et al., 2005; Minho et al., 2008; Molan, 2014; Quijada et al., 2015; Desrues et al., 2016a). Similarly, species-specific differences in the susceptibility of different GIN to anthelmintics have been documented *in vitro* (Gill and Lacey, 1993; 1998; Bartley et al. 2016).

**Table 14** $IC_{50}$ of CT containing browse plants with antiparasitic efficacy *in vitro* against common GIN in ruminants.

| Plant species | Parasite | Methods | $IC_{50}$ [µg DM/mL] | $IC_{50}$ [µg CT/mL] | %CT in extracts | Reference |
|---|---|---|---|---|---|---|
| *Lotus* spp., *Hedysarum coronarium, O. viciifolia, Dorycnium* spp., *Rumex obtusifolius* | *T. colubriformis* | EHA, LDA | NA | 36-92 | NA | Molan et al., 2002 |
| Flavan-3-ol gallate monomers | *T. colubriformis* | EHA, LDA | NA | 32-365 | 100 | Molan et al., 2003 |
| *O. viciifolia, Lotus* spp. | *C. oncophora, O. ostertagi* | LFIA, LEIA | 2.6-16.1 | 0.5-1.5 | 9.4-19.4 | Novobilsky et al., 2011 |
| *P. radiata* | *T. circumcincta, T. colubriformis* | EHA, LDA, LMIA | 157-1114 | 55-390 | 35 | Molan, 2014 |
| 18 plant materials, e.g., *P. sylvestris, B. pendula, Salix* spp. | *H. contortus, T. colubriformis* | LEIA | 63-300 | 8-159 | 13-83 | Quijada et al., 2015 |
| 10 East African browse plants | *H. contortus* | LEIA | 127-2037 | NA | NA | Mengistu et al., 2017 |
| *O. viciifolia, Acacia pennatula* | *H. contortus* | LEIA | 36.6-1003 | NA | NA | Chan-Pérez et al., 2017 |
| *L. corniculatus* | *H. contortus* | EHA | 660-9360 | NA | 0.15-6.02* | Barone et al., 2019 |

EHA: egg hatch assay; LDA: larval development assay; LFIA: larval feeding inhibition assay; LEIA: larval exsheathment inhibition assay, LMIA: larval migration inhibition assay. NA: Data not available.
*There was no correlation between CT content in the plant extracts and the antiparasitic activity.

### 5.3. *In vivo* experiments

The reduction in oocyst counts and faecal consistency in *Eimeria*-infected pre-ruminant lambs treated with water extracted spruce bark (SpruceS-H2O, Table 4, **Paper III**) is in line with findings from previous trials where CT rich supplements reduced FOC in *Eimeria* infected goats and lambs (Hur et al., 2005; Saratsis et al., 2012; Fraquelli et al., 2015). Lower FCS in treated lambs compared to infected untreated lambs during the post treatment period is to be expected since a high FOC is correlated with damage to the intestinal mucosa and a higher risk of diarrhoea in ruminants (Enemark et al., 2013).
We saw no reduction in parasitological parameters in mice infected with *H. bakeri*, but the growth and FI:BW-ratio were improved in the infected slow responder mice treated with Pine-W-Ac (**Paper IV**). Furthermore, the bark extract treated, infected slow responder mice demonstrated a higher tolerance to the parasite infection (i.e. had a higher carcass weight despite carrying a significant number of parasites), compared to the fast responder mice. Previous studies vary in their conclusions when assessing different plant extracts against GIN *in vivo*. Enejoh et al. (2015) and Gutu (2017) experienced a reduction in FEC and/or TWC when assessing the antiparasitic effect of plant extracts of Nigerian and Ethiopian origin in *H. bakeri* infected mice, while Githiori et al., 2003a, 2003b, observed significant but unsatisfactory antiparasitic effects (below the decided cut-off point of 70% reduction) when treating *H. contortus* infected sheep and *H. bakeri* infected mice with plant extracts of Kenyan origin. The plant extract doses used in these studies were in a range similar to the doses used in **Paper III** and **IV**, but as the active compounds in these studies were not known, it is not possible to make meaningful comparisons with the studies presented here.
In contrary to the increase in the performance in infected, slow responder mice in **Paper IV**, lambs in the *Eimeria* trial (**Paper III**) experienced reduced milk intake and growth and showed signs of constipation and diarrhoea during the treatment period. We assumed that the negative reactions were due to the bark extract treatment, which was confirmed by the cessation of the symptoms and increased appetite when discontinuing the treatment. Negative effects due to the administration of PSM rich extracts have been reported previously. Several studies report of detrimental reactions upon the intake of CT rich plant extracts, and some have reported increased mortality (Joslyn and Glick, 1969; Barry and McNabb, 1999; Githiori et al., 2003a; Gutu, 2017). Athanasiadou et al. (2001b) found that lambs fed a high dose of CT rich plant extract exhibited reduced FI and growth,

suggesting that the level of detrimental reactions upon the plant extracts may be dose dependent. These detrimental effects might be due to toxic reactions induced by the bark extracts consequently leading to a too high dose of detrimental compounds. It may also be related to the treatment method (drenching through a stomach tube *vs.* administration in the feed). When CT are administered by the feeding of CT-rich forage, e.g., sainfoin, CT may bind to salivary proteins and rumen digesta, which may protect the proximal part of the digestive tract from the astringent effects of CT, thereby releasing them more slowly into the digestive tract. This is not taking place when they are drenched directly into the stomach via a gastric tube (Luck et al., 1994; Athanasiadou et al., 2001a; Saratsis et al., 2012; Saratsis et al., 2016; Gutu, 2017). It has also been reported that ruminants are able to tolerate potential toxic effects of plant extracts by slowly adapting their ruminant microbiota (Smith et al., 2005). Consequently, pre-ruminant lambs will most likely be more sensitive to the detrimental effects of CT than adult ruminants.

Trade-off is defined as the act of balancing two features opposed to each other (Oxford University Press, 2023). It appears that it is possible to obtain a benefit from the administration of PSM rich extracts if the positive effects of the treatment (e.g., anti-parasitic) outweigh the negative effects (e.g., anti-nutritional) (Athanasiadou and Kyriazakis, 2004). In **Paper IV**, only the slow responder mice, i.e. the animals most susceptible to the parasite, benefited from the bark (i.e. a positive trade-off), whereas all the other treatment groups experienced a penalty from the bark extract administration (negative trade-off), confirming our hypothesis saying that infected slow responder mice would benefit more from the bark extracts than the fast responder mice. Although the lambs that received the bark extract (**Paper III**) experienced a lower parasite output compared to the controls, the trade-off was negative: the cost of the anti-nutritional effect outweighed the benefits of the antiparasitic treatment.

The difference in the impact of bark administration on the performance of animals could be attributed to the different compounds in the extracts. Although the two *in vivo* experiments were performed with different extracts in two different hosts and caution should be exercised in conclusions, we hypothesise that the CT dose required to achieve a positive trade-off (x mg CT/kg BW) is somewhere between the doses used in these two *in vivo* trials (250>x>42 mg CT/kg BW). Further assessment of the bark extracts in different concentrations against ovine *Eimeria* spp. in lambs and against GIN in ruminants *in vivo* would be required prior to draw final conclusions on the optimum concentration inclusion of CT in the diets.

## 5.4. General points across experiments

The outcomes presented in this thesis are in line with previous studies that highlight different conclusions when extracts are tested *in vitro* and *in vivo*. Although the water extracts did not show promising effects against *Cryptosporidium in vitro* (**Paper I**), SpruceS-H2O tested against another apicomplexan, *Eimeria* spp., in young lambs was found to exhibit a significant anticoccidial effect *in vivo* (**Paper III**). In contrast, Pine-W-Ac showed antiparasitic properties against nematodes *in vitro* (**Paper II**), but there was no antiparasitic effect *in vivo* (**Paper IV**). *In vitro* findings do not always agree with results of *in vivo* experiments. Compounds in the extracts not exhibiting antiparasitic effects *in vitro* may have synergistic effects with compounds in the host, showing their effect only after entering the animal. The difference in pH, microbiome, digestive enzymes, and metabolic processes in the various compartments of the intestinal tract may explain the variation in antiparasitic effect. PSM may undergo transformation as they pass through the intestinal tract. Hydrolysation by intestinal enzymes or modification by the intestinal microbiota might make them bioavailable or even absorbable. This may explain why crude extracts or even bark powder might show antiparasitic properties in parasitised animals although the same plant materials show no effect *in vitro*, or *vice versa* (Matthews et al., 1997; Touriño et al., 2009; Pérez-Jiménez and Torres, 2011). Furthermore, differences in the digestive systems of mice and ruminants may lead to outcomes more challenging to compare when assessing bark extracts against parasites *in vivo*. Factors such as variations in salivary proteins, intestinal microbiota, digestive enzymes, etc. will often influence on the efficacy but also on the level of unwanted side-effects following bark extract treatment (Robbins et al., 1991). Thus, further *in vivo* studies of bark extracts against *C. parvum* in calves and GIN in cattle or sheep are needed.

In summary, our data showed that the use of organic solvents for extraction results overall in more efficacious extracts, however antiparasitic activity was also present in some of the water extracts. Acetone and methanol are strictly regulated, highly flammable, and toxic organic solvents potentially harmful to humans (Klima- og miljødepartementet, 2004). Large scale production of bark extracts with the use of organic solvents is costly. The consequence of having to use organic solvents in the extraction process would be a more expensive product and could lead to the development of a less economically and environmentally sustainable product. Water extracts may be the easier and less costly direction when further exploring the antiparasitic potentials of bark extracts.

# 6. Conclusions and perspectives

Today, the common way of controlling parasite infections in livestock is by routine treatments with antiparasitic pharmaceuticals, often without preceding diagnostic. This uncritical drug use has contributed to development of widespread resistance against common antiparasitic drugs globally. When evaluating the efficacy of anthelmintic drugs for commercial use, it is desirable with a reduction in parasite load of >90% when defining it as efficacious (Geurden et al., 2022). However, model studies have indicated that reductions of around 75% may still achieve acceptable levels of control of GIN in sheep (Barnes et al., 1995). Several studies deemed PSM to be idle although the tested substances significantly reduced the FEC up to 20-50% (Githiori et al., 2003a, 2003b). Implementing such PSM as e.g., feed supplements in livestock may contribute to the reduction of the parasite load and reduce parasite contamination of pastures and environment. By decreasing the infection pressure on susceptible animals, PSM might mitigate the need of routine treatment with antiparasitic drugs and thus contribute to a decrease in the development of antiparasitic resistance. Still, the continuous provision of crops or other products with antiparasitic effect is difficult to envisage and is, like the sole reliance on drug use, not a sustainable way of controlling parasites. Future control of gastrointestinal parasites needs to include a diversity of strategies, including management, immunological, and biological approaches when available.

The results obtained in this project add essential knowledge to the insight of bioactive compounds in bark extracts of coniferous trees. Our findings confirm the hypothesis that bark extracts are active against several gastrointestinal parasites and that variation in the bark extract composition impact the antiparasitic activity measured.

Based upon the results of our experiments, the conclusions can be summarised as follows:

- Acetone and methanol extracted bark from *P. sylvestris* exhibited anti-cryptosporidial properties by inhibiting the development of *C. parvum* in cell cultures.
- Several bark extracts demonstrated high antiparasitic activity against *T. colubriformis* and *T. circumcincta in vitro*, inhibiting >90% of the egg hatching. The bark extracts with the highest inhibiting effect on egg hatching were those extracted with organic solvents, the bark from the sawmill (ring debarked), and the

bark harvested during the winter. Furthermore, pine bark extracts had a higher level of inhibition compared to the spruce bark extracts. *T. colubriformis* egg hatching was more affected than that of *T. circumcincta*, while the opposite was the case for larval motility.

- In contrast to EHA where bark extracted with organic solvents had a higher inhibitory effect, more of the water extracts inhibited larval motility to the level of the dead controls compared to acetone and methanol extracts.
- There was a significant negative correlation between CT and EHA $IC_{50}$ for *T. colubriformis*, which could not be seen for *T. circumcincta*
- Novel compounds with putative antiparasitic effect against ovine GIN *in vitro* included the monoterpenoid eugenol and a sesquiterpenoid, e.g., calacorene.
- Water extracted bark from *P. abies* collected in winter had anticoccidial properties against ovine *Eimeria* spp. *in vivo* but caused detrimental effects in the lambs, displayed as reduced feed intake, reduced body weight, and acute gastrointestinal signs like colic and diarrhoea.
- Acetone extracted bark from *P. sylvestris* harvested during the winter did not reduce parasitological parameters in mice infected with *H. bakeri*. The bark extract increased the performance and did not have a negative effect on the tolerance in the *H. bakeri* infected slow responding line (C57BL/6), which confirmed the hypothesis saying that infected slow responding mice would have a higher tolerance compared to fast responding mice (**Paper IV**).

Future research is needed to i) gain further knowledge about the actual antiparasitic compounds and their mode of action, alone or synergistically, ii) assess if *in vivo* experiments follow the results gained in the *in vitro* trials, iii) determine if the observed effect in mice applies to ruminants, iv) optimise the bark extract doses, timing and duration of the treatment, v) assess the long-term effect of the bark extracts on parasitism and performance by following the treated parasitised animals over a longer time span after discontinuation of the treatment, and vi) develop economic and practical sustainable application methods to facilitate the use of plant products against gastrointestinal parasites. We recommend assessing bark extracts, preferably extracted with water, against GIN in ruminants, against *C. parvum* in calves, and against coccidiosis in lambs.

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# 8. Publications and manuscripts

## Paper I

**Extracts of pine bark (*Pinus sylvestris*) inhibit *Cryptosporidium parvum* growth in cell culture**

**Berit M. Blomstrand**, Heidi L. Enemark, Øivind Øines, Håvard Steinshamn, Inga M. Aasen, Karl-C. Mahnert, Kristin M. Sørheim, Spiridoula Athanasiadou, Stig M. Thamsborg, Ian D. Woolsey. Parasitology research vol 120.8 (2021).

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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

# Extracts of pine bark (*Pinus sylvestris*) inhibit *Cryptosporidium parvum* growth in cell culture

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## Abstract

The widespread apicomplexan parasite *Cryptosporidium parvum* is responsible for severe gastrointestinal disease in humans and animals. The treatment options are limited, and the efficacy of available drugs is low. Bark contains condensed tannins (CT), which are bioactive compounds previously shown to inhibit parasite development. Here, we examined the anti-cryptosporidial properties of bark extract of Scots pine (*Pinus sylvestris*) against *C. parvum* by means of an in vitro growth inhibition test. We hypothesised that bark extracts would have dose-dependent inhibitory effects on the development of *C. parvum* in cell culture. Bark extracts from Scots pine extracted with acetone, methanol, and water as solvents were investigated using human colorectal adenocarcinoma cells infected with *C. parvum*. Oocysts were inoculated onto the cell monolayer and bark extract was added at seven different concentrations. Parasite growth inhibition was quantified by qPCR.

The acetone and methanol extracts demonstrated a sigmoid dose-dependent inhibition of *C. parvum*. The $IC_{50}$ values were 244.6 and 279.1 µg dry matter extract/mL, and 25.4 and 24.1 µg CT/mL, for acetone and methanol extracts, respectively. The $IC_{50}$ for both extracts were similar, both with regard to the dry matter concentration of each extract and to CT concentrations. Given the limited treatment options available for *Cryptosporidium* spp., the evidence generated in our study encourages further investigation into the in vitro and in vivo effects of pine bark extracts against *C. parvum.*

**Keywords** *Cryptosporidium parvum* · Pine bark · Condensed tannins

## Introduction

*Cryptosporidium parvum* is an apicomplexan parasite with a worldwide distribution and a high zoonotic potential. Oocysts excreted in the faeces are immediately infective. Transmission is via the faecal-oral route, directly from host to host, or indirectly via the environment, by ingestion of contaminated food or water (Kosek et al. 2001) or possibly via insect vectors (Graczyk et al. 2003). Globally, cryptosporidiosis is a significant cause of diarrhoeal disease in humans and animals, and *C. parvum* is one of the most frequently diagnosed agents, with possible fatal consequences in children and immunocompromised individuals (Innes et al. 2020). *Cryptosporidium* spp. may lead to chronic joint

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pain, fatigue, and post-infectious irritable bowel syndrome in humans (Carter et al. 2019). Similarly, *C. parvum* causes diarrhoea, loss of condition, and reduced growth rate in new-born calves, lambs, and other vertebrates (Smith 2008). Cryptosporidiosis in Norway is generally believed to be underdiagnosed (Nygård et al. 2003). A survey between 2001 and 2003 demonstrated *C. parvum* infection in 53% of the dairy farms included in the study ($n = 136$) (Hamnes et al. 2006a), and a study on wild cervids completed in the same time period concluded that *C. parvum* is widespread among the cervid population in Norway (Hamnes et al. 2006b). An outbreak of human cryptosporidiosis in Norway was first reported in 2006 and was related to contamination from calves (Robertson et al. 2006). In 2011 and 2014, two additional animal associated human outbreaks were reported (Rimšelienė et al. 2011; Lange et al. 2014).

The options for treatment and prevention of cryptosporidiosis in both animals and humans are limited and, in many cases, suboptimal. Presently, there are to our knowledge only two moderately effective pharmaceuticals applied for metaphylactic treatment of calves: halofuginone lactate and paromomycin (The European Commission 2020; US food and drug administration 2020). Nitazoxanide is the only licenced pharmaceutical for use in humans, and the drug has limited effect in immunocompromised patients. Currently, there are no vaccines against *C. parvum*, and the robust oocysts are highly resistant to the most commonly used disinfectants (Innes et al. 2020).

There is evidence that dietary plant secondary metabolites (PSM) possess antiparasitic properties, both in vitro and in vivo (Anthony et al. 2005; Hoste et al. 2015). For instance, condensed tannins (CT) have proven antiparasitic effects (Dhakal et al. 2015; Desrues et al. 2016b; Spiegler et al. 2017). Few studies have tested the activity of different PSM against *C. parvum* (Sreter et al. 1999; Shahiduzzaman et al. 2009; Teichmann et al. 2012, 2016; Gaur et al. 2018; Woolsey et al. 2019b), and even fewer have tested CTs against *C. parvum* in animals (Kim and Healey 2001; Derbakova et al. 2016). To our knowledge, bark extracts from pine (*Pinus sylvestris*) have not previously been tested systematically against *C. parvum* in cell cultures in vitro.

The current insufficient treatment options against cryptosporidiosis and the possibility to exploit large amounts of excess bark from the Norwegian forest industry offer a strong incentive to further explore novel approaches to control cryptosporidiosis including bioactive compounds from bark extracts. In this study, we assessed the anti-cryptosporidial properties of bark extract of Scots pine against *C. parvum* by means of an in vitro growth inhibition test (Slifko et al. 1997; Woolsey et al. 2019a). We hypothesised that bark extracts with high CT concentration would have dose-dependent inhibitory effects on the intracellular development of *C. parvum* in cell culture.

## Materials and methods

### Parasite material

*Cryptosporidium parvum* oocysts (Iowa strain) purchased from Bunch Grass Farm (ID, USA) harvested from suckling calves and isolated by sucrose gradient centrifugation within 14 days prior to delivery to the institute were stored at $2 \times 10^7$ oocysts/mL in 50 mL phosphate-buffered saline (PBS) with penicillin 1000 IU and streptomycin 1000 µg (2–7 °C). The viability of the oocysts was assessed within a week before the assays by staining oocysts in wet mounts with 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Campbell et al. 1992; Petersen and Enemark 2018). The oocysts ($\geq 100$) were counted using immunofluorescence microscopy, and the viability percentage was calculated (90%). This percentage was taken into account when calculating the concentration of viable oocyst in the stock solution.

The oocyst concentration was assessed within a week prior to the trial by diluting 10 µL stock solution with 990 µL PBS. Subsequently, 10 samples of 5 µL diluted stock solution were stained with Crypt-a-Glo (Waterborne Inc, LA, USA) according to the product instructions and counted using an immunofluorescence microscope fitted with a fluorescein isothiocyanate (FITC) filter.

### Cell culture

Human ileocecal colorectal adenocarcinoma (HCT-8) cells (ECACC, Salisbury, UK) (Upton et al. 1994; Joachim et al. 2018) were maintained as described by Woolsey et al. (2019a). A cell subcultivation was performed twice a week, when 80% confluent, using trypsin/EDTA (Sigma Aldrich, MO, USA). The cell cultures were incubated at 37 °C, 5% $CO_2$ and 100% humidity. HCT-8 cells ($2 \times 10^5$ per well) were seeded onto Nunclon® 96-well plates (Sigma Aldrich, MO, USA) and incubated till 80% confluence (24 h).

### Bark extraction and determination of the concentration of condensed tannins (CT)

Bark from *P. sylvestris* was ring debarked and collected in a sawmill in eastern Norway (Bergene Holm AS, Kirkenær) in March 2017 and stored at − 20 °C until use. The bark was milled to chips of 0.5–2 cm in a hammer mill (Schutte Mini Mill, Buffalo, NY, USA), freeze-dried, and ground to particle sizes of approximately 2 mm in a coffee grinder. Aqueous acetone (70%), aqueous methanol (80%), and water were used as solvents producing three different extracts, PS-Ac, PS-Me, and PS-H2O, respectively. PS-Ac and PS-Me were prepared by adding 200 mL solvent to 10 g fine-ground

bark, in an ultrasonic cleaning bath at full power (60 W) for 20 min (temperature increasing from 20 to 30 °C). For PS-H2O, 100 ml water was added to 10 g fine-ground bark, the extract was separated, and the procedure repeated. The temperature was 65 °C, and the time was 1 h for each of the two steps. The extracts were isolated by centrifugation and filtration (Whatman no 1) using vacuum suction. The organic solvents (acetone or methanol) were removed by evaporation (Rotavapor, 38 °C) before freezing and freeze-drying. For PS-H2O, the combined extracts were concentrated in a vacuum centrifuge at 65 °C (Thermo Scientific Savant SC250EXP SpeedVac Concentrator) to approximately 50% of the volumes, before freezing and freeze-drying. The dried extracts were stored at − 20 °C until further use.

Total CTs were quantified by the butanol-HCl assay. Freeze-dried extracts were dissolved in methanol (80% in water) and analysed with cyanidin-HCl as standard (Grabber et al. 2013), using the conventional reagent without acetone, 2.5 h, and absorbance reading at 545 nm.

To determine the relative monomer composition, the mean degree of polymerisation (mDP), and cis–trans-ratio, the methanol extracts were thiolysed with cysteamine hydrochloride and analysed by HPLC (Bianchi et al. 2015) using an Ascentis Express C18 column (15 cm × 2.1 mm, 2.7 µm, Supelco) and a flow rate of 0.3 mL/min.

Immediately prior to use, the dried extracts were dissolved in 100% dimethyl sulfoxide (DMSO), vortexed > 1 min, and diluted down to final extract concentrations of 300 µg/mL, 250 µg/mL, 200 µg/mL, 150 µg/mL, 100 µg/mL, and 50 µg/mL dry matter extract in 1% DMSO using maintenance medium (MM; RPMI-1640 (Biowest, France) supplemented with 5% v/v bovine foetal serum, 5% v/v horse serum, 1 mM sodium pyruvate, penicillin 100 U/mL, streptomycin 100 µg/mL, and amphotericin B 0.25 µg/mL (all from Sigma Aldrich, MO, USA)).

## Parasite inoculation onto cell monolayer and addition of extract

Before inoculation, the oocysts went through an excystation protocol as described earlier (Slifko et al. 1997; Woolsey et al. 2019a): $2 \times 10^6$ viable oocysts (120 µL) and $2 \times 10^6$ inactivated oocyst (IO) solution (120 µL stock solution previously incubated at 70 °C for 30 min) were suspended in bleach (120 µL 5.35% sodium hypochlorite and 960 µL MilliQ water) for 10 min on ice, and then centrifuged at 4000 × g for 4 min at 4 °C. The supernatant was aspirated, and 14 mL MilliQ water was added to each of the suspensions. The solutions were vortexed for 10 s and spun again (4000 × g, 4 min, 4 °C). The supernatant was aspirated down to 200 µL and the oocysts were re-suspended in 9.8 mL pre-warmed MM (37 °C), which gave a final oocyst concentration of $2 \times 10^5$ oocysts per millilitre.

Oocyst solution (100 µL) was added to each monolayer well with a multichannel pipette with IO and MM as controls ($n = 3$). The plates were incubated for 4 h (37 °C, 5% $CO_2$, 100% humidity), subsequently the wells were washed 5 min with PBS, and bark extracts were added to the live oocyst wells at the extract concentrations as mentioned above (biological repeat, $n = 3$ for each concentration). DMSO (1%) in MM was added to the negative MM controls and the IO wells, and paromomycin (500 µg/mL) in MM was added as positive control ($n = 3$) (see supplementary material for a plate overview). Each plate was duplicated.

## Cell viability

After 44-h incubation (37 °C, 5% $CO_2$, 100% humidity) (Slifko et al. 1997), the cell viability was determined using an integrated non-destructive water-soluble tetrazolium salt assay (WST-1 cell proliferation assay kit; Roche, Basel, Switzerland): MM was aspirated from the wells and 10-µL WST-1 10% working solution was added to each well. The plates were incubated for > 30 min and the optical density was read at 450 nM ($OD_{450}$). The plates were read every 15 min until all negative control wells had reached an $OD_{450}$ value of > 1. Monolayers were considered viable if OD values in the extract wells were > 75% of the negative controls (Teichmann et al. 2012).

## DNA extraction

The wells were washed (3 × 5 min) with 100 µL PBS (37 °C), and 20 µL proteinase K and 180 µL ATL buffer (Qiagen, Hilden, Germany) were added to each well. Then, the plates were incubated (56 °C, 90 min) and the content of each well was aspirated into 1.5 mL Eppendorf tubes. DNA extraction was performed by means of QIAcube DNeasy® blood and tissue kit (Qiagen, Hilden, Germany) (tissues and rodent tails protocol, elution volume: 200 µL).

## qPCR assay

The growth of *C. parvum* in the cell cultures was assessed by measuring the amount of *C. parvum* specific DNA. Quantitative polymerase chain reaction (qPCR) was performed using 18S primers (Morgan et al. 1997) (Cryp18S_Frt 5'-AGTGACAAGAAATAACAATACAGG and Cryp18S_Rrt 5'-CCTGCTTTAAGCACTCTAATTTTC-3') with hydrolysis probe (Amann et al. 1990; Keegan et al. 2003) (Integrated DNA Technologies, Coralville, IA, USA). Quantitative PCR reactions were conducted in a total volume of 25 µL containing 3 µL of each forward and reverse primers, 0.6 µL probe, 12.5 µL 2 × Brilliant III Fast Master Mix (Agilent Technologies, Santa Clara, CA, USA), 5.4 µL $dH_2O$ (nuclease-free water, Integrated DNA Technologies,

Coralville, IA, USA), and 0.5 µL DNA template. Reactions were run on 96-well white-bottomed qPCR plates (Bio-Rad, Hercules, CA, USA).

From each biological replicate plate, extracted DNA from each biological repeat ($n = 3$) was run in duplicate (2 technical repeats) on the qPCR plate. DNA template from a separate oocyst titration study (cell monolayer infected with $5 \times 10^4$–50 oocysts/well, 2 biological and 2 technical repeats per biological repeat per concentration) was added to each qPCR run to serve as DNA standards (Woolsey et al. 2019a).

The cycling conditions were initially 95 °C for 2 min followed by 44 cycles (95 °C for 10 s, 58 °C for 10 s, 72 °C for 20 s) on C1000 Touch (BioRad, Hercules, CA, USA). The definition of the cycle threshold was set at the beginning of the rise of the log-linear phase, and a quantification cycle (Cq) above 40 cycles was defined as negative based on the non-template controls included on each qPCR plate (Bustin et al. 2009). To analyse the amplification curves, BioRad CFX manager v3.1 software was used and the Cq values from the standard templates with known oocyst number were used to calculate the DNA level of the test templates. The data used in the subsequent analyses were defined as *C. parvum* DNA quantity relative to the DNA standard templates ($DNA_{rel}$).

### Statistics

qPCR standard curves were produced in BIORAD CFX Manager v3.1. For each extract (PS-Ac, PS-Me, and PS-H2O), data used for statistical analyses was collected from two independent parasite growth inhibition assays with three biological repeats per extract concentration and two technical repeats per well per assay. By plotting the seven extract concentrations as independent variables and $DNA_{rel}$ as dependent variable, it appeared that the relationship was inverse sigmoid for PS-Ac and PS-Me. We therefore analysed the data using a non-linear regression procedure (NLIN) in SAS (SAS release 9.4, SAS Institute, Cary, NC), and applied this to calculate the half maximal inhibitory concentration ($IC_{50}$) for both PS-Ac and PS-Me. Two-sided $p$ values of $\leq 0.05$ were considered significant. The results from the PS-H2O assay were plotted into a simple (x,y)-plot and fitted a linear regression model.

## Results

### Characterisation of bark extracts

The extract DM yield per gram bark was 55, 47, and 54 mg/g for PS-Ac, PS-Me, and PS-H2O, respectively. The mean CT concentrations of PS-Ac, PS-Me, and PS-H2O were $95.3 \pm 3.0$, $90.9 \pm 2.2$, and $33.3 \pm 2.5$ mg/g DM extract ($\pm$ s.d.) (9.5, 9.1, and 3.3%), respectively. The final CT concentrations in the dilutions thus ranged from 4.8 to 28.6, 4.5 to 27.3, and 1.7 to 10.0 µg/mL in PS-Ac, PS-Me, and PS-H2O, respectively. For the methanol extract, the PC:PD ratio was > 99:1, cis:trans ratio 79:21, and mDP 5.8.

### Cell viability

HCT-8 monolayer viability was within threshold limits of 75% of the negative controls in all wells on all plates except for two wells (yielding 70.0 and 73.8% of the negative controls), which were excluded from the later calculations.

### C. parvum growth inhibition assay

Based on model property and the result of the statistical analysis (model mean square error and the value of the parameter estimates relative to their respective standard error), the following model (Model 1) was chosen for the extracts PS-Ac and PS-Me when testing the extract dry matter (DM) concentrations for anti-cryptosporidial effect ($p < 0.01$):

$$\mu_y(x) = \frac{\gamma}{1 + \left(\frac{x}{\delta}\right)^{\theta}}$$

$\mu_y(x)$ is the $DNA_{rel}$ of a given value $x$, $x$ is the extract DM concentration, $\gamma$ is the value of $\mu_y(x)$ when no extract is applied ($x = 0$), $\delta$ is the $x$ value at the inflection point (concentration of the strongest decline in $DNA_{rel}$), which is equivalent to $IC_{50}$, and $\theta$ describes the slope of the curve, i.e., the reduction in $DNA_{rel}$ per unit increase in extract (Table 1). Based on the calculation, we found a dose-dependent relationship between the extract DM concentration (PS-Ac and PS-Me) and $DNA_{rel}$ (Fig. 1a and 1b).

We used the same principles to investigate the relationship between CT concentration and $DNA_{rel}$. Data from PS-Me (CT) converged using Model 1, and its estimated parameters are presented in Table 1. The data from PS-Ac (CT) converged when using the following Model 2 ($p < 0.0001$):

$$\boldsymbol{E(y) = \alpha \cdot e^{-\beta \cdot e^{k \cdot x}}.}$$

$E(y)$ is the oocyst concentration of a given value $x$, $x$ is the extract CT concentration, α and $\beta$ describe the $DNA_{rel}$ when no extract was applied ($x = 0$), and $\beta$ and $k$ form the slope of the curve. Table 2 lists the estimated parameters for the PS-Ac CT curve.

The $IC_{50}$ for PS-Ac and PS-Me extracts were estimated to 244.6 and 279.1 µg DM/mL (24.1 and 25.4 µg CT/mL), respectively.

**Table 1** Parameter estimates describing inhibition related to dry matter (DM) and CT concentrations

| Parameter | Extract (DM) | | Extract (CT) | Extract (CT) |
|---|---|---|---|---|
| | PS-Ac | PS-Me | PS-Me | PS-$CT_m$ |
| Model $p$ value | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| $\gamma$ | 24,125.4 (1926.5) | 20,103.8 (2755.8) | 20,103.8 (2755.8) | 21,632.5 (1448.6) |
| $\delta$ ($IC_{50}$) (μg/mL) | 244.6 | 279.1 | 25.4 | 26.2 |
| $\theta$ | 5.2594 (2.3817) | 14.6109 (15.1513) | 14.611 (15.1514) | 11.2822 (8.015) |

Data converged when using the function $\mu_y(x) = \frac{\gamma}{1+\left(\frac{x}{\delta}\right)^{\theta}}$, where $x$ is the concentration, $\gamma$ is the value of $\mu_y(x)$ when $x = 0$, $\delta$ is the $x$ value giving $\mu_y(x) = \frac{1}{2} \cdot \mu_y(0)$ ($IC_{50}$), and $\theta$ gives the slope of the curve

For PS-Ac, $DNA_{rel}$ values were not significantly different from the positive control for concentrations higher than 200 μg/mL ($P > 0.05$). For PS-Me, this was observed in concentrations above 250 μg/mL ($P > 0.05$).

The results from the PS-H2O assay could not be fitted in any reasonable way by any of the sigmoid models we tried. A simple (x,y) plot of the observed data together with a fitted linear model shows no statistically significant relationship between PS-H2O extract concentration and $DNA_{rel}$ (Fig. 1c).

The CT data from all three extracts were merged (PS-$CT_m$) and the relationship between PS-$CT_m$ and $DNA_{rel}$ was estimated with extract as random variable. The data converged using Model 1, and estimated parameters can be seen in Table 1. We found a dose-dependent negative correlation between the PS-$CT_m$ concentration and $DNA_{rel}$ (Fig. 1d). The $IC_{50}$ for PS-$CT_m$ was estimated to 26.2 μg CT/mL.

## Discussion

This study revealed a dose-dependent anti-cryptosporidial effect of acetone and methanol bark extracts (PS-Ac and PS-Me) from *P. sylvestris*. Our findings support the results of other studies addressing the possible effects of pine extracts against protozoa. Kim and Healey (2001) demonstrated in vivo that ethanol extracted bark extracts from *Pinus pinaster* reduced the oocyst shedding and improved the overall health of mice infected with *C. parvum*. Similarly, a study of goats infected with *Eimeria* spp. demonstrated that feeding pine (*Pinus densiflora*) needles significantly reduced the oocyst excretion compared to untreated controls. In the treated goats, oocyst excretion 10 days post-treatment was reduced by 93%, relative to the pre-treatment oocyst excretion (Hur et al. 2005). In vitro antiprotozoal effect of bark extract has also been shown against *Eimeria* spp. of poultry, where water extracted pine bark reduced the oocyst sporulation by 77.2–86.4% relative to untreated controls (Molan et al. 2009).

Importantly, we showed that both PS-Ac and PS-Me were, in the highest concentrations, no different from their respective positive controls. This shows that the extracts have an in vitro anti-cryptosporidial effect close to that of paromomycin, one of the few pharmaceuticals registered for use in animals.

Furthermore, merging results of PS-Ac and PS-Me, we described a dose-dependent relationship between CT concentration and $DNA_{rel}$. Previous evidence is not conclusive on whether the CTs in the plant extracts are the compounds responsible for their antiparasitic activity. In some cases, the observed activity is closely linked to CT concentration (Novobilsky et al. 2011, 2013; Desrues et al. 2016a), while others have been unable to demonstrate such a link (Castañeda-Ramírez et al. 2017; Hernández-Bolio et al. 2018; Esteban-Ballesteros et al. 2019). Possible explanations to this divergence include the degree of purity of the extracts, and it is evident that bioactivity of CTs is strongly related to chemical structure, e.g., type of monomers, mDP and degree of galloylation (Mueller-Harvey et al. 2019).

In this trial, the extracts with the highest CT concentration also demonstrated the highest antiprotozoal effect; thus, it is likely that CTs play an important role in the interaction between parasites and extracts. Nevertheless, the possibility that other PSM, such as other polyphenols or non-phenolic compounds like sesquiterpene lactones, may also have a role in the activity observed in this study cannot be excluded as previously suggested (Barone et al. 2019).

In our study, PS-Ac exhibited anti-cryptosporidial effect at a lower dose but had a lower rate of reduction compared to PS-Me (cf.$\theta$, Table 1). Additionally, the $IC_{50}$ was lower for PS-Ac than for PS-Me. The CT concentration of both extracts was similar and showed approximately the same inhibition of the parasitic development. Yet, it was not possible to fit the data generated from both extracts to the same model. This may support the theory that unknown, non-CT components present had a clear influence on the anti-cryptosporidial effect and/or that differences in chemical composition were related to the extraction method.

To our knowledge, $IC_{50}$ values for pine bark extracts tested against *C. parvum* have not been calculated before. Our findings were in accordance with Williams et al. (2014) who tested purified and fractionated acetone pine bark extracts against *Haemonchus contortus* in a larval migration

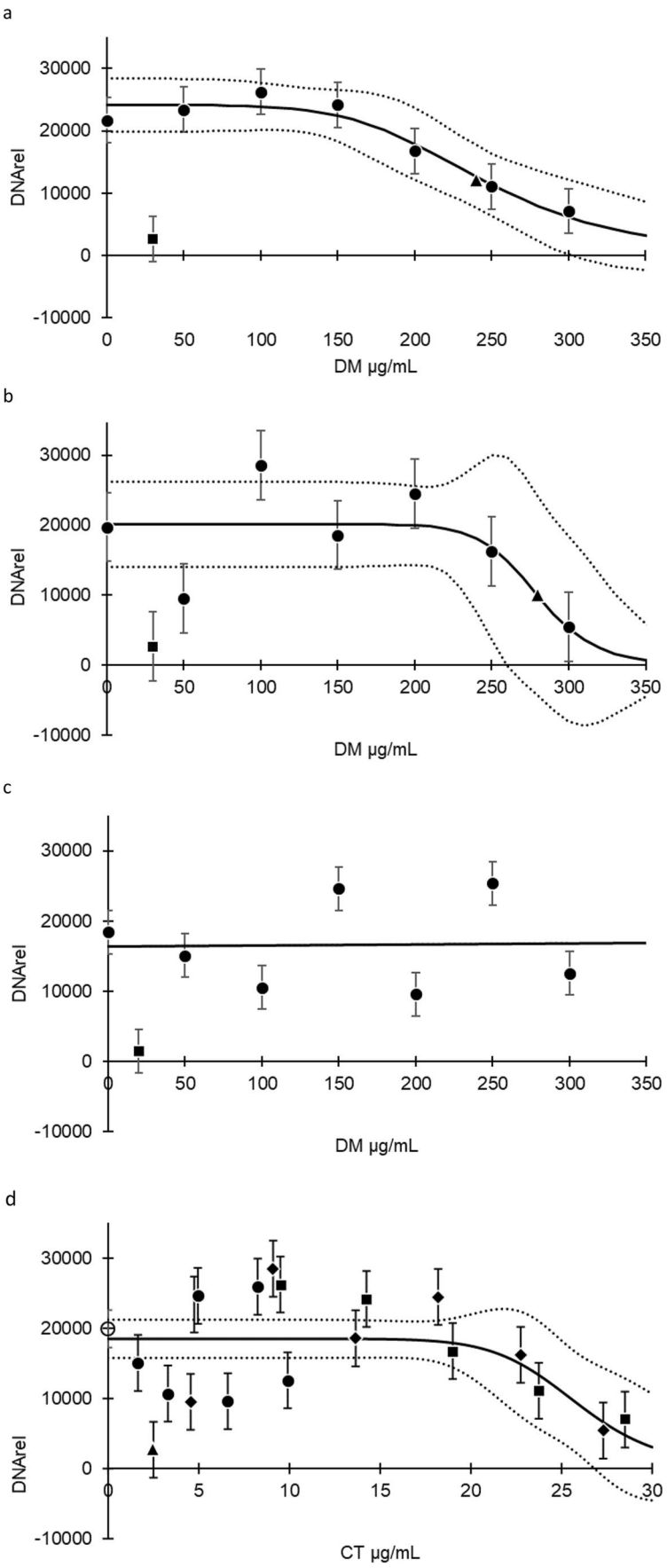


**Fig. 1** Inhibition of *Cryptosporidium parvum* development (black line) with increasing dry matter (DM) (**a**–**c**) or condensed tannins (CT) (**d**) concentrations from acetone (**a**), methanol (**b**), and water (**c**) based bark extracts from Scots pine (*Pinus sylvestris*). Black circles, mean DNA content relative to the standards ($DNA_{rel}$) of observed values for each extract dose. Square, positive control (paromomycin). Triangle, half maximal inhibitory concentration ($IC_{50}$). Error bars on observed values represent standard error of the mean (SEM). Area between dashed lines: 95% confidence interval. (**d**) DM concentrations was converted to CT concentrations, and all extracts were merged ($PS\text{-}CT_m$). $PS\text{-}CT_m$ exhibited a negative correlation with $DNA_{rel}$. The symbols are mean of observed values of PS-Ac (squares), PS-Me (routes), and PS-H2O (black circles). The open circle displays the mean of the negative controls and the triangle the positive control

**Table 2** Parameter estimates describing inhibition related to CT concentration for PS-Ac

| Parameter | Extract (CT) PS-Ac |
|---|---|
| Model $p$ value | $<0.0001$ |
| $\alpha$ | 24,674.7 (2828.3) |
| $\beta$ | 0.0114 (0.026) |
| $k$ | 0.1713 (0.0865) |
| $IC_{50}$ (µg/mL) | 24.1 |

Data converged when using the function $\mu_y(x) = \alpha \cdot e^{-\beta \cdot e^{k \cdot x}}$, where $x$ is the CT concentration; $\alpha$ and $\beta$ give the oocyst level when $x$=0; and $\beta$ and $k$ describe the slope of the curve. $IC_{50}$ is the half maximal inhibitory concentration for each extract

inhibition test and estimated $IC_{50}$ to be 40.4 µg CT/mL. Molan (2014) found $IC_{50}$ values of 69 µg CT/mL in a *Trichostrongylus colubriformis* larval development inhibition assay with purified acetone pine bark extracts containing 35% CT. It has previously been shown that a high mDP and low PC:PD ratio are of importance when it comes to the antiparasitic activity (Mueller-Harvey et al. 2019). PS-Ac and PS-Me had a low CT percentage, low mDP, and negligible amount of PDs, which was generally in accordance with previous studies (Matthews et al. 1997; Bianchi et al. 2015, 2019; Desrues et al. 2016a), yet the anti-cryptosporidial effect was significant with a low $IC_{50}$. This supports the assumption that other components may have contributed to the inhibition of the development of *C. parvum*.

For PS-H2O, there was no statistically significant relationship between the extract DM concentration and $DNA_{rel}$ (Fig. 1c). Barone et al. (2019), on the other hand, tested 51 *Lotus corniculatus* water extracts in vitro and found dose-dependent anthelmintic activity in several extracts, demonstrating that the use of water as a solvent does not preclude the extraction of active antiparasitic compounds. The lack of biological activity can be explained by the fact that we were not able to achieve a high enough concentration (DM or CT) or that the active components cannot be extracted using water as solvent.

Our results confirmed that acetone and methanol solvents have a higher ability to extract proanthocyanidins (CTs) from pine bark compared to water. This solvent-dependent difference in CT extractability is in accordance with Ramos et al. (2013), who found that water extracted bark extract from *P. sylvestris* had a high extract yield but a low concentration of total phenol and CT compared to extracts produced with methanol and acetone as solvents (Ramos et al. 2013). A possible reason for the low CT yield when using water as solvent is explained by Matthews et al. (1997) who describe direct linkages between non-extractable CTs and the cell wall matrix and weaker CT-CT linkages extractable with aqueous methanol but not with water. Despite the lack of anti-cryptosporidial effect of PS-H2O at the concentrations tested, it is important not to dismiss water extracts as inactive. Acetone and methanol are strictly regulated (Klima- og miljødepartementet 2004), highly flammable, toxic organic solvents potentially harmful to humans. Large scale production of bark extracts with the use of organic solvents is costly, and potential production sites are hard to come by. Water, on the other hand, is non-toxic to living organisms, leaves a lower environmental footprint and is less costly and easier to use for large scale production of extracts. We recommend further in vitro studies to test pine water extracts against *C. parvum* in higher concentrations.

Cryptosporidiosis is considered a neglected disease and reducing oocyst shedding in animals is an important One Health goal as it reduces the risk of disease in humans (Innes et al. 2020). If pine bark extracts can be used to combat livestock cryptosporidiosis, they may be an environmentally friendly alternative to the current pharmaceuticals and constitute a step towards minimising contamination of drinking water and subsequent human cryptosporidiosis.

## Conclusion

Both acetone and methanol extracts of *P. sylvestris* showed marked anti-cryptosporidial properties by inhibiting the development of *C. parvum* in HCT-8 cell cultures. The effect of the bark extracts was dose-dependent with $IC_{50}$ values for CT almost similar between extracts (approximately 25 µg CT/mL). At the highest concentrations, the inhibitory activities were similar to that of existing drugs. A dose-dependent effect could not be confirmed for the water extract. Ultimately, in vitro testing should be followed by in vivo experiments with pine bark extracts to assess their applicability and relevance for disease control in animals. We also suggest further exploration of the anti-cryptosporidial effect of water extracts.

## Data and materials availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00436-021-07220-w.

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## Declarations

**Conflicts of interest** The authors declare no competing interests.



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## Paper II

### The antiparasitic activity of Nordic conifer bark against gastrointestinal nematodes: condensed tannins, novel compounds, and sources of variation

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(Manuscript)

# The antiparasitic activity of Nordic conifer bark against gastrointestinal nematodes: condensed tannins, novel compounds, and sources of variation

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**Keywords**: Pine, spruce, bioactive, egg hatch inhibition, larvae motility, LC-MS, extraction solvents; ovine

**Abstract:**

Parasitic gastrointestinal nematodes (GIN) threaten the health and productivity of small ruminant productions systems worldwide. As resistance against anthelmintic drugs continues to spread, alternative control options are urgently needed. Plant-based solutions may offer a viable alternative, where certain plant molecules, such as condensed tannins (CT), have demonstrated antiparasitic activity against GIN. Leveraging the antiparasitic potential of these bioactive molecules is, however, complex, where plant species, extraction process and seasonality can impact bioavailability and efficacy. The objective of this study was to assess the impact of a comprehensive set of factors on the antiparasitic activity of bark extracts of Norwegian conifers *in vitro* and identify compounds that may be associated with the antiparasitic activity of bark. The study used *in vitr*o assays targeting morphologically distinct life stages of ovine GIN: the egg hatch assay (EHA) and the larval motility assay (LMA). In depth characterisation of the chemical composition of the bark extracts was carried out based on chromatographic separation, UV-absorbance, and molecular mass profiles, to identify compounds that may be responsible for the activity. Three key findings emerged: i) there was variation in the activity of bark extracts observed attributed to tree species, extraction process and seasonality, from low to high reaching up to 100% of efficacy. These results confirm the potential of Norwegian bark extracts as a tool in GIN control; ii) variation in susceptibility of the two nematode species and parasite stages tested was also evident, with *Trichostrongylus colubriformis* egg hatching being affected more than *Teladorsagia circumcincta;* motility of L3 was differentially affected; iii) the presence of CT and numerous other compounds was associated with the observed anthelmintic activity. These findings add new insights to a growing toolkit of alternative parasite control strategies that can complement the use of anthelmintic drugs for effective parasite control, to reduce the rate of the development of anthelmintic resistance in livestock.

## Introduction

Gastrointestinal nematode (GIN) infections are a key threat to the health and productivity of small ruminant farming systems throughout the globe. With widespread resistance against all major antiparasitic drug classes, the current parasite control situation is tenuous. No new drug families have reached the livestock market since monepantel (trade name Zolvix®) was launched in 2009 (Bartley et al. 2015) and novel compounds are urgently needed. Increasingly, antiparasitic discovery is looking towards biopharmaceuticals, including the broad repository of plants that produce antiparasitic molecules (Trowbridge, 2014). A group of plant secondary metabolites known as condensed tannins (CT) are of particular interest; several CT-rich plants have exhibited high antiparasitic activity against key GIN stages *in vitro* (reviewed by Hoste et al. 2012). *In vivo* studies using CT-rich forages, such as *Sericea lespedeza* and *Onobrychis viciifolia* Scop., have further demonstrated that moderate consumption by small ruminants can reduce GIN burden (Hoste et al. 2006; 2015).

The opportunity to cultivate such forages can, however, be limited in regions with cooler climates, such as the Scandinavian countries. Instead, the bark of Scandinavian coniferous trees has been documented to contain CT (Matthews et al. 1997) and the antiparasitic potential of bark extracts has recently been demonstrated *in vitro* (Athanasiadou et al. 2021). For countries with a substantial sawmill industry where bark is a by-product, the CT contained within may offer a novel GIN control solution as a feed additive, while reinforcing the circularity of local economy. Recent findings documented considerable variability in the antiparasitic activity of bark extracts, as a function of different tree species, their age at processing, CT type and relative CT content against the ovine GIN *Teladorsagia circumcincta* (Athanasiadou et al. 2021). The production of specific plant compounds has also been found to vary across a range of other factors including rainfall, soil, temperature, use of pesticides, etc, (Nixon et al. 2020), with their relative bioavailability depending on the extraction process used (Ali et al. 2021); all of these are having an impact on compound composition in bark extracts, which may influence their antiparasitic activity.

In bark, a small part of the CT is free and can be extracted, and these CT constitute only a small part of the total extractives (Athanasiadou et al. 2021; Bianchi, 2016; Matthews et al., 1997). Polar solvents, such as water and water-miscible solvents like methanol, ethanol, or acetone, are used to extract CT. These solvents also extract other polyphenols, including hydrolysable tannins and a wide range of other flavonoids, some of which may be bioactive against parasites. Chemical characterisation of bark extracts is often limited to analyses of total phenolics, CT, sugars etc., but a more comprehensive characterisation of the complex extracts can be obtained by liquid chromatography coupled with mass spectrometry (LC-MS), as recently reported for bark of Norway spruce (*Picea abies*) (Brennan et al., 2020) and African birch (*Anogeissus leiocarpus*) (Orlando et al., 2019). Yet, correlating bioactivity to specific compounds in the chemical profile can be challenging, where more than one compound, or synergies between compounds, may be responsible for the observed effects (e.g., Valente et al. 2021).

The present study aimed to build upon results obtained by Athanasiadou et al. (2021) by assessing the impact of a comprehensive set of factors on the antiparasitic activity of spruce and Scots pine bark extracts from Norway, including collection season, extraction solvent, and level of inclusion. The bark extracts were characterized, and bark compound abundance was quantified using LC-UV and LC-MS

analyses. Their relative antiparasitic activity was assessed *in vitro* against both the eggs and the infective larval stages of two economically important ovine GIN species, *Trichostrongylus colubriformis* and *T. circumcincta*. The associations between compound abundance and antiparasitic efficacy have resulted in identification of candidate compounds in bark and provided new insights in the potential use of plants in the sustainable control of GIN.

## Materials and methods

### Bark sampling and preparation of bark extracts

Six batches of bark were collected over two collection periods: summer (S), covering July-August 2017, and winter (W), covering February-March 2018. Bark from Norway spruce (*P. abies* L.) was sampled from a sawmill (S1) and a pulp mill (S2), while bark from Scots pine (*Pinus sylvestris L.*) (P) was from a sawmill. In the pulp mill, the logs were sprayed with cold water prior to debarking. Water was then removed from the bark by pressing before the bark was shredded. The debarking process at the sawmills did not include water, and the bark was not shredded. Each batch contained equivalent amounts of bark from three partial samples. The partial samples were collected at the mills' debarking plant once a week for a three-week period. About 10 kg of each batch were stored in plastic bags and frozen at -15°C. The six batches were abbreviated to reflect the tree species (spruce or pine), mill (1 or 2) and season (summer or winter) and referred to as spruce1-summer (S1-S), spruce1-winter (S1-W), spruce2-summer (S2-S), spruce2-winter (S2-W), pine-summer (P-S) and pine-winter (P-W).

For preparation of the bark extracts, the bark was milled, and extracts were produced as described by Blomstrand et al. (2021) using three different solvents: water, 70% acetone in water, and 80% methanol in water. This produced three different extracts from each of the six bark batches mentioned above and included in the bark code with the addition of -water, -acetone and -methanol. Prior to use, the dried extracts were reconstituted in 50% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS), vortexed >1 min, and diluted with PBS to reach a final extract level of 1000, 500, 250, 125, and 62.5 µg dry matter (DM)/ml in 1% DMSO.

### Quantification and characterisation of condensed tannins

Total CT were quantified by the butanol-hydrochloric acid (butanol-HCl) assay. The freeze-dried extracts were dissolved in methanol (80% in water) and the analyses were carried out using the conventional reagent without co-solvents, 2.5 h incubation, and absorbance reading at 545 nm, with cyanidin-HCl as standard (Grabber et al. 2013). The relative contents of procyanidins (PC) and prodelphinidins (PD), which are the two main types of CT, the mean degree of polymerisation (mDP), and cis-trans-ratio were determined for the methanol extracts by thiolysis with cysteamine hydrochloride and analysis by High Performance Liquid Chromatography (HPLC) (Bianchi et al. 2015) using an Ascentis express C18 column (15 cm x 2.1 mm, 2.7 µm, Supelco) and a flow rate of 0.3 ml/min.

Characterization of bark extracts by Liquid Chromatography – Mass Spectrometry

The bark extracts were analysed using an Agilent 1200 Series Liquid Chromatography – Mass Spectrometry (LC-MS) system equipped with an Ascentis express C18 column (15 cm x 2.1 mm, 2.7 µm, Supelco) connected to a Diode Array Detector (DAD) and an Agilent 6520 Quadruple Time-of-Flight (QTOF) mass spectrometer. 25 mM formic acid [A] and acetonitrile [B] were used as mobile phases. The concentration of mobile phase [B] was increased from 0% to 90% over a period of 17 min by the following steps: 0% for the first 3 min, then linear gradients from 0 to 8.5% in 2 min, to 50% in 10 min and to 90% in 2 min. The concentration of [B] was then reduced to 0% over a 0.5 min period. The flow rate was 0.3 ml/min, and the column thermostat was maintained at 30 °C. The QTOF was equipped with an electrospray- or a Jetstream ionization source operated in positive mode. The gas temperature was set to 345 °C, drying gas flow was 9 L/min, nebulizer pressure 45 psi, fragmentor voltage 150 V, skimmer 47 V and capillary voltage 4000 V. Data was processed with MassHunter qualitative analysis B.06.00 and MassProfiler professional 12.6.1. Putative annotations of identified masses were obtained by automatic annotation or manual search using the Metlin PLCL database (https://metlin.scripps.edu/). For all putative annotations, the observed isotopic distributions were compared with the theoretical distribution.

Egg Hatch Assay (EHA)

The activity of the bark extracts against GIN egg hatching was tested using an EHA, as described in von Samson-Himmelstjerna et al. (2009). In brief, GIN eggs were isolated from freshly collected faeces of donor sheep, mono-specifically infected with either *T. colubriformis* or *T. circumcincta* using a flotation technique (Christie et al. 1982). Eggs were washed with distilled water to remove debris, quantified, adjusted to obtain 100 – 150 eggs in 250 µl, then added to the wells of a 24-well plate. Control wells received 6 µl of distilled water, and bark treatments received 6 µl of the relevant bark extract, tested at the following levels in each well: 1000, 500, 250, 125 and 62.5 µg DM/mL. Within each assay, the controls and bark extracts were tested in triplicate. The plates were incubated at 20 °C for 48h. Hatching was stopped by adding a drop of helminthological iodine (10g iodine, 50g potassium iodine (KI), 100ml deionized water in a ¼ dilution) to the samples. The number of eggs and first-stage larvae (L1) present in each well were counted under an inverted microscope at 400 x magnification. The inhibition efficacy ($x$), for each bark extract was calculated as:

$$\boldsymbol{x = (\frac{a}{a+b})\, x\, 100}$$

$x$ is the percentage of unhatched eggs, $a$ is number of eggs, and $b$ is the number of L1 in the sample.

Larval Motility Assay (LMA)

The activity of the bark extracts against L3 (third stage) larval motility was measured using a high-throughput LMA, as described in Athanasiadou et al. (2021). In brief, the DP xCELLigence Real Time Cell Analyzer, which measures the electrical impedance-based signals across interdigitated

microelectrodes integrated on the bottom of tissue culture e-plates, had previously been adapted to diagnose antiparasitic resistance (Smout et al. 2010). Prior to the addition of L3, 50 µl of a 50% phosphate-buffered saline (PBS) in distilled water solution was added to each well for calibration of the assay. The L3 were recovered from faecal cultures of donor sheep, mono-specifically infected with either *T. colubriformis* or *T. circumcincta*, after a 10-day incubation period at 23 °C, and extracted using the Baermann technique (Baermann, 1917). The larval suspension was washed twice in the 50% PBS solution and adapted to obtain 3000 L3 in 146 µl which were then added to each well of the e-plate. Three controls were included in each assay: i) technical control, using a 50% DMSO in PBS solution; ii) positive control, with alive L3 and iii) negative control, with dead L3. The dead L3 larvae were obtained by incubating them for 30 minutes in a 1/10 dilution of 2% sodium hypochlorite sterilising fluid, followed by repeated washing in 50% PBS in distilled water. A light-proof box was placed over the e-plates to exclude potential light interference on L3 motility. The impedance was recorded for each well every 15 seconds, for 24 hours, at 20°C. Twenty-four hours after the assay began, 4 µl of the bark extracts were added to each well, tested at a single level of 1000 µg DM/mL final concentration. Controls received 4 µl of 50% PBS solution. All bark treatments and controls were tested in triplicate. For each GIN species, all extracts were tested in a single assay. Impedance data were converted into a motility index based on the curve scatter as described by Smout et al. (2010) prior to statistical analysis.

Statistical Analyses

EHA data: Prior to analysis, the residuals of all egg hatch data were tested, and normality was confirmed. Data for each of the GIN species, *T. circumcincta* and *T. colubriformis*, were analysed separately using the software package Genstat (Version 18; VSN international, 2020). Significant differences in the data were tested using ANOVA, including interactions between each of the variables in the bark extracts. The extract level of extract required to inhibit 50% hatching ($IC_{50}$) for each of the bark extracts was calculated using probit analysis. A targeted, Pearson correlation analysis was performed using the 'corr' procedure in SAS (SAS release 9.4, SAS Institute, Cary, NC) to associate CT content in the extracts to the $IC_{50}$-values for each GIN species.

LMA data: Larval motility was measured for 24 hours prior to the addition of the bark extracts, and for 24 hours following the addition of the extracts (48 hours total). To quantify the antiparasitic activity of the extracts, two six-hour windows were selected: 15 – 21 hours after the start of the assay, and 15 – 21 hours following the addition of the bark extracts to the wells. These six-hour windows were selected to minimise any potential impact of the assay procedures (e.g., addition of extracts) on larvae motility readings. Motility data following the addition of the bark extract were analysed using multiple comparison ANOVA, with bark, solvent and season included in the model as factors. Motility data prior to the addition of the bark extracts were used as a covariate. Bonferroni multiple comparison correction threshold was set at $P<0.05$. Statistical analyses were carried out separately for each GIN species; our null hypothesis was that the motility of the larvae exposed to bark extracts was significantly different ($P < 0.05$) than that of the dead control larvae, thus, a significant effect would indicate the lack of antiparasitic activity from the specific bark extract. Previous experience indicated that the variation of the three technical replicates is minimal in this assay (Athanasiadou et al, 2021)

Principal component analysis (PCA) of mass spectroscopy data was performed to characterise the compound variation between the extracts. PCA plots were produced using Mass Profiler Professional, version 12.6.1, Agilent Technologies. Following the identification of bark compounds with LC-MS, a Pearson correlation was performed to identify potential candidate-compounds responsible for the observed anthelmintic activity. To achieve this, the log transformed abundance of each of the LC-MS determined masses in the extracts was associated with the inhibition ($IC_{50}$) of *T. colubriformis* and *T. circumcincta* egg hatching.

**Results**

Season, tree species, and solvent contribute to the variation observed in the CT yield and concentration in bark samples

The CT extraction yields varied between 2 and 16 mg/g dry bark, and the CT concentration in each extract ranged from 17 to 153 mg/g extract DM (Table 1). Overall, acetone extracted the greatest concentration of CT. The water and acetone pine extracts had lower CT yields than the two spruces. Further, only pine extracts had higher CT concentration in summer than winter, irrespective of the solvent used in the extraction. Of note, the pine also contained the highest (winter) and lowest (summer) wood content compared to the other barks (Supplementary Table S1). Of the two spruce barks, spruce2 (S2), which originated from a pulp mill and used water in the debarking process, had lower DM-yields and CT-concentrations than spruce1 (S1), which came from a sawmill where water was not used. The mDP for the CT of the methanol extracts was similar for all bark sources and varied in the range 5.7-7.8. Prodelphinidins were not detected in the pine bark extracts (100% PC) and constituted 2-3% of the total CTs in the spruce extracts (97-98% PC). The cis-isomer (epicatechin) constituted 87% for spruce and 79-83% for pine (lowest in summer).

CT content significantly associated with a reduction in *T. colubriformis* egg hatching, but not in *T. circumcincta*

A proportion of the control eggs did not hatch for both *T. colubriformis* (14%) and *T. circumcincta* (8%) (Table 2). Most of the bark extracts significantly inhibited egg hatching for both GIN, when tested at the top level. All bark extracts that were highly effective or effective against *T. circumcincta* showed a similar pattern of efficacy also against *T. colubriformis*; in general, the latter were more susceptible to inhibition against a greater range of extracts, and at lower levels. For the majority of acetone and methanol extracts a dose response was evident whereas there was little or no evidence of a dose response for the water extracts.

Of the variables associated with the anti-parasitic activity in the EHA, there was a significant effect of the tree species, ($P < 0.001$), season ($P < 0.001$), solvent ($P < 0.001$) and GIN species ($P < 0.001$) (Table 3), with significant interactions observed between GIN and solvent ($P < 0.001$), GIN and season ($P < 0.001$), bark and season ($P = 0.042$). Pine was more effective than either of the spruces against both GIN species, but the two spruces did not differ significantly. Overall, bark collected in winter was more

effective than the summer. The methanol and acetone extracts were more effective than water against *T. colubriformis* eggs. Similarly, methanol extracts were more effective against *T. circumcincta* eggs than water extracts, but the impact of acetone extracts did not differ significantly from either of the other extracts.

The pine-winter-acetone extract showed the highest efficacy at the lowest levels against both GIN species, which was lower in *T. colubriformis* than *T. circumcincta* (100% efficacy at 125 µg DM/mL and 250 µg DM/mL, respectively) (Table 2). Indeed, the pine-winter-acetone extract had the lowest lethal dose in both GIN species, which was almost four-fold lower in *T. colubriformis* (48 µg DM/mL) than *T. circumcincta* (221 µg DM/mL) (Table 4). In *T. circumcincta*, spruce2-winter-acetone had an equally low $IC_{50}$ (221 µg DM/mL); the $IC_{50}$ of the other bark extracts were considerably higher.

The targeted Pearson correlation revealed a significant negative correlation between the CT content in the different bark extracts and the *T. colubriformis* egg hatching inhibition ($IC_{50}$) $r = -0.54$ ($P < 0.021$), while the respective correlation for *T. circumcincta* was not significant ($r = -0.14$, $P = 0.579$).

Water bark extracts were the most efficient in reducing the motility of L3 in ovine GIN.

Throughout the LMA assays, the motility of the alive control larvae was consistently higher than that of the dead control larvae. Motility values of alive larvae were not included in the statistical analysis but were only used as technical controls. Our data showed that six out of the 18 bark extracts reduced the motility of *T. colubriformis* larvae to that of the dead controls: four water and two methanol-based extracts (Table 5). For *T. circumcincta*, our data showed that larvae motility was reduced to that of dead controls by four water, four acetone, and three methanol-based extracts. In some cases, motility of larvae following the addition of the extract was lower compared to that of the alive controls, but still significantly different from dead controls.

Four of these extracts demonstrated strong anthelmintic efficacy against larvae of both GIN. Three of these were from spruce (S1) and one from pine (P). In both GIN species, water extracts were more frequently effective at reducing larvae motility at the level of control dead larvae (8 extracts), with methanol extracts coming second (4 extracts) and the acetone extracts being the least effective (2 extracts) in reducing the motility of larvae to that of dead larvae.

Significant variation in the compound profiles of the bark extracts tested by liquid chromatography - UV - mass spectrometry

The LC-DAD-MS-analyses were performed with a diode array detector (UV) connected upstream of the MS. The UV-absorbance of the extracts, presented as LC-DAD-isoplots, revealed that the extracts from the two spruce samples, contained the same UV-absorbing compound classes, but with an overall lower concentration of compounds in spruce2. The concentration of UV-absorbing compounds from spruce1 was considerably higher in the bark collected in winter than in summer (Figure 1). The winter extracts from both spruce sources also contained hydrophilic compounds not visible in the summer

extracts (Figure 1, retention time below eight minutes). The lower concentrations of UV absorbing compounds in spruce2 (data not shown) were in accordance with the DM and CT-yields and could be attributed to the use of water during the debarking process.

The majority of the UV-absorbing compounds in the spruce extracts eluted between 8-11 min (Figure 1). The concentrations of this group of compounds were higher in the winter compared to the summer extracts. This characteristic group of compounds was not present in the pine extracts. Instead, the pine extracts contained UV-absorbing compounds that, due to different UV-profile and elution over a wider time range, most likely belong to different chemical groups. In addition, the compounds in the pine methanol extract eluting later than 10 min in the chromatograms, were not extracted with water. By comparing the UV- and MS-data, molecular masses of some of the UV-absorbing compounds of the extracts could be identified and the compounds putatively annotated. The UV-absorbing compounds from spruce bark eluting between 8 and 11 min all had UV spectra similar to flavones and hydroxycinnamic acid derivatives, while the MS-data identified these more specifically as flavones (Supplementary Table S2).

Whilst UV analysis demonstrated that the extracts tested showed differences in 20-50 compounds, the MS analysis detected 2299 different compounds in the 18 extracts. In agreement with the UV data, the extracts from bark collected in winter contained more compounds than bark harvested in the summer. This was particularly evident for the water extracts from pine, where 480 compounds were detected in the winter sample compared to 248 compounds in the summer one (Figure 2). For both spruce bark sources, methanol extracts showed the highest number of masses whereas for pine, the acetone extract showed the highest compound diversity. The extracts from pine bark contained a higher number of compounds than the bark from both spruces. The total number of compounds detected in the acetone, methanol, and water extracts of winter samples of spruce1, spruce2, and pine were 1098, 829 and 1511, respectively (Figure 2). The PCA of the complete MS data set showed that pine-winter extracts were separated from the other samples (Supplementary Figure S1). The corresponding score plot (data not shown) showed that the 97 masses with the strongest impact on the separation of pine-winter extracts eluted early in the chromatogram, indicating that they are hydrophilic compounds. The pine-winter bark had the highest wood content (Supplementary Table S1).

Presence of CT molecules with three or less monomers (DP≤3) were detectable in the MS-analysis, eluting at retention times between 6 and 8 min. They occurred with higher abundance in the pine than in the spruce extracts and were not detected in the spruce2 samples. Three CTs were identified in the extracts from spruce and/or pine with less than 5 parts per million (ppm) mass error and a correlating isotopic distribution. These compounds were procyanidin C1 (CT trimer), procyanidin B1/B2 (CT dimer), and catechin/epicatechin (CT monomer).

Novel compounds significantly associated with inhibition of GIN egg hatching activity

A correlation analysis between the 2299 detected masses and EHA $IC_{50}$ of *T. colubriformis* and *T.*

*circumcincta* was performed. Since the acetone and methanol extracts had a higher overall bioactivity than the water extracts, it is not unlikely that different compounds were responsible for the strong effect of the organic solvent extracts and the weaker effect of the water extracts. Therefore, correlation analysis was performed using (i) all extracts and (ii) only methanol and acetone extracts (Supplementary Table S3). When all extracts were included in the analysis, only one compound of unknown id ($r = -0.54$, $P = 0.02$) was significantly negatively associated with $IC_{50}$ of *T. colubriformis* egg hatching. When only the methanol and acetone extracts were included, a total of 69 compounds were significantly negatively associated with *T. colubriformis* egg hatching $IC_{50}$. Most of the masses correlating with *T. colubriformis* inhibition were detected only in pine. For *T. circumcincta*, four compounds were negatively associated with $IC_{50}$ on egg hatching when all extracts were included, and five compounds when only methanol and acetone extracts were included in the correlation analysis. Since the methanol and acetone extracts showed relatively similar efficacy that was consistently higher than the water extracts, an additional analysis of the methanol extracts were conducted. A total of 1316 compounds were identified in the six methanol extracts (i.e., spruce1-summer, spruce1-winter, spruce2-summer, spruce2-winter, pine-summer, and pine-winter) with this method; importantly, 89 new compounds were identified that we had not identified previously. A Pearson correlation analysis of the detected masses revealed that 46 of these had a significant negative correlation to the $IC_{50}$ from the egg hatch assay of *T. colubriformis* (Supplementary Table S3).

For a visual assessment of the relationship between the candidate compounds that originated from the statistical analysis and the anthelmintic activity, the relative abundance of each of the candidate masses was plotted against the anthelmintic activity given as $1/IC_{50}$. Supplementary Figure S2 shows this relationship for seven masses regarded as particularly interesting based on this visual inspection. These masses were all correlated to the inhibition of *T. colubriformis*, as this was the GIN that appeared to be most susceptible by the extracts. Two compounds that were found in both pine and spruce with masses 164.0832 and 200.1557 were the only two ones with $r < -0.90$ and $p < 0.01$. Based on accurate molecular masses and isotopic distribution observed with MS (Supplementary Table S3), the molecular formulas of these compounds are likely to be $C_{10}H_{12}O_2$ and $C_{15}H_{20}$, respectively. A high number of benzene ring containing molecules with these formulas exist, and possible annotations for these molecules are eugenol (a monoterpenoid) for the first one, and a sesquiterpenoid (e.g., calacorene) for the other. The mass M=226.1004 eluted at the retention time that corresponded to a UV peak found in pine extracts (Fig. 1; Supplementary Table S2, Table S3) and corresponds to the basic unit of the flavanols ($C_{15}H_{14}O_2$), the compound group where the tannins belong. The mass 300.2089 could potentially be a naphthalene derivative.

## Discussion

The objective of this study was to assess the impact of a comprehensive set of factors on the antiparasitic activity of bark extracts from Norwegian conifer trees *in vitro* and identify compounds that may be associated with the antiparasitic activity of bark. Three key findings emerged: i) certain bark extracts demonstrated very high antiparasitic activity, inhibiting 100% of eggs from hatching and/or reducing the L3 larvae motility to levels indicative of death. These results confirm the potential of using

Norwegian bark extracts in GIN control; ii) variation in susceptibility of the two species tested was evident, with *T. colubriformis* egg hatching being affected more than that of *T. circumcincta* ones; the opposite was the case for the impact of bark extracts on larvae motility; iii) the presence of CT and numerous other compounds was significantly associated with the observed antiparasitic activity.

The yield and concentration of CT in our bark extracts were similar to those observed in other studies using sawmill by-products (Matthews et al. 1997; Bianchi, 2016; Athanasiadou et al. 2021). Overall, the dry matter and CT-yields were lower in extracts from the summer bark, which may be explained by spraying of the logs and leakage of water-soluble compounds. The relatively high CT-content of the pine summer extracts, combined with the lower dry matter, indicate that fewer other compounds were extracted from this bark. The pine-winter bark had the highest wood content, which offers a likely explanation for the high number of compounds detected in the extracts from this bark and the separation in the PCA-plot. As none of these masses were significantly more abundant in the acetone and methanol extracts than in the water extract, they cannot explain the high bioactivity of the acetone and methanol extracts from pine. While CT are water-soluble and should be extractable using all three solvents, the greater yields obtained by acetone and methanol compared to water is likely to be attributable to the organic solvents capacity to disrupt bonds to other compounds, thus releasing more of the bound CT (Bianchi et al. 2019). The characterisation of the chemical composition of the bark extracts that was carried out based on chromatographic separation, UV-absorbance, and molecular mass profiles, offered a lot of novel data. The spruce and pine extracts had distinctly different UV-profiles, and the MS-analysis detected 878 compounds that were unique to pine when compared to spruce1, debarked by the same method, while 595 compounds were unique to spruce1, compared with pine. Published LC-MS identification of bark compounds is scarce, but clear differences in the MS-profiles of bark extracts from different conifer tree species, including Norway spruce and Silver fir (*Abies alba*) were also observed by Brennan et al. (2020).

Certain bark extracts inhibited egg hatching of both GIN species up to 100%, although *T. colubriformis* exhibited greater susceptibility to lower levels of extracts ($IC_{50}$) than *T. circumcincta.* The associations of EHA $IC_{50}$ with concentrations of different compounds in extracts showed that CT were at least partly responsible for the *T. colubriformis* egg hatching inhibition (r = -0.54). These results support that CT play a role in the egg hatching inhibition, at least of the *T. colubriformis* eggs. Although some of the variation in the anthelmintic activity against *T. colubriformis* egg hatching is explained with variation in CT content, this is not the case for *T. circumcincta*. This observation is in agreement with previous evidence where Athanasiadou et al. (2021) observed variation in the anthelmintic efficacy of bark extracts which was not always associated with overall CT content. Indeed, additional CT characteristics are thought to impact antiparasitic effects of CT, such as the degree of polymerisation and type of monomeric units (Williams et al. 2014). The pine and spruce bark contain almost solely PC, but as the thiolysis only captures the average degree of polymerisation (DP), it cannot be excluded that some of the extracts have larger variation in DP and thus contain larger CT-molecules. However, since the CT constituted less than 15% of the extract DM, a more likely explanation for the variation in the efficacy observed is contributions from other compounds. In support of this, the association of MS-data with the inhibition of egg hatching in *T. colubriformis* (Supplementary Figure S2) showed other compounds that likely have a role to play in the antiparasitic activity of bark extracts. Several plant secondary

metabolites have been related to such activity, including terpenoids, saponins, flavonoids, hydroxycinnamic acid derivatives and other polyphenolic compounds (Williams et al. 2014; von Son-de Fernex et al. 2015), such as flavanol derivatives (Shepherd et al, 2022) which may act synergistically to achieve higher antiparasitic activity (Klongsiriwet et al. 2015). Indeed, Valente et al (2021) is a good example of verified interactions. The use of polyvinylpolypyrrolidone (PVPP), which blocks CT, has been particularly useful in demonstrating that CT are not solely responsible for antiparasitic activity, and some studies have even observed improved antiparasitic activity following PVPP incubation, indicating potentially antagonistic effects between plant secondary metabolites (Vargas-Magaña et al. 2014; Castañeda-Ramírez et al. 2020). In our study, that the pine extracts had comparatively lower $IC_{50}$ than the spruce extracts, indicates that some of the compounds that were absent in the spruce, for example those that have retention time over 14 min (Figure 1), may be responsible for the antiparasitic activity. However, as both spruces still demonstrated strong antiparasitic activity at higher levels, it seems possible that different compounds may contribute to the bioactivity of spruce and pine.

In relation to the latter, indeed, highly polar solvents, such as methanol, have been known to release more bioactive compounds from plants, which are specific to ovicidal activity, or they may result in releasing fewer compounds which antagonizing activity (Altemimi et al. 2017; Castañeda-Ramírez et al. 2020; Ali et al. 2021). Species-specific differences in the susceptibility of GIN to both plant compounds (Costa et al. 2008; Rahman et al. 2011; Al-Rofaii et al. 2012; Athanasiadou et al. 2021) and broad-spectrum anthelmintics (Smith-Buijs and Borgsteede, 1986; Gill and Lacey, 1993; Gill and Lacey, 1998; Bartley et al. 2016) have been previously documented *in vitro*, where the pharmacological activity can depend upon interactions with species – specific enzymes, proteins, nucleic acids, biomolecules and receptors (Roy, 2011). Similarly, the antiparasitic efficacy of the bark extracts varied depending on the GIN life stage they were targeted against. This is likely owing to the distinct structural and compositional characteristics of the egg membrane compared to the larval cuticle, which may interfere with the compounds mechanism of action and resulting antiparasitic activity (de Paula Carlis et al. 2019). The GIN eggshell is composed of three layers; the inner lipid, medial chitin and outer vitelline (Bird and Bird, 1991). The L3 larvae stage possesses a double cuticle sheath, consisting of four parts: a triple layered epicuticle at the external surface and the inner-most stratum, primarily composed of collagen (insoluble in detergent), soluble proteins and components of low molecular weight, such as lipids (Decraemer et al. 2003; Rocha et al. 2020). It has been suggested that the GIN eggshell is comparatively more permeable to some antiparasitic compounds than the L3 larvae cuticle (Rocha et al. 2020), however, further studies would be required to establish an $IC_{50}$ for the L3 larvae motility before any conclusions could be drawn for the bark extracts.

The most abundant masses that were strongly correlating with the bioactivity of the extracts, were annotated by search in databases. However, as bark contain a high number of bioactive phenolic compounds with very similar structure (Metsämuuronen and Sirén; 2019), a reliable identification was not possible. This would require MS/MS-analysis or NMR. Nevertheless, two masses were present in both pine and spruce, and were negatively associated with $IC_{50}$. One mass was putatively annotated as monoterpenoid eugenol, previously reported to significantly reduce the total worm burden in humans (El-kady et al. 2019). The molecular mass of the other corresponded to several sesquiterpenoids; one of them, calacorene, has previously been detected in plant extracts active against the filaroid nematode

*Onchocerca* (Metuge et al. 2014).

In conclusion, this paper has presented abundant *in vitro* evidence to support the possible exploitability of Norwegian sawmill by-products as a tool in the control of ovine GIN. Importantly, it has identified novel sources of variation associated with antiparasitic activity and specific compounds that warrant further study. This is the first *in vitro* study conducted on such a scale, where 18 different extracts were studied, and solid associations were made between compounds and efficacy. From the 18 extracts tested, many showed activity against one or more of the nematode stages and species. Although CT showed a significant association with antiparasitic activity against *T. colubriformis*, other compounds have contributed to the activity observed. These findings add new insights to a growing library of bioactive plants that contain key compounds for the future control of ovine GIN in a time of anthelmintic resistance. Additional characterization studies with MS/MS fragmentation and NMR would be necessary to identify the specific compounds that are responsible for the activity. Validation of the results *in vivo* would further ascertain host tolerance to the bark based on production parameters and indicators of toxicity. Further exploitation will largely depend on the possibility for upscaling; as we present evidence of antiparasitic activity in water extracts, upscaling appears to be a realistic option.

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**Author Contribution**

HS initiated the study. HS, SA, HLE and SMT designed the study. KFD performed the MS-analyses and processed the data, IMA and KM conducted the sampling, preparation of the bark extracts and the bark analysis. CC, SP and BB performed the parasitological assays. CC, SA, and HS analysed the results. CC, SA, IMA, KFD, drafted the manuscript. All authors read and approved the manuscript.

**Conflict of Interest**

None.

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**Table 1** Condensed Tannin yields and concentrations for 18 bark extracts, according to tree species, origin, season, and solvent used in extraction.

| Tree species and origin | | CT yield per gram dry bark (mg/g bark) | | CT concentration in extracts (mg/g DM) | |
|---|---|---|---|---|---|
| | Solvent | Summer | Winter | Summer | Winter |
| S1 | Water | 2.7 | 8.7 | 70 | 80 |
| S2 | | 2.5 | 3.5 | 17 | 48 |
| P | | 1.5 | 1.8 | 65 | 33 |
| S1 | Acetone | 11.0 | 15.7 | 107 | 122 |
| S2 | | 7.8 | 8.7 | 91 | 101 |
| P | | 5.4 | 5.3 | 153 | 95 |
| S1 | Methanol | 5.2 | 10.3 | 98 | 106 |
| S2 | | 3.6 | 3.8 | 68 | 68 |
| P | | 3.8 | 4.2 | 142 | 91 |

Quantification was carried out on bark extracts at the highest level tested in the GIN assays (1000 µg extract dry matter (DM)/mL). Bark extracts include: (S1) spruce, sawmill, ring debarking; (S2): spruce, pulp mill, drum debarking; (P): pine, sawmill, ring debarking. Season bark was collected, summer (S) and winter (W).

**Table 2** Mean egg hatch inhibition (%) from bark extracts against two ovine GIN species.

| | | Solvent | Water | | | | | Acetone | | | | | Methanol | | | | | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GIN | Bark | Season | 1000 | 500 | 250 | 125 | 62.5 | 1000 | 500 | 250 | 125 | 62.5 | 1000 | 500 | 250 | 125 | 62.5 | |
| *T. colubriformis* | | | | | | | | | | | | | | | | | | |
| | S1 | S | **25** | **16** | **15** | 12 | 14 | **100** | **100** | **54** | **28** | **24** | **100** | **95** | **56** | **18** | 13 | 14 |
| | | W | **32** | **20** | **26** | **15** | **15** | **100** | **100** | **85** | **35** | 12 | **100** | **91** | **37** | **22** | **20** | |
| | S2 | S | 10 | 11 | **16** | 11 | 7 | **100** | **93** | **69** | **34** | **16** | **100** | **91** | **50** | **29** | 14 | |
| | | W | **59** | **40** | **21** | **18** | **21** | **100** | **100** | **99** | **56** | **30** | **100** | **100** | **68** | **29** | **25** | |
| | P | S | **34** | **28** | **29** | **14** | 11 | **100** | **100** | **96** | **74** | **26** | **100** | **97** | **95** | **50** | **39** | |
| | | W | **59** | **55** | **40** | **22** | **18** | **100** | **100** | **100** | **100** | **94** | **100** | **100** | **100** | **92** | **50** | |
| *T. circumcincta* | S1 | S | 7 | 6 | 2 | 4 | 2 | **62** | 4 | 2 | 4 | 1 | **11** | 4 | 5 | 3 | 1 | 8 |
| | | W | **38** | **21** | **15** | **16** | **15** | **98** | **56** | **14** | **14** | **19** | **97** | **28** | **17** | **10** | **22** | |
| | S2 | S | 4 | 4 | 5 | 3 | 5 | **25** | 5 | 3 | 4 | 3 | **16** | 3 | 4 | 4 | 2 | |
| | | W | **63** | **18** | **15** | **13** | **18** | **100** | **96** | **27** | **20** | **18** | **100** | **39** | **15** | **16** | **16** | |
| | P | S | **35** | 4 | 3 | 4 | 4 | **95** | **44** | 5 | 4 | 3 | **93** | **24** | 2 | 4 | 3 | |
| | | W | **85** | **43** | **20** | **16** | **13** | **100** | **100** | **100** | **35** | **20** | **100** | **94** | **60** | **22** | **14** | |

Mean egg hatch inhibition (%) of bark extracts (n = 3) tested against both *T. colubriformis* and *T. circumcincta* eggs. S1: spruce, sawmill, ring debarking; S2: spruce, pulp mill, drum debarking; P: pine, sawmill, ring debarking. Collection seasons from summer (S) and winter (W). Results in bold were significantly different from the negative control treatments (one-way ANOVA and post-hoc Bonferroni test for multiple comparisons, where $p \leq 0.05$). Results highlighted in dark grey were considered highly active (≥ 99%), and those in grey considered active (90 – 98%).

**Table 3** Impact of bark extract variables on the anti-parasitic activity observed in egg hatch assay (EHA) against two ovine GIN species.

| Variable | *T. colubriformis* | | *T. circumcincta* | |
|---|---|---|---|---|
| | p value | Significance | p value | Significance |
| Tree species | <.001 | $S1^{b}$, $S2^{b}$, $P^{a}$ | <.001 | $S1^{b}$, $S2^{b}$, $P^{a}$ |
| Season | 0.025 | $S^{b}$, $W^{a}$ | <.001 | $S^{b}$, $W^{a}$ |
| Solvent | <.001 | $Wa^{b}$, $Ac^{a}$, $Me^{a}$ | <.001 | $Wa^{b}$, $Ac^{ab}$, $Me^{a}$ |
| Level (µg DM/mL) | <.001 | $1000^{a}$, $500^{ab}$, $250^{b}$, $125^{c}$, $62^{c}$ | <.001 | $1000^{a}$, $500^{b}$, $250^{c}$, $125^{c}$, $62^{c}$ |

Variables of bark extracts tested within GIN species included: Bark (S1): spruce, sawmill, ring debarking; (S2): spruce, pulp mill, drum debarking; (P): pine, sawmill, ring debarking; Season summer (S) and winter (W); solvent used in extraction (Wa) water, (Ac) acetone, (Me) methanol; tested at five different levels (µg DM/mL). Significant differences (one-way ANOVA, $p \leq 0.05$) denoted with letters in superscript, where $^{a}$ has a higher antiparasitic effect than $^{b}$, which has a higher effect than $^{c}$ ($^{a} > ^{b} > ^{c}$).

**Table 4** $IC_{50}$of the 18 bark extracts to inhibit egg hatching, ordered from the lowest to the highest concentration against two ovine GIN species.

| ***T. colubriformis*** | | | | ***T. circumcincta*** | | | |
|---|---|---|---|---|---|---|---|
| Bark | Season | Solvent | $IC_{50}$ (µg DM/mL) | Bark | Season | Solvent | $IC_{50}$ (µg DM/mL) |
| P | W | Ac | 48 | P | W | Ac | 221 |
| P | W | Me | 63 | S2 | W | Ac | 221 |
| P | S | Ac | 89 | S1 | W | Ac | 353 |
| P | S | Me | 93 | S2 | W | Me | 407 |
| S2 | W | Ac | 95 | P | W | Me | 407 |
| S2 | W | Me | 145 | P | W | Wa | 459 |
| S2 | S | Ac | 159 | S1 | W | Me | 475 |
| S1 | W | Ac | 162 | P | S | Ac | 492 |
| S2 | S | Me | 193 | P | S | Me | 596 |
| S1 | S | Me | 196 | S1 | S | Ac | 1050 |
| S1 | W | Me | 208 | S2 | W | Wa | 1282 |
| S1 | S | Ac | 400 | P | S | Wa | 3587 |
| P | W | Wa | 495 | S2 | S | Ac | 5908 |
| S2 | W | Wa | 856 | S1 | W | Wa | 7093 |
| P | S | Wa | 3,498 | S2 | S | Me | 20733 |
| S1 | W | Wa | 16,203 | S1 | S | Me | 83,393 |
| S1 | S | Wa | 212,179 | S1 | S | Wa | 4,236,058 |
| S2 | S | Wa | $1.8\times10^{14}$ | S2 | S | Wa | $1.2\times10^{11}$ |

$IC_{50}$: dose required to inhibit 50% of the GIN eggs from hatching. Calculated based on results from dose-response egg hatch assay (EHA). Bark extracts tested included: (S1): spruce, sawmill, ring debarking; (S2): spruce, pulp mill, drum debarking; (P): pine, sawmill, ring debarking and were collected during summer (S) and winter (W) seasons. Each of the barks were extracted using water (W), acetone (A), and methanol (M) as the solvent.

**Table 5** L3 larvae motility following incubation with bark extracts at 1000µg/mL dry weight in two GIN species.

| | | Solvent | Water | | | | Acetone | | | | Methanol | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GIN species | Bark | Season | Alive | Dead | Bark | $H_0$ Rejected | Alive | Dead | Bark | $H_0$ Rejected | Alive | Dead | Bark | $H_0$ Rejected |
| *T.colubriformis* | S1 | S | 0.0043 | 0.0022 | 0.0035 | Y | 0.0043 | 0.0022 | 0.0042 | N | 0.0043 | 0.0022 | 0.0038 | Y |
| | | W | 0.0043 | 0.0022 | 0.0037 | Y | 0.0043 | 0.0022 | 0.004 | N | 0.0043 | 0.0022 | 0.0039 | Y |
| | S2 | S | 0.0042 | 0.0008 | 0.0037 | N | 0.0042 | 0.0008 | 0.0038 | N | 0.0042 | 0.0008 | 0.0038 | N |
| | | W | 0.0042 | 0.0008 | 0.0037 | N | 0.0042 | 0.0008 | 0.0041 | N | 0.0042 | 0.0008 | 0.004 | N |
| | P | S | 0.0043 | 0.0009 | 0.0033 | Y | 0.0043 | 0.0009 | 0.0044 | N | 0.0043 | 0.0009 | 0.0042 | N |
| | | W | 0.0043 | 0.0009 | 0.0033 | Y | 0.0043 | 0.0009 | 0.0035 | N | 0.0043 | 0.0009 | 0.0046 | N |
| *T.circumcincta* | S1 | S | 0.0088 | 0.0038 | 0.0038 | Y | 0.0088 | 0.0038 | 0.0041 | Y | 0.0088 | 0.0038 | 0.004 | Y |
| | | W | 0.0088 | 0.0038 | 0.0042 | Y | 0.0088 | 0.0038 | 0.0039 | Y | 0.0088 | 0.0038 | 0.0041 | Y |
| | S2 | S | 0.0077 | 0.0031 | 0.004 | Y | 0.0077 | 0.0031 | 0.0041 | N | 0.0077 | 0.0031 | 0.0041 | N |
| | | W | 0.0077 | 0.0031 | 0.0045 | N | 0.0077 | 0.0031 | 0.0038 | Y | 0.0077 | 0.0031 | 0.0046 | N |
| | P | S | 0.0077 | 0.0048 | 0.0067 | Y | 0.0077 | 0.0048 | 0.0038 | Y | 0.0077 | 0.0048 | 0.0041 | Y |
| | | W | 0.0077 | 0.0048 | 0.0043 | N | 0.0077 | 0.0048 | 0.0042 | N | 0.0077 | 0.0048 | 0.0047 | N |

Values for the 'alive' L3 control, 'dead' L3 control, and 'bark' treated L3 represent mean (n = 3) larval motility adjusted for the covariate (motility of larvae prior to the addition of bark extracts). Bark treatments included: S1: spruce, sawmill, ring debarking; S2: spruce, pulp mill, drum debarking; P: pine, sawmill, ring debarking and were collected during summer (S) and winter (W) seasons. Each of the bark samples was extracted using water, acetone, or methanol as the solvent. The null hypothesis ($H_0$) tested was that the motility of the larvae in the bark extract treatment differed significantly from the dead controls; rejected null hypothesis (Y) was indicative of strong antiparasitic activity, whereas not-rejected null hypothesis (N) was indicative of variable and/or no strong antiparasitic activity

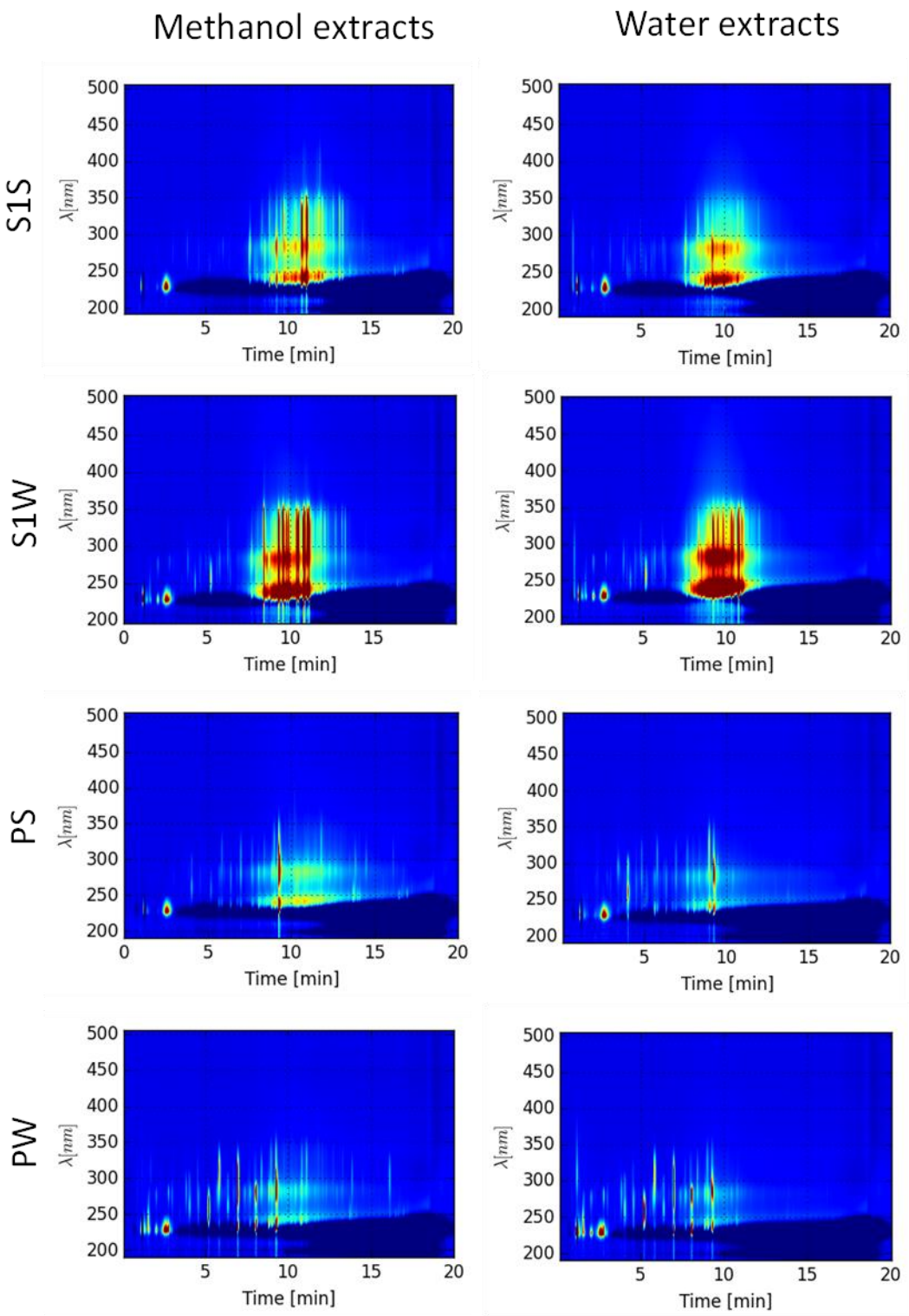



**Figure 1** LC-DAD isoplots of eight bark extracts. The analysis was performed with reverse phase chromatography, where hydrophilic compounds elute first and the more hydrophobic compounds elute at higher retention time. UV wavelengths is shown on the y-axis and the high-performance liquid chromatography (HPLC) retention time on the x-axis. The colour represents a relative concentration of the UV absorbing compounds where red is high concentration, yellow is medium concentration, and blue is low concentration/absence. S1-S: spruce1 summer; S1-W: spruce1 winter; P-S: pine summer; P-W: pine winter.

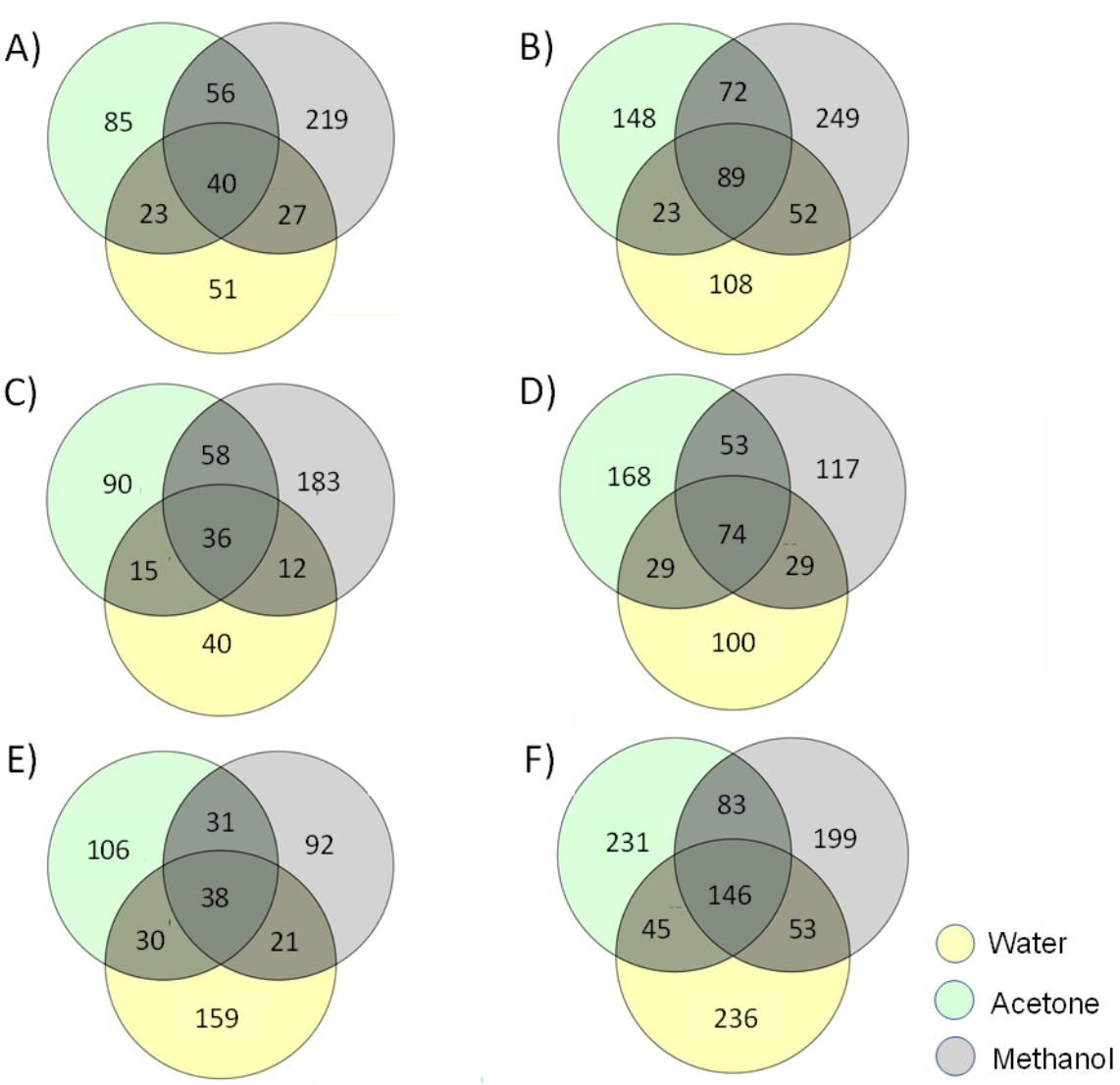



**Figure 2** Venn diagram displaying the number of compounds (entities) detected with high resolution mass spectrometry in the bark batches extracted using water, acetone, and methanol. A: spruce1 summer; B: spruce1 winter; C: spruce2 summer; D: spruce2 winter; E: pine summer; F: pine winter.

**Supplementary Table S1** Moisture content and wood percentage of the bark batches

| Bark batch | Wood species | Location Norway | Debarking method | Moisture content (% of wet mass) | | Wood percentage (% of dry mass) | |
|---|---|---|---|---|---|---|---|
| | | | | S | W | S | W |
| S1 | Spruce | Brandval | Ring | 54 | 59 | 19 | 19 |
| S2 | Spruce | Halden | Drum | 50 | 67 | 17 | 20 |
| P | Pine | Kirkenær | Ring | 63 | 56 | 6 | 25 |

S: Summer, W: Winter (bark harvesting time)

**Supplementary Table S2** Masses of compounds with high UV-absorbance (Fig. 1), identified based on retention time and comparison of UV and MS data

| Extract source | Retention time [min] | Monoisotopic mass [Da] | Putative annotation (Compound or chemical group) |
|---|---|---|---|
| Spruce[1] | 8.53 | 488.1457 | flavone |
| | 8.54 | 423.1521 | flavone |
| | 9.41 | 456.1564 | flavone |
| | 11.03 | 504.1445 | flavone |
| | 11.15 | 514.1675 | trihydroxy-flavan-dimer |
| Pine | 5.8 | 146.0367 | Coumarin |
| | 7.0 | 218.0554 and 507.2309 | Coumarin derivatives |
| | 16.2 | 226.1004 | Flavanol base unit |

1: Annotation based on UV-spectra (Zhang et al, 2017; Miyagusuku-Cruzado et al., 2020; Spectrabase.com) and occurrence only in the spruce bark extracts.

**Supplementary Table S3** Pearson's correlation between abundance (log2) of each of the LC-MS determined masses in the extracts and the estimated egg hatching $IC_{50}$ values of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*.

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| All extracts | 18 | CT | | 89.306 | 33.6586 | 16.700 | 153.300 | -0.54 | 0.021 |
| | 18 | 662.533 | 18.37 | 4.620 | 2.1290 | 0 | 5.660 | -0.54 | 0.020 |
| Methanol and acetone | 12 | CT | | 103.425 | 55.1611 | 67.700 | 153.300 | -0.32 | 0.310 |
| | 12 | 164.083 | 8.2 | 1.724 | 2.5581 | 0 | 5.600 | -0.82 | 0.001 |
| | 12 | 196.073 | 7.1 | 1.820 | 2.6986 | 0 | 5.890 | -0.82 | 0.001 |
| | 12 | 379.199 | 10.9 | 1.812 | 2.6842 | 0 | 5.870 | -0.82 | 0.001 |
| | 12 | 475.205 | 10.0 | 1.916 | 2.8353 | 0 | 6.110 | -0.82 | 0.001 |
| | 12 | 146.037 | 6.0 | 1.254 | 2.2725 | 0 | 5.210 | -0.72 | 0.008 |
| | 12 | 650.585 | 18.4 | 6.483 | 0.2992 | 6 | 6.780 | -0.72 | 0.008 |
| | 12 | 480.161 | 10.0 | 1.404 | 2.5430 | 0 | 5.800 | -0.72 | 0.008 |
| | 12 | 524.241 | 11.7 | 1.188 | 2.1502 | 0 | 4.950 | -0.72 | 0.008 |
| | 12 | 362.173 | 10.9 | 1.423 | 2.5778 | 0 | 5.960 | -0.72 | 0.009 |
| | 12 | 380.109 | 6.8 | 1.277 | 2.3111 | 0 | 5.250 | -0.72 | 0.009 |
| | 12 | 518.179 | 8.4 | 1.159 | 2.0971 | 0 | 4.670 | -0.71 | 0.009 |
| | 12 | 358.141 | 12.4 | 1.331 | 2.4077 | 0 | 5.360 | -0.71 | 0.009 |
| | 12 | 486.203 | 9.2 | 1.188 | 2.1503 | 0 | 4.960 | -0.71 | 0.010 |
| | 12 | 332.199 | 13.0 | 1.786 | 2.6393 | 0 | 5.580 | -0.70 | 0.011 |
| | 12 | 494.179 | 10.2 | 1.165 | 2.1076 | 0 | 4.690 | -0.69 | 0.012 |
| | 12 | 298.193 | 13.6 | 2.138 | 2.6497 | 0 | 5.630 | -0.67 | 0.017 |
| | 12 | 359.142 | 1.3 | 2.593 | 2.7211 | 0 | 5.740 | -0.66 | 0.020 |
| | 12 | 638.622 | 17.8 | 6.914 | 0.2539 | 6.490 | 7.200 | -0.65 | 0.022 |

**Supplementary Table S3 Cont.**

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| Methanol and acetone | 12 | 375.168 | 12.4 | 1.676 | 2.4899 | 0 | 5.360 | -0.65 | 0.022 |
| | 12 | 456.140 | 7.4 | 0.774 | 1.8082 | 0 | 4.690 | -0.64 | 0.026 |
| | 12 | 269.149 | 3.0 | 0.731 | 1.7069 | 0 | 4.420 | -0.64 | 0.026 |
| | 12 | 498.171 | 6.3 | 0.917 | 2.1409 | 0 | 5.500 | -0.64 | 0.026 |
| | 12 | 396.082 | 6.8 | 0.775 | 1.8100 | 0 | 4.650 | -0.64 | 0.026 |
| | 12 | 514.146 | 6.3 | 0.799 | 1.8665 | 0 | 4.800 | -0.64 | 0.026 |
| | 12 | 182.094 | 8.2 | 0.814 | 1.9015 | 0 | 4.890 | -0.64 | 0.026 |
| | 12 | 778.313 | 9.4 | 0.799 | 1.8665 | 0 | 4.800 | -0.64 | 0.026 |
| | 12 | 530.159 | 9.0 | 0.763 | 1.7808 | 0 | 4.580 | -0.64 | 0.026 |
| | 12 | 235.142 | 1.6 | 0.763 | 1.7828 | 0 | 4.590 | -0.64 | 0.026 |
| | 12 | 182.094 | 6.1 | 0.858 | 2.0027 | 0 | 5.160 | -0.64 | 0.026 |
| | 12 | 150.036 | 7.1 | 0.814 | 1.9015 | 0 | 4.900 | -0.64 | 0.026 |
| | 12 | 218.056 | 7.1 | 0.825 | 1.9268 | 0 | 4.970 | -0.64 | 0.026 |
| | 12 | 382.104 | 6.1 | 0.806 | 1.8821 | 0 | 4.860 | -0.64 | 0.026 |
| | 12 | 136.052 | 8.2 | 0.914 | 2.1351 | 0 | 5.520 | -0.64 | 0.026 |
| | 12 | 486.115 | 5.3 | 0.790 | 1.8451 | 0 | 4.770 | -0.64 | 0.026 |
| | 12 | 429.114 | 1.4 | 0.785 | 1.8334 | 0 | 4.740 | -0.64 | 0.026 |
| | 12 | 496.137 | 10.0 | 0.871 | 2.0339 | 0 | 5.260 | -0.64 | 0.026 |
| | 12 | 144.043 | 1.3 | 0.786 | 1.8354 | 0 | 4.750 | -0.64 | 0.026 |
| | 12 | 385.195 | 4.5 | 0.822 | 1.9191 | 0 | 4.970 | -0.64 | 0.026 |
| | 12 | 541.253 | 9.9 | 0.813 | 1.8996 | 0 | 4.920 | -0.64 | 0.026 |
| | 12 | 560.121 | 1.2 | 0.723 | 1.6875 | 0 | 4.370 | -0.64 | 0.026 |
| | 12 | 361.174 | 6.1 | 0.930 | 2.1721 | 0 | 5.630 | -0.64 | 0.026 |

**Supplementary Table S3 Cont.**

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| Methanol and acetone | 12 | 200.104 | 7.8 | 0.758 | 1.7712 | 0 | 4.590 | -0.64 | 0.026 |
| | 12 | 486.208 | 9.4 | 0.819 | 1.9133 | 0 | 4.960 | -0.64 | 0.026 |
| | 12 | 746.327 | 10.9 | 0.814 | 1.9016 | 0 | 4.930 | -0.64 | 0.026 |
| | 12 | 507.233 | 7.1 | 0.766 | 1.7888 | 0 | 4.660 | -0.64 | 0.026 |
| | 12 | 489.221 | 10.3 | 0.900 | 2.1023 | 0 | 5.490 | -0.64 | 0.026 |
| | 12 | 294.097 | 9.7 | 0.789 | 1.8435 | 0 | 4.820 | -0.64 | 0.026 |
| | 12 | 286.106 | 5.3 | 0.768 | 1.7929 | 0 | 4.690 | -0.64 | 0.026 |
| | 12 | 445.195 | 10.0 | 0.867 | 2.0246 | 0 | 5.300 | -0.64 | 0.026 |
| | 12 | 435.175 | 5.7 | 0.789 | 1.8435 | 0 | 4.830 | -0.64 | 0.026 |
| | 12 | 194.077 | 1.2 | 0.747 | 1.7443 | 0 | 4.570 | -0.64 | 0.026 |
| | 12 | 523.230 | 5.6 | 0.758 | 1.7697 | 0 | 4.650 | -0.64 | 0.026 |
| | 12 | 459.211 | 10.1 | 0.821 | 1.9178 | 0 | 5.050 | -0.64 | 0.026 |
| | 12 | 493.217 | 6.9 | 0.794 | 1.8558 | 0 | 4.910 | -0.64 | 0.026 |
| | 12 | 400.150 | 9.4 | 4.395 | 2.0681 | 0 | 5.730 | -0.62 | 0.031 |
| | 12 | 697.586 | 18.4 | 4.755 | 2.2222 | 0 | 5.820 | -0.62 | 0.032 |
| | 12 | 294.219 | 13.4 | 1.194 | 2.1610 | 0 | 4.920 | -0.62 | 0.033 |
| | 12 | 136.053 | 3.0 | 1.598 | 2.3629 | 0 | 4.970 | -0.61 | 0.036 |
| | 12 | 367.236 | 15.4 | 1.283 | 2.3254 | 0 | 5.430 | -0.60 | 0.040 |
| | 12 | 690.411 | 15.4 | 1.293 | 2.3423 | 0 | 5.410 | -0.60 | 0.040 |
| | 12 | 866.207 | 8.3 | 1.171 | 2.1196 | 0 | 4.880 | -0.59 | 0.042 |
| | 12 | 866.205 | 6.9 | 1.188 | 2.1527 | 0 | 5.040 | -0.59 | 0.042 |
| | 12 | 320.200 | 15.0 | 1.227 | 2.2210 | 0 | 5.130 | -0.59 | 0.043 |
| | 12 | 369.251 | 14.0 | 2.030 | 2.5103 | 0 | 5.040 | -0.59 | 0.044 |

**Supplementary Table S3 Cont.**

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| Methanol and acetone | 12 | 316.204 | 15.9 | 2.137 | 2.6449 | 0 | 5.440 | -0.58 | 0.048 |
| | 12 | 674.668 | 18.3 | 2.193 | 2.7107 | 0 | 5.400 | -0.58 | 0.049 |
| Methanol | 6 | CT | | 95.367 | 27.6327 | 62.000 | 215.000 | -0.32 | 0.534 |
| | 6 | 200.160 | 17.89 | 17.178 | 0.8444 | 16.404 | 18.504 | -0.99 | <.0001 |
| | 6 | 498.170 | 7.14 | 6.017 | 9.3839 | 0 | 19.767 | -0.93 | 0.007 |
| | 6 | 136.050 | 9.36 | 6.146 | 9.5688 | 0 | 19.938 | -0.93 | 0.007 |
| | 6 | 150.030 | 8.19 | 5.605 | 8.7130 | 0 | 17.945 | -0.93 | 0.007 |
| | 6 | 196.070 | 8.09 | 6.649 | 10.3324 | 0 | 21.240 | -0.93 | 0.007 |
| | 6 | 466.120 | 10.45 | 5.694 | 8.8453 | 0 | 18.127 | -0.93 | 0.007 |
| | 6 | 146.040 | 7.13 | 5.884 | 9.1404 | 0 | 18.709 | -0.93 | 0.007 |
| | 6 | 360.160 | 11.73 | 5.900 | 9.1630 | 0 | 18.730 | -0.93 | 0.007 |
| | 6 | 132.060 | 9.39 | 8.383 | 9.2491 | 0 | 18.778 | -0.93 | 0.007 |
| | 6 | 475.210 | 10.59 | 5.841 | 9.0680 | 0 | 18.451 | -0.93 | 0.007 |
| | 6 | 470.140 | 6.29 | 5.979 | 9.2790 | 0 | 18.793 | -0.93 | 0.008 |
| | 6 | 226.100 | 17.37 | 6.203 | 9.6244 | 0 | 19.450 | -0.93 | 0.008 |
| | 6 | 480.160 | 10.58 | 6.672 | 10.3518 | 0 | 20.893 | -0.93 | 0.008 |
| | 6 | 578.210 | 10.74 | 5.805 | 9.0047 | 0 | 18.128 | -0.93 | 0.008 |
| | 6 | 154.060 | 6.18 | 5.296 | 8.2148 | 0 | 16.538 | -0.93 | 0.008 |
| | 6 | 380.110 | 7.6 | 6.176 | 9.5773 | 0 | 19.187 | -0.93 | 0.008 |
| | 6 | 230.080 | 1.51 | 5.393 | 8.3613 | 0 | 16.676 | -0.93 | 0.008 |
| | 6 | 274.160 | 13.95 | 5.344 | 8.2831 | 0 | 16.486 | -0.93 | 0.008 |
| | 6 | 524.240 | 12.19 | 5.346 | 8.2868 | 0 | 16.491 | -0.93 | 0.008 |
| | 6 | 342.010 | 10.28 | 5.541 | 8.5862 | 0 | 16.974 | -0.93 | 0.008 |

**Supplementary Table S3 Cont.**

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| Methanol | 6 | 256.110 | 17.2 | 5.351 | 8.2899 | 0 | 16.230 | -0.92 | 0.009 |
| | 6 | 262.120 | 10.43 | 5.332 | 8.2597 | 0 | 16.051 | -0.92 | 0.009 |
| | 6 | 332.130 | 9.14 | 5.254 | 8.1402 | 0 | 15.801 | -0.92 | 0.009 |
| | 6 | 164.080 | 10.45 | 5.430 | 8.4118 | 0 | 16.371 | -0.92 | 0.009 |
| | 6 | 590.180 | 12.69 | 5.365 | 8.3126 | 0 | 16.297 | -0.92 | 0.010 |
| | 6 | 218.170 | 10.1 | 5.514 | 8.5433 | 0 | 16.768 | -0.92 | 0.010 |
| | 6 | 550.180 | 12.69 | 5.477 | 8.4868 | 0 | 16.669 | -0.92 | 0.010 |
| | 6 | 646.060 | 10.27 | 5.239 | 8.1180 | 0 | 15.970 | -0.92 | 0.010 |
| | 6 | 510.210 | 11.82 | 5.453 | 8.4489 | 0 | 16.627 | -0.92 | 0.010 |
| | 6 | 632.070 | 10.27 | 5.117 | 7.9302 | 0 | 15.681 | -0.92 | 0.010 |
| | 6 | 276.240 | 17.7 | 5.554 | 8.6136 | 0 | 17.300 | -0.91 | 0.011 |
| | 6 | 200.160 | 16.81 | 5.624 | 8.7262 | 0 | 17.626 | -0.91 | 0.012 |
| | 6 | 332.090 | 11.2 | 5.152 | 7.9958 | 0 | 16.223 | -0.91 | 0.012 |
| | 6 | 332.200 | 13.96 | 5.972 | 9.2560 | 0 | 18.325 | -0.91 | 0.012 |
| | 6 | 202.170 | 15.87 | 5.571 | 8.6497 | 0 | 17.620 | -0.91 | 0.012 |
| | 6 | 448.120 | 10.33 | 5.549 | 8.6209 | 0 | 17.673 | -0.91 | 0.013 |
| | 6 | 152.120 | 5.08 | 8.161 | 8.9473 | 0 | 16.976 | -0.90 | 0.014 |
| | 6 | 208.130 | 6.54 | 8.136 | 8.9131 | 0 | 16.434 | -0.90 | 0.015 |
| | 6 | 538.180 | 8.91 | 7.883 | 8.6380 | 0 | 15.975 | -0.89 | 0.016 |
| | 6 | 298.190 | 17.96 | 10.303 | 11.2912 | 0 | 21.129 | -0.89 | 0.017 |
| | 6 | 154.030 | 4.91 | 8.159 | 8.9395 | 0 | 16.638 | -0.89 | 0.018 |

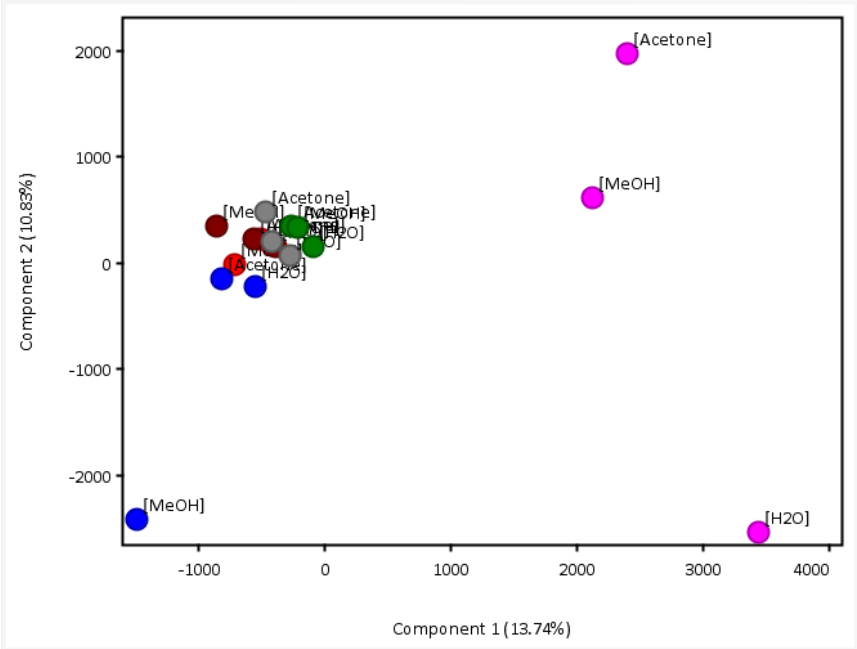
**Supplementary Table S3 Cont.**

*Trichostrongylus colubriformis*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| Methanol | 6 | 117.080 | 1.28 | 19.337 | 0.3936 | 18.828 | 19.963 | -0.88 | 0.021 |
| | 6 | 304.060 | 10.27 | 19.141 | 1.3041 | 17.635 | 20.936 | -0.86 | 0.026 |
| | 6 | 578.210 | 11.23 | 18.860 | 0.2985 | 18.571 | 19.365 | -0.85 | 0.033 |
| | 6 | 114.100 | 15.46 | 19.260 | 0.0562 | 19.214 | 19.367 | -0.82 | 0.044 |
| | 6 | 300.140 | 12.06 | 15.893 | 0.8354 | 14.941 | 17.192 | -0.81 | 0.050 |

*Teladorsagia circumcincta*

| Extract | n | Mass [Da] | Retention time [min] | Abundance (log2) | | | | Pearson's correlation | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Min | Max | r | *P* value |
| All extracts | 18 | CT | | 89.306 | 33.6586 | 17 | 153.300 | -0.14 | 0.579 |
| | 18 | 148.017 | 17.93 | 4.722 | 1.1798 | 0 | 5.090 | -1.00 | <.0001 |
| | 18 | 618.519 | 18.37 | 4.941 | 1.7991 | 0 | 5.660 | -0.68 | 0.002 |
| | 18 | 278.154 | 17.93 | 4.094 | 1.8850 | 0 | 5.040 | -0.54 | 0.021 |
| | 18 | 256.131 | 7.84 | 4.708 | 2.1848 | 0 | 6.080 | -0.54 | 0.022 |
| Methanol and acetone | 12 | CT | | 103.425 | 55.1611 | 68 | 153.300 | -0.19 | 0.565 |
| | 12 | 386.134 | 9.17 | 4.381 | 1.4214 | 0 | 5.390 | -0.98 | <.0001 |
| | 12 | 390.278 | 18.07 | 5.047 | 1.5903 | 0 | 5.620 | -0.97 | <.0001 |
| | 12 | 400.150 | 9.44 | 4.395 | 2.0681 | 0 | 5.730 | -0.82 | 0.001 |
| | 12 | 697.586 | 18.39 | 4.755 | 2.2222 | 0 | 5.820 | -0.63 | 0.028 |
| | 12 | 638.622 | 17.83 | 6.914 | 0.2539 | 6 | 7.200 | -0.60 | 0.038 |

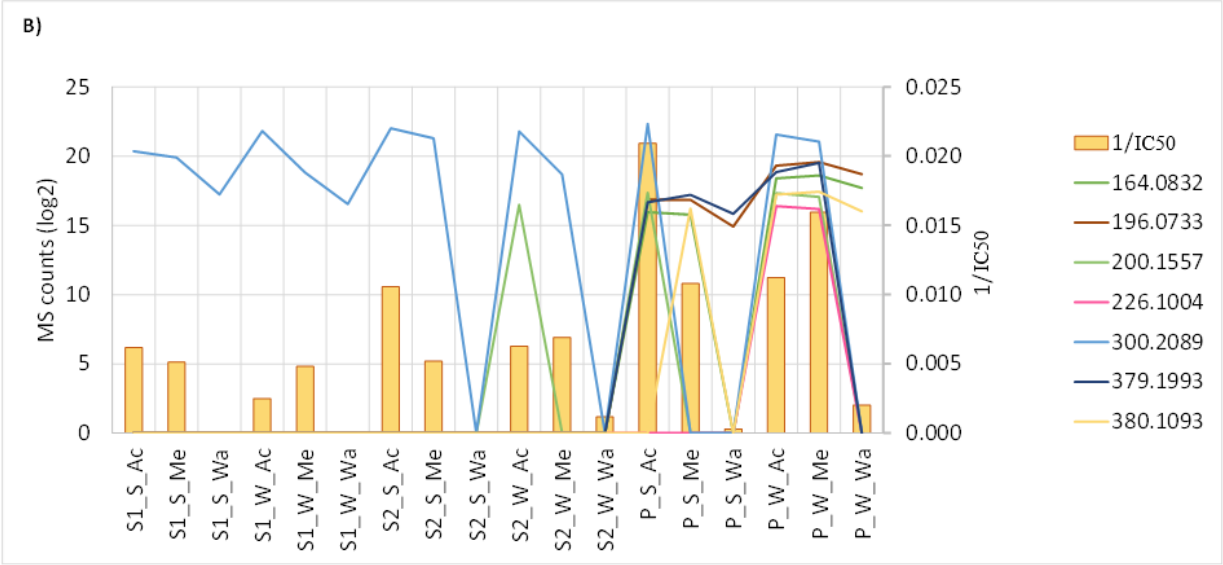



**Supplementary Figure S1**: Principal component analysis of all 2299 compounds that were detected in the 18 bark extracts from mass-spectrometry. Bark extracts are colour coded as follows: spruce 1 (S1: sawmill, ring debarking) from summer (red) and winter (blue); spruce 2 (S2: pulp mill, drum debarking) from summer (brown) and winter (grey); and pine (P: sawmill, ring debarking) from summer (green) and winter (pink). The bark extraction solvents are noted next to the dots; water (H2O), Acetone and methanol (MeOH).

**A)**

| Monoisotopic mass [Da] | Retention time [min] | Molecular formula | Putative annotation | Compound class | Occurrence in species | Correlation analysis | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Me | | Me_Ac | |
| | | | | | | r | *P* value | r | *P* value |
| 164.0832 | 8.18 | $C_{10}H_{12}O_2$ | Eugenol, thujaplicin, thymoquinone | Phenol, monoterpenoid | Both | -0.92 | 0.01 | -0.82 | 0.001 |
| 196.0733 | 7.05 | $C_{10}H_{12}O_4$ | Acetosyringone, atraric acid, brevifolin | Benzoic acid derivative | Pine | -0.93 | 0.01 | -0.82 | 0.001 |
| 200.1557 | 15.4 | $C_{15}H_{20}$ | Corocalene, calacorene | Sesquiterpenoids | Both | -0.99 | <.0001 | | |
| 226.1004 | 16.24 | $C_{15}H_{14}O_2$ | Flavanol (basic unit) | Flavanols | Pine | -0.93 | 0.01 | | |
| 300.2089 | 16.7 | $C_{20}H_{28}O_2$ | Several options | | Both | | | -0.71 | 0.005 |
| 379.1993 | 10.86 | $C_{24}H_{29}NOS$ | Several options | Opioid | Pine | | | -0.82 | 0.001 |
| 380.1093 | 6.8 | $C_{22}H_{20}O_4S$ | Several options | Substituted furan | Pine | -0.93 | 0.01 | -0.72 | 0.009 |

**B)**




**Supplementary Figure S2**: Selected masses where the abundance in the extracts from two independent MS-analyses correlated negatively with $IC_{50}$ (the half maximum inhibitory concentration) of hatching of eggs of *T. colubriformis* (low r value, low *P* value). **A):** Molecular formulas for the selected masses generated based on the molecular mass and MS isotopic distribution, and putative annotations based on data base search. **B)** The abundance of the selected masses (MS counts) plotted against *T. colubriformis* $1/IC_{50}$ to visualize how abundance correlated with bioactivity in EHA. All extracts were analysed using an Electrospray Ionization (ESI) Source, while the Me-extracts were additionally analysed using a more sensitive "Jet Stream" ion source. The masses observed in the two analyses (Me_Ac and Me) were correlated with bioactivity against *T. colubriformis* using Pearson's correlation. Masses that correlated with the inhibition of *T. colubriformis* were manually evaluated by comparing the mass profile (abundance *vs.* sample) with the bioactivity ($1/IC_{50}$).

## Paper III

**Administration of spruce bark (*Picea abies*) extracts in young lambs exhibits anticoccidial effects but reduces milk intake and body weight gain**

**Berit M. Blomstrand**, Heidi L. Enemark, Håvard Steinshamn, Inga M. Aasen, Juni R. E. Johanssen, Spiridoula Athanasiadou, Stig M. Thamsborg, Kristin M. Sørheim. Acta Veterinaria Scandinavica vol 64.10 (2022).

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Acta Veterinaria Scandinavica

RESEARCH

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# Administration of spruce bark (*Picea abies*) extracts in young lambs exhibits anticoccidial effects but reduces milk intake and body weight gain

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## Abstract

**Background:** *Eimeria* spp. are widespread apicomplexan parasites known to cause coccidiosis in livestock, resulting in reduced animal welfare and productivity, particularly in sheep. The treatment options are limited, and there is an emerging development of resistance against registered pharmaceuticals. Spruce bark is rich in plant secondary metabolites (PSM), such as condensed tannins, which are bioactive compounds previously shown to have antiparasitic activity. Here, we examined the anticoccidial properties of bark extract of Norway spruce (*Picea abies*) against a field isolate of ovine *Eimeria* spp. by treating *Eimeria*-infected pre-ruminant lambs with water-extracted bark daily for 12 days. We hypothesised that the bark extract would reduce the faecal oocyst excretion and, consequently, the severity of diarrhoea.

**Results:** Oral administration of spruce bark extract significantly reduced the excretion of *Eimeria* oocysts in milk-fed lambs post treatment till the end of the trial 22 days post infection. This difference in oocyst excretion between the treated and the untreated infected animals increased with time.

Compared to the untreated and the sham-infected control group, the group treated with bark extract had softer faeces and reduced milk intake during the treatment period. After discontinuing the treatment, the treated animals got a more solid and formed faeces compared to that of the untreated control group, and the milk intake increased to the level of the sham-infected, untreated control group. The bark extract treated animals had a lower body weight and a lower mean daily body weight gain throughout the whole duration of the experiment.

**Conclusions:** Bark extract from Norway spruce showed marked anticoccidial properties by reducing the faecal oocyst count and associated diarrhoea in young lambs. Simultaneously we experienced detrimental effects of the treatment, displayed as reduced feed intake and daily body weight gain. Therefore, we suggest conducting similar studies with lower bark extract dosage to explore the possibilities of a better trade-off to reduce the negative impact while maintaining the antiparasitic effect.

**Keywords:** Coccidia, Coccidiocide, *Eimeria*, Industrial by-products, Sheep

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## Background

Coccidiosis is a common disease of livestock caused by various protozoa genera belonging to the phylum *Apicomplexa*. In small ruminants, the monoxenous parasites of the genus *Eimeria* have the potential to negatively impact animal welfare and productivity by causing diarrhoea, reduced growth, and increased mortality, particularly in young animals [1]. Oocysts excreted in the faeces are infective within a few days depending on the parasite species and the temperature, and naïve animals are infected by ingesting sporulated oocysts.

*Eimeria* spp. are common causes of diarrhoea in lambs in Norway [2, 3]. Eleven ovine *Eimeria* spp. have been described, of which *E. crandallis* and *E. ovinoidalis* are considered major pathogens [4]. In Norway, coccidiosis is mainly related to spring grazing and appear 2–3 weeks after release to pasture [3, 5].

To our knowledge only two pharmaceuticals are available for treatment of coccidiosis in mammalian livestock in Europe: Toltrazuril and diclazuril, and they are mainly meant for metaphylactic use [6]. A survey conducted in 2017 showed that anticoccidials were applied in > 80% of Norwegian sheep flocks, and most lambs were treated without a laboratory diagnosis or clinical signs of coccidiosis [3]. Odden et al. [7] hypothesised that uncontrolled and exaggerated use of anticoccidials in lambs may promote the development of drug resistance as previously shown for anthelmintics, and in 2018 anticoccidial resistance against toltrazuril was detected for the first time in ovine coccidia.

Several review articles have pointed out that plant secondary metabolites (PSM) possess antiparasitic properties, both in vitro and in vivo [8–15]. For instance, condensed tannins (CT) have proven antiparasitic effects [16–21]. Few studies have tested the activity of different PSM against *Eimeria* spp. in vivo [10, 22–26], and even fewer have tested CTs against coccidia infections of sheep [27–29]. Bark extracts from coniferous trees are rich in CT, and a recent study has shown that pine extracts have in vitro activity against *Cryptosporidium parvum*, another apicomplexan parasite [16, 30]. To our knowledge, CT-containing bark extracts from Norway spruce (*Picea abies*) have not previously been tested systematically against ovine *Eimeria* spp. in vivo in milk feeding lambs this young.

Norway has a large forestry industry. A production volume of 7.21 million $m^3$ spruce (*P. abies*) and pine (*Pinus sylvestris*) logs utilised in the sawmill and wood processing industry was estimated for 2019, which is equivalent to a total of 721,000 $m^3$ wet bark [31]. The possibility to exploit large amounts of excess bark from the Norwegian forestry industry offers a strong incentive to further explore novel approaches to control coccidiosis. In this study, we assessed the anticoccidial effect of a water-based extract of bark from Norway spruce (*Picea abies*) in milk-fed lambs. We hypothesized that bark extract would reduce the faecal oocyst excretion and consequently reduce the severity of diarrhoea in lambs infected with mixed *Eimeria* spp.

## Methods

### Animals

To ensure parasite-free experimental animals, a total of 24 lambs of the Norwegian White Sheep breed ("Norsk kvit sau") where removed from their mothers at birth in April 2019, immediately washed with Optima pH 4 soap (Optima Produkter AS, Norheimsund, Norway) and dried with clean towels before being placed in a parasite free, indoor housing area (Deluxe SL calf hutches, Agri-Plastics, Galway, Ireland) [32]. Mean body weight (BW) of all lambs at birth was 4.7 kg ± 0.18 (mean ± SEM).

All experimentation was conducted in line with FOTS Norwegian Food Safety Authority, license number 18555, according to The Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations.

### Study design

The lambs were grouped in blocks by sex and birth weight and randomly allocated into three experimental groups (n = 8) within a week after birth: IB (infected animals treated with bark extract), IC (infected, untreated control group), and SC (sham-infected, untreated control group; for milk consumption and BW comparison). There was no difference in mean birth weight between the groups ($P > 0.05$). Animals in IB and IC, housed in pairs or three together (0.7–1 $m^2$ per lamb) and the lambs in SC in one group of eight (1 $m^2$ per lamb), were acclimatised in their respective huts prior to infection.

The lambs were observed twice daily with regards to health status and adverse reactions to infection and bark extract treatment. In case of adverse reactions to the bark extract treatment, we would reduce the bark extract concentration and/or the treatment frequency. If considered necessary, the animals would receive the appropriate treatment, i.e. electrolyte solutions per os. If no response was obtained, the animals would be removed from the trial.

### Parasite infection

The field isolate of mixed ovine *Eimeria* spp. was obtained from 3 to 6 weeks old, naturally infected lambs housed at the Norwegian University of Life Sciences in Sandnes, Norway. The oocysts were recovered from lambs 10 months prior to this study and stored at 2–7 °C until use. The oocysts were purified according

to Eckert et al. with some modifications [32]. In short, faeces were mixed with tap water (1:10) for 30 s in a blender and filtered through a sieve (250 µm pore size). The fluid was collected in 50 mL tubes and centrifuged (1550×*g*, 5 min). Then, the top 35 mL was removed with a syringe and discarded, and flotation fluid (concentrated salt-sucrose fluid, specific gravity 1.28) was added to the precipitate (3:1) and thoroughly mixed. The mixture was left for 30 min for the oocyst to float. Subsequently, the upper 15 mL containing the oocysts were collected with a syringe and washed twice in tap water (1550×*g*, 5 min). Finally, the oocysts were washed in phosphate buffered saline (PBS) (1550×*g*, 5 min), poured into a borosilicate bottle, and left to sporulate for 10 days under constant aeration at room temperature using an aquarium aeration device.

The oocyst concentration and degree of sporulation was calculated using a modified McMaster method with a sensitivity of 5 oocysts per gram, and the solution was diluted down to 20,000 sporulated oocysts/mL in tap water and stored at 2–7 °C until use the following day [33, 34]. Speciation of the inoculum was done by examining > 500 oocysts and revealed 62% highly pathogenic *Eimeria* spp.: 54% *E. ovinoidalis* and 8% *E. crandallis*. The remaining 38% consisted of *E. parva* (18%), *E. faurei* (16%), *E. pallida* (1%), *E. ahsata* (1%), *E. weybridgensis* (< 1%), *E. bakuensis* (< 1%), and *E. intricata* (< 1%).

At the age of 23–26 days (D0), IB and IC were infected for three consecutive days by oral gavage (gastric tube) with 100.000 *Eimeria* oocysts (5 mL) per day [32]. We aimed for the experimental animals to have mild to moderate clinical symptoms of coccidiosis. SC was drenched with 5 mL tap water (Table 1).

### Feeding

The lambs were fed high-quality sheep colostrum stored frozen (− 80 °C) from the previous lambing season. Good quality barn dried hay (grass-clover mixture) harvested in 2018 was purchased from a local farmer, allocated in plastic bags of 10 kg and frozen at − 25 °C for eight weeks (decontamination). The fodder was stored in the bags until feeding. The milk replacer (Pluss Ulla) and concentrates (FORMEL Lam) were purchased from the feed supplier Felleskjøpet (Oslo, Norway). Each lamb was dosed with 150 mL colostrum within 1 h post-partum, followed by five meals of 150 mL within the first 24 h of

**Table 1** Experimental timeline showing time of infection, bark extract drenching, weighing, and faecal sampling and scoring

| Project day | *Eimeria* oocyst infection | Bark extract drenching | Weighing | Faecal sampling | Faecal scoring |
|---|---|---|---|---|---|
| 0 | IB, IC | IB | All | All | All |
| 1 | IB, IC | IB | | | |
| 2 | IB, IC | IB | | | |
| 3 | | IB | | | |
| 4 | | IB | | | |
| 5 | | IB | | | |
| 6 | | IB | | | |
| 7 | | IB | IB, IC | | |
| 8 | | IB | | | |
| 9 | | IB | SC | All | All |
| 10 | | IB | | | IB, IC |
| 11 | | IB | | | IB, IC |
| 12 | | | | All | IB, IC |
| 13 | | | | | |
| 14 | | | | All | IB, IC |
| 15 | | | | IB, IC | IB, IC |
| 16 | | | | IB, IC | IB, IC |
| 17 | | | | IB, IC | IB, IC |
| 18 | | | | IB, IC | IB, IC |
| 19 | | | | IB, IC | IB, IC |
| 20 | | | | All | All |
| 21 | | | All | | |
| 22 | | | Slaughter | All | All |

*IB* infected lambs treated with bark extracts, *IC* infected, untreated control animals, *SC* sham-infected, untreated control animals

**Table 2** Quantity (L/meal and L/d) of milk replacer offered to the lambs during the experiment

| Week number | 1 | 2 | 3 | 4 | 5–7 |
|---|---|---|---|---|---|
| L/meal | 0.3 | 0.5 | 0.7 | 0.5 | 0.4 |
| L/d | 0.9 | 1.5 | 2.1 | 1.5 | 1.2 |

their lives. From the second day after birth, the lambs were fed milk replacer at the amounts shown in Table 2, and water, hay, and concentrate ad libitum.

#### Bark extraction and determination of CT concentration

Bark from *Picea abies* (Norway spruce) was collected from a sawmill located in Møre og Romsdal, Norway (Bøfjorden sag AS, Surnadal) in March 2019. The bark was obtained by ring debarking, air dried to approximately 40% dry weight and milled to 10–30 mm particle size with an apple grinder.

The dry bark (66 kg) was divided in two batches and each batch was extracted twice for 1–1.5 h in a stirred tank at 80 °C, using 1000 L water in step 1 and 850 L in step 2, which corresponded to 28 L tap water per kg dried bark. After each extraction step, stirring was turned off to let the bark sink. The liquid phase was collected from the top by pumping and transferred to a holding tank. The combined extract (approximately 1300 L) was evaporated in a mechanical vapour recompression evaporator with forced recirculation (Epcon, Epcovap MVR 4) to a final concentrate volume of 82 L before freeze-drying.

Total CT in the extract was quantified by the butanol-HCL assay with cyanidin-HCl as standard: the freeze-dried extract and cyanidin-HCl (standard) were dissolved in methanol (80% in water) and analysed using the conventional reagent without acetone, 2.5 h, and absorbance reading at 545 nm [35]. The extract yield was 118 mg dry matter (DM)/g dry bark, and the analysed CT yield was 5.8 mg CT/g dry bark. The concentration of CT in the bark extract was 49 mg CT/g DM extract.

The dried extract was stored at − 20 °C until use.

#### Bark extract drenching

Based upon previous trials, we decided to dose the lambs with a bark extract equivalent to 0.1% CT/kg metabolic bodyweight (mBW)/d [24, 28]. Initially, we mixed 17 g dry bark extract (equivalent to 0.85 g CT, ¼ of the planned daily dose) with 250 mL milk replacer and offered this to the lambs to drink voluntarily. As the animals refused to drink this mixture, we chose to administer the bark extract dissolved in tap water with a stomach tube. Immediately prior to use, the bark extract was dissolved in tap water. From D0, IB lambs were drenched daily for 12 days. Each treatment was planned to be given as ca 250 mL (depending on BW) dissolved extract at the dose shown in Table 3. D0, IB was drenched once with CT equivalent to 0.05% of mBW in one meal (~250 mL dissolved extract). This dose was repeated in the morning on D1. The lambs showed reduced appetite and experienced discomfort, hence on the evening of D1 we reduced the volume given by half. This clearly reduced the discomfort of the animals. Because of the high viscosity of the extract, the solution was difficult to administer, and for the fourth extract administration (D2) we decided to dilute the extract by half. To acclimatise the animals to the treatment, this was given once daily D2–D4. From D5, this amount of extract was given twice daily and consequently the final volume per day was approximately 500 mL with a CT concentration of approximately 7.6 mg CT/mL, a CT dose of 0.05% of mBW (Table 3). The dosage was adjusted with each weighing of the animal. IC and SC were given the equivalent volume of tap water.

#### Sampling and laboratory analysis

Individual faecal samples from each lamb were collected directly from rectum on D0, D9, D12 and daily from d14 (Table 1). The faecal consistency was evaluated D0 and daily from D9 on a scale from one to five (1 = hard pellets, 2 = soft, sticky pellets, 3 = soft, paste-like with no pellet structure, 4 = watery, 5 = watery with blood and/or intestinal casts) [36]. A score > 3 was considered as diarrhoea. The faecal samples were stored in 40 mL polypropylene screw-cap containers (VWR, Avantor®) at 2–7 °C until analysis. The oocyst excretion was quantified as mentioned above using a modified McMaster method [33, 34]. All lambs were weighed at birth (D − 26 to D − 23) and on D − 6, D0, D7, and D21. Due to practical challenges, SC was weighed at D9 instead of D7. The appetite was determined by registering the daily individual milk intake (Fig. 1). The health status of the lambs was evaluated twice daily by visual inspection. We judged the animals' posture at resting and at movement, and behaviour was evaluated on the animals' preference for playing, eating, exploring the environment, and other physiological habits. If the general constitution of the animal was assessed to be

**Table 3** Concentration of condensed tannins (CT) in bark extracts dosed (mg/mL) and the daily dosing of CT as percentage of lamb metabolic body weight (mBW)

| Experiment day | Number of daily doses | Daily dose in % CT of mBW | CT concentration, mg/mL |
|---|---|---|---|
| 0 | 1 | 0.05 | 15.4 |
| 1 | 2 | 0.075 | 13.0 |
| 2–4 | 1 | 0.025 | 6.7 |
| 5–6 | 2 | 0.05 | 6.7 |
| 7–10 | 2 | 0.05 | 7.3 |
| 11 | 1 | 0.025 | 10.3 |

within the physiological limits, we judged the animal to be healthy. At any deviation from expected physiological posture, behaviour, or habitus, we examined the animal more thoroughly by measuring the rectal body temperature, evaluating the pulse frequency and quality, appraise the mucus membranes, auscultating thorax and abdomen, and palpating abdomen and relevant superficial lymph nodes. All deviations were registered, and appropriate measures were taken with regards to animal health and welfare. On D22, the animals were transported to a nearby abattoir and euthanised using a captive bolt gun followed by exsanguination.

treatment-time interaction as fixed effects, and pen and individual as random effects. We used the least square means procedure to estimate mean values with 95% confidence intervals (CI) at each time point.

For SC, the values of faecal consistency score (FCS) were $\leq 2$ for all animals and days, and there was practically no variation, hence SC was not included in the statistical modelling of FCS.

FCS was modelled as an ordinal response variable and day was treated as a continuous variable. We ended up using a statistical model for the cumulative probabilities of the response variable, FCS:

$$P\left(y_{ijk}(d) \leq q | B_{j(i)}, I_{k(ij)}\right) = \frac{\exp\left(\mu_q + \alpha_i + \beta \cdot d + \gamma_i \cdot d + B_{j(i)} + I_{k(ij)}\right)}{1 + \exp\left(\mu_q + \alpha_i + \beta \cdot d + \gamma_i \cdot d + B_{j(i)} + I_{k(ij)}\right)}, q = 1, 2, \ldots, 5$$

### Statistical methods

The faecal excretion of *Eimeria* oocysts in infected lambs generally follows an exponential curve before it flattens out and finally starts declining [37]. We assumed we could describe the faecal oocyst excretion per g of faeces (OPG) by the following logistic equation: $Y = Ce^{k\left(\frac{1}{t}\right)}$, where $C$ is the constant, e is the natural constant, $k$ is the growth rate, and $t$ is the time (day). We transformed the exponential expression into a linear equation: $Y1 = a + b * t1$, where $Y1$ is $\log(Y+1)$, $a$ is $\log(C)$, $b$ is $k$ depending on treatment (IB, IC or SC), and $t1$ is $\frac{1}{t}$. Data for oocyst excretion was analysed with a repeated-measure analysis for infected groups with and without bark extract, with time ($t1$ as D0 of experiment) and

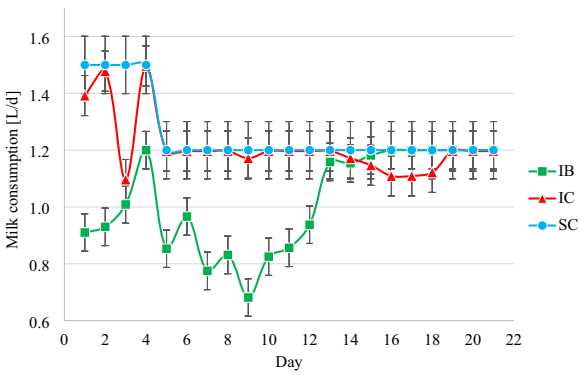


**Fig. 1** Mean daily milk consumption for all three treatment groups. *IB* Infected lambs treated with bark extract, *IC* Infected, untreated negative control group, *SC* Sham-infected, untreated control group. Error bars standard error of the means

where $y_{ijk}(d)$ is FCS for individual $k$, from housing $j$ within treatment $i$ on day $d$. $\alpha_i$ is the main effect of treatment $i$, $\beta$ is the main effect of day, $\gamma_i$ is the interaction effect between treatment $i$ and day, $i$ = IB, IC. $B_{j(i)}$ is the random effect of house $j$ within treatment $i$, and $I_{k(ij)}$ is the random effect of individual $k$ within house $j$ and treatment $i$. The $\mu_q$'s are intercepts, where $\mu_1 \leq \mu_2 \leq \mu_3 \leq \mu_4 \leq \mu_5$ ($\mu_5 = \infty$). Our null hypothesis ($H_0$) was that there was no effect of treatment or of treatment by day interaction on FCS: $H_0 : \alpha_1 = \alpha_2$ and $\gamma_1 = \gamma_2$. Sex had no impact on FCS and was not included in the model. The model was estimated, and the hypothesis tested using the glimmix procedure in SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA). *The null* hypotheses were tested using Bonferroni adjustment.

BW and milk consumption were analysed using the glimmix procedure in SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA). Treatment (IB, IC, SC), sex (male or female), and day in experiment (continuous) and their interactions were included as fixed effects and pen within treatment as random effect. Sex had no effect and was removed from the final model. For BW, we included BW at the start of experiment (D = 0) as a co-variate. The body weight gain for each treatment was calculated from the solutions for the fixed effects (estimated regression coefficients). Bonferroni's test was used for pairwise comparisons of LSmeans ($P \leq 0.05$).

## Results

### Clinical observations

One lamb in the IB group showed mild signs of colic on D1, which subdued after a few minutes. D7 and D8, two lambs (IB) refused to drink milk. Instead, they were offered an electrolyte solution, which they drank

voluntarily. D9, one lamb in the SC group developed mild signs of colic, which abated after a few minutes of massaging the abdomen. On D4 of the experiment, a lamb (IB) died shortly after bark extract drenching. Necropsy results suggested an intrapulmonary rather than transoesophageal administration of the bark extract, resulting in death due to asphyxiation. This lamb was immediately replaced by a lamb from SC (lamb 10), which was infected for three consecutive days and given bark extract daily for the following 12 days. Data was tested statistically both with and without data from lamb 10, with no difference in outcome, hence we decided to include data from lamb 10 in the analyses. One lamb (IB) was diagnosed with polyarthritis on D20 and was euthanised D21 using a captive bolt gun and subsequent exsanguination.

### Faecal oocyst count (FOC)

The lambs in IC started excreting oocysts on D14, and those in IB on D15, with 88% and 63% of the lambs excreting oocysts in group IC and IB, respectively. There was an extract by day interaction where the difference in oocysts per gram (OPG) between IB and IC increased with time ($P<0.05$, Fig. 2). Compared to IC, IB had a lower mean oocyst count on D14 and onwards ($P<0.001$), with an arithmetic mean oocyst count on D22 of 25,838 OPG in IB and 613,250 OPG in IC (SEM 16,854 and 173,190, respectively).

### Faecal consistency score (FCS)

Before the start of the experiment (D0), all animals had pelleted, dry faeces. During the trial period (D0–D22), FCS differed between the two infected groups. From D0 to D11, IB lambs had higher FCS compared to IC, with 4 lambs (13%) in IB having FCS > 3 (i.e. diarrhoea) on D11 vs. 0 lambs in IC. For the post treatment period (D12-D22), the number of incidents with FCS > 3 was 1 vs. 22 in the IB lambs compared to IC lambs (Additional file 1). Thus, during the post treatment period, the animals treated with bark extracts (IB) were less prone to diarrhoea compared to the untreated animals (IC). This is illustrated in Fig. 3, which shows the estimated probability for FCS being less than or equal to a certain value. For IB, the probability of a decrease in FCS (i.e. more solid faeces) was lower at the beginning of the experiment compared to after D11, and the probability of a lower FCS increased with time. For lambs in IC, on the other hand, we observed a high probability of low FCS during the prepatent period (before D11), but this probability decreased with time (i.e. the animals were more likely to experience diarrhoea as time passed). The estimated parameters of treatment effect ($\alpha_{IB}$) and effect of treatment-day interaction ($\gamma_{IB}$) for IB were − 6.2 and 0.5, respectively, and day effect ($\beta$) for both IB and IC was -0.4. The equivalent parameters for IC, $\alpha_{IC}$ and $\gamma_{IC}$, were 0. Our null hypothesis ($H_0 : \alpha_1 = \alpha_2$ and $\gamma_1 = \gamma_2$) that treatment with bark extract had no effect on FCS and that there was no extract-day interaction, had to be rejected ($P<0.001$).

### Weight gains and milk consumption

There was a significant day by extract interaction on mean BW ($P<0.05$). At D0, there was no difference in BW between the groups ($P>0.05$). On D21, IB had a lower mean BW compared to IC and SC ($P<0.05$). From D0 to D22, the estimated mean daily weight gain was 292 g/D, 387 g/D, and 415 g/D for IB, IC, and SC, respectively, with IB lambs having a lower weight gain than SC ($P<0.05$).

There was a significant day by extract interaction on milk consumption ($P<0.05$). IB had a lower milk consumption compared to IC during the whole treatment period (D0–D11) (Fig. 1). Compared to SC, IC experienced a reduction in milk consumption during D0–D2 when they were inoculated with *Eimeria* oocysts (Fig. 1),

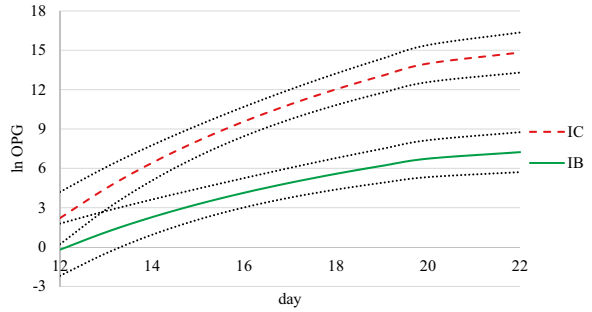


**Fig. 2** Estimated values of *Eimeria* oocysts per gram faeces (OPG) over time in lambs infected with *Eimeria* spp. (D0–D2), treated with spruce bark extract (IB) or treated with water (IC). The bark extract was given D0–D11. Area between dashed lines: 95% confidence interval. The figure shows data transformed with the natural logarithm (ln)

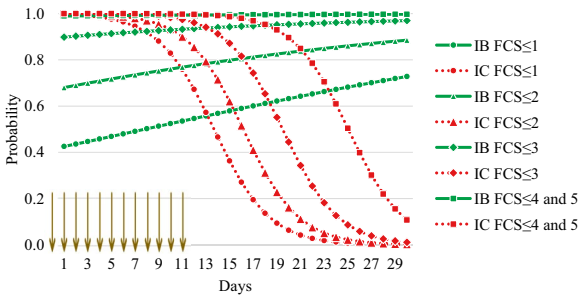


**Fig. 3** Estimated probability for faecal consistency scores (FCS) being less than or equal to 1, 2, 3, or 4 and 5 in lambs infected with *Eimeria* spp., treated (IB) or not treated with (IC) bark extract. The vertical arrows symbolise the time when bark extract was administered (D0–D11). IB and IC were infected for three consecutive days (D0–D2). FCS 1: hard pellets, 2: soft, sticky pellets, 3: soft, paste-like faeces with no pellet structure, 4: watery faeces, 5: watery faeces with blood and/or intestinal tissue

although this difference was not significant ($P > 0.05$). Additionally, this group had a minor drop in milk consumption from D13 to D19, also not significant ($P > 0.05$). SC consumed the milk they were offered during the whole experimental period.

## Discussion

This study showed that bark extract from Norway spruce had anticoccidial properties in milk-fed lambs infected with *Eimeria* spp. The regression analyses support the hypothesis that oral administration of bark extract reduces the excretion of *Eimeria* oocysts in lambs for the period up until 22 days after infection, corresponding to 12 days after the last day of bark extract administration. We also demonstrated that the difference in oocyst excretion between the treated and the untreated group increased with time, past the time of the extract administration. These findings agree with the results of other studies addressing the possible effects of CT containing PSM against protozoa. In a study where experimentally infected weaned lambs were fed CT-rich sainfoin (*Onobrychis viciifolia*), they found reduced faecal oocyst excretion in treated lambs compared to untreated control lambs [24]. Similarly, in an experiment with naturally infected goats it was found that animals receiving pine needles had significantly lower FOC than the untreated control group [29]. Quebracho extract supplemented to the diet reduced the *Eimeria* oocyst excretion in naturally infected goats [28]. Providing DM digestibility of a diet does not change, reduced feed intake will result in a reduced amount of faeces. Therefore, FOC might appear to increase as it is a concentration number. Similarly, diarrhoea will have a diluting effect on the oocyst concentration, as FOC is estimated on fresh matter basis. To reduce this type of biases, it would have been ideal to calculate faeces dry matter content and express the oocyst number per gram of dry matter faeces. However, it is important to point out that both a lower feed intake and lower FCS in group IB would increase FOC and not decrease it, as we observed.

After discontinuation of the treatment (from D12 onwards), the lower FCS of IB coincided with the lower FOC, compared to IC. From D14, we saw that IC had a higher FCS compared to IB and SC, which coincided with the prepatent time of ovine *Eimeria* infections, which is 12–20 days for most ovine species, including the pathogenic species *E. ovinoidalis* and *E. crandallis* that constituted ≈ 62% of the infection dose used in our study [32]. A high oocyst excretion correlates with damage to the intestinal mucosa and diarrhoea in ruminants [38].

The IC lambs had a reduction in milk consumption during the oocyst inoculation period (D0–D2), which can be expected for animals exposed to parasites [39]. Additionally, this group had a drop in milk intake during D13–D19. This period coincides with the end of the prepatent period of several ovine *Eimeria* spp., which is when we would expect damage to the intestinal mucosa and a subsequent reduction in appetite [38]. IB lambs also had a reduction in milk intake in the same time frame, but to a much lesser degree and on D13–D14 only.

Shortly after bark extract treatment, several of the animals in the IB group had reduced milk intake and showed symptoms of obstipation and colic, in some cases with subsequent diarrhoea. We also observed lower mean daily weight gain for IB compared to IC and SC lambs. We assumed that the elevated FCS and the reduction in milk consumption during the treatment period (D0–D11) was a consequence of the bark extract administration, which was confirmed by the fact that FCS decreased, and the appetite returned to normal after discontinuation of the extract treatment. The reduced growth we experienced in this trial was probably due to the reduction in voluntary feed intake, likely attributed to the high astringency of CTs, hence the low palatability of the extract [40]. Additionally, direct toxic effects of the extract, or impact on the dry matter digestibility of the feed, can have a negative impact on the animal's growth [41, 42].

Detrimental effects of plant extracts have been reported previously. For instance, CT-rich quebracho extracts have shown to cause anorexia, diarrhoea, and reduced growth in lambs [43, 44]. The observed difference in BW between the treatment groups in these two studies was nullified at the end of the experiment, day 39–67. In the present trial, the animals were euthanised at D22, hence we were unable to evaluate the long-term effect of the bark extract on the BW.

In another experiment, extracts of Ethiopian plants caused anorexia and near moribund behaviour in house mice infected with *Heligmosomoides bakeri* [45]. Loose stool in sheep and goats treated with wattle tannin extracts containing 70% CT was attributed to the low molecular weight of hydrolysable tannins in the extract, the ability of hydrolysable tannins to desquamate the intestinal surface epithelium and in this way cause diarrhoea [46]. Contradictory to these findings, in trials where lambs were naturally infected with *Eimeria* spp. were fed CT-rich sainfoin diets, there were no differences between the groups regarding FCS and weight gain [24, 27]. Although the effective CT dose in our trial (2.4–4.8 g CT/20 kg BW) was lower than that used in other studies (10–58 g CT/20 kg BW) [24, 47, 47], our IB lambs still exhibited detrimental effects of the bark extract, i.e. lower body weight gain, reduced milk consumption, and increased FCS during the treatment, compared to IC. Possible reasons for this might be the different composition of the plant materials (spruce bark *vs.* sainfoin vs.

wattle vs. quebracho), the route of administration of the CT-rich supplement (extract vs. in-feed), or the age of the animals (pre-weaned *vs.* weaned). There might be other, non-CT, components in the respective plants contributing to the antiparasitic effect.

A reduction in the levels of parasitism is often followed by improvement on the performance of the host. In this trial, although we saw a significant reduction in OPG and a lower FCS of IB lambs in the post treatment period, we also observed detrimental effects of the extracts, such as higher FCS during the treatment period and a significant reduction in milk consumption and weight gain. It has been previously reported that CT consumption can have positive (antiparasitic) and negative (anti-nutritional) consequences when consumed by parasitised host [48]. Although IB had significantly lower overall FOC (D1–D22) compared to IC (98.0% lower), the anti-nutritional effects of the bark extracts on performance (10.3% lower BW for IB at the end of experiment and 29.0% lower milk intake for IB for the treatment period (D0–D11), compared to IC) and the initial problems of indigestion outweighed the pathological effects of the coccidia infection, thus the trade-off resulted in an overall cost for the parasitised animals. A reduction in the extract dose, in volume and/or in CT content per kg BW, or in the duration of administration, would most likely have resulted in a better trade-off between anti-parasitic efficacy and adverse effect induced by the supplementation in the treated lambs. Nevertheless, the possibility that the BW of the treated lambs may have caught up with or surpassed that of lambs in the sham-infected control group, if the experiment was prolonged, cannot be excluded.

CT are components important for the plant's protection; their consumption can penalise herbivores and act as feeding repellents. The consumption of tannins has been associated with a reduction in feed intake and dry matter digestibility when administered at 7.5–10% of the dry matter [42]. High concentrations of Quebracho extract are considered toxic to ruminants which can be attributed to CTs' ability to bind to proteins [49]. It seems that CT administered via the feed, e.g., as browse, hay, or pelleted feed, may have fewer negative effects on faecal consistency and feed intake compared to crude plant extracts administered through drenching [24, 27, 43, 45, 46, 50]. The animals in our trial were young lambs without a fully developed ruminating function. It has been reported that ruminants are able to tolerate potential toxic effects of CT by slowly adapting their ruminal bacteria through shifting the microbial population towards microbes able to alter the CT [51]. It is likely that young lambs with undeveloped rumen may not have this ability, which would make them more susceptible to an astringent, toxic component. Another mechanism for tolerating the anti-nutritional effects of CT is by excreting CT-binding proteins in the saliva [40]. Little research has focused on CT-binding proteins in ruminant saliva, and it is plausible that the protein composition in pre-ruminant lambs is different from adults.

It is improbable that the reduction in milk intake in IB lambs was due to the total volume of extract offered. According to Large, 1964, the volume of omasum-abomasum measured in ≥ 3 weeks old lambs of a similar breed is > 250 mL, which was the amount of bark extract given in our trial [52, 53]. This was indeed confirmed by the fact that IC receiving 250 mL water consumed all the milk replacement they were offered. Nevertheless, the bark extract had a higher viscosity compared to water, which might have influenced the passing time of the drenched substance (i.e. the time it takes for the extract to pass the abomasum). Furthermore, the bark extract contains components (e.g., CT) which might influence the abomasum and the animal negatively, e.g., by causing nausea.

In this trial, the bark extract was administered by oral gavage for 12 days. More research is needed regarding modes of administration to make it practicable to use bark extract as an agent to control parasites in young lambs.

We suggest that future research should focus to improve the trade-off between the negative side effects *vs.* the benefits of the treatment to assess if the trade-off could be better utilised at a lower bark extract concentration. We also recommend conducting a dose–response study and to evaluate the outcome with subsequent necropsy. Furthermore, we suggest additional studies with higher parasite load and a longer follow-up period, e.g., from birth till slaughtering at 5 months of age, which is common in Norway, to assess if the bark extract treated group will benefit from the lower parasite burden in a longer perspective.

Coccidiosis is considered an important disease in sheep production worldwide. If spruce bark extracts can be used to control coccidiosis in sheep, it will help reducing oocyst shedding and prevent the contamination of pastures. This will decrease the need of metaphylactic treatment, which again will reduce the development of anticoccidial resistance.

## Conclusion

The water-based extract of bark from Norway spruce (*P. abies*) showed anticoccidial properties by reducing the oocyst excretion in milk-fed lambs. Additionally, after treatment with bark extract, the lambs experienced a lower incidence of diarrhoea. We observed unwanted side effects of the bark extract, expressed as cases of indigestion, reduced milk consumption and body weight gain.

**Abbreviations**
BW: Body weight; CT: Condensed tannins; DM: Dry matter; FCS: Faecal consistency score; FOC: Faecal oocyst count; IB: Infected lambs treated with bark extract; IC: Infected, untreated control animals; SC: Sham-infected, untreated control animals; mBW: metabolic body weight; OPG: Oocysts per gram faeces; PSM: Plant secondary metabolite.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13028-022-00629-y.

**Additional file 1** Faecal consistency score of every individual on each sampling day. IB and IC were infected for 3 consecutive days (D0–D2), with 100,000 sporulated *Eimeria* oocysts. IB was treated with spruce bark extract for 12 consecutive days (D0–D11). IC and SC were given the equivalent amount of tap water. SC was not sampled on D10–D19. FCS: faecal consistency score; IB: infected lambs treated with bark extract; IC: infected, untreated control animals; SC: sham-infected, untreated control animals. n is the number of samples collected on the specific sampling day.

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**Prior to publication**
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**Author contributions**
BMB, HLE, and KMS designed the experiment, BMB, HLE, JREJ, and KMS performed the experiment, including collecting all samples, IMA and KMS prepared bark and produced and analysed the bark extracts, HS, KMS, IMA, BMB, SMT, and SA analysed and interpreted data. All authors read and approved the final manuscript.

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**Availability of data and materials**
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate**
All experimentation was conducted in line with FOTS Norwegian Food Safety Authority, license number 18555, according to The Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations.

**Consent for publications**
Not applicable.

**Competing interests**
The authors declare that they have no conflict of interest.

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## Publisher's Note

## Paper IV

# Bark extract from Scots pine (*Pinus sylvestris*) reduces the impact of *Heligmosomoides bakeri* infection on C57BL/6 but not on BALB/c mice (*Mus musculus*)

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(Manuscript)

# Bark extract from Scots pine (*Pinus sylvestris*) reduces the impact of *Heligmosomoides bakeri* infection on C57BL/6 but not on BALB/c mice (*Mus musculus*)

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## 1. Abstract

Plant secondary metabolites may improve gut health by exerting immunomodulatory, anti-inflammatory, and antiparasitic effects. Bark extracts from coniferous tree species have previously shown to reduce the burden of a range of parasite species in the gastrointestinal tract, with condensed tannins as the suspected active ingredients. Here, we investigated the impact of an acetone extract from pine bark (*Pinus sylvestris*) on the resistance, performance, and tolerance of genetically diverse mice (*Mus musculus*). Mice able to clear an infection quickly (fast responders, BALB/c) or more slowly (slow responders, C57BL/6) were infected orally with 200 infective 3$^{rd}$ stage larvae of the parasitic nematode *Heligmosomoides bakeri* or were left uninfected by dosing with water only. Each infection group of mice were gavaged for three consecutive days daily from day 19 post infection with either bark extract or dimethyl sulfoxide as vehicle control.

Oral administration of pine bark extract did not exert any significant effects in the parasitological parameters measured, though it increased the body weight and carcass weight and reduced the FI-BW ratio in the infected slow responder mice only. Furthermore, bark extract administration reduced the tolerance of the fast responder mice only. Thus, our results imply that host responses to plant secondary metabolites may be sensitive to variation in genetic resistance to parasites.



## 2. Keywords

Gastrointestinal nematodes, plant secondary metabolites, condensed tannins


## 3. Introduction

Gastrointestinal nematode (GIN) infections cause a decrease in animal health and welfare, productivity, and farm profitability worldwide (Charlier *et al.*, 2020). Anthelmintic pharmaceuticals (AH) are crucial for the control of GIN and often represent the most easily directly available option (Molento, 2009). However, extensive use of AH has led to a widespread anthelmintic resistance in GIN (Rose Vineer *et al.*, 2020). Though this worldwide challenge has been known for some time, there remains thus a pressing need for alternative options for the control of GIN. Amongst the alternatives currently under investigation, plant secondary metabolites (PSM) have been extensively researched *in vitro* and *in vivo* for their

antiparasitic properties (Anthony *et al.*, 2005; Hoste *et al.*, 2015; Spiegler *et al.*, 2017). Some studies have shown that condensed tannins (CT) have anthelmintic activity (Hoste *et al.*, 2006; Desrues *et al.*, 2016b; Mengistu *et al.*, 2017), whereas other studies have revealed other components like sesquiterpene lactones or fatty acids to be responsible for the antiparasitic attributes of plant extracts (Valente *et al.*, 2021; Bonde *et al.*, 2021).

Bark extracts from coniferous trees are rich in PSM such as CT, and recent investigations have shown that bark extracts have antiparasitic properties *in vitro* (Athanasiadou *et al.*, 2021; Blomstrand *et al.*, 2021). Here for the first time, we tested the anthelmintic activity of bark extract *in vivo* in mice infected with the intestinal nematode *Heligmosomoides bakeri.* *H. bakeri* is a trichostrongyloid nematode of the house mouse, *Mus musculus*, placed phylogenetically in the same order as some of the most pathogenic nematode species in humans and livestock (Ancylostoma duodenale, Necator americanus, *Ostertagia* spp., *Haemonchus contortus, Trichostrongylus spp*., amongst others) (Gouÿ De Bellocq *et al.*, 2001; Reynolds *et al.*, 2012). It is widely used as a laboratory model of chronic helminthiasis of human and veterinary importance, and to investigate mechanisms of host resistance and tolerance to GIN infections (Monroy and Enriquez, 1992; Behnke *et al.*, 2009).

Host resistance (ability to clear the pathogen) to *H. bakeri* is, at least partly, genetically controlled (Ayres and Schneider, 2012; Råberg, 2014). Behnke *et al.*, 2006, demonstrated that there is variation in the susceptibility of specific mouse lines towards *H. bakeri*. Mice strains such as BALB/c are characterised as fast responders to *H. bakeri*, as they have the ability to quickly clear out the infection relative to that of others, including C57BL/6, known therefore as slow responders. The latter are maintaining the parasite population in their intestine for many weeks prior to expulsion, which is a suitable trait to mimic long term GIN infections in livestock. Athanasiadou *et al.*, 2015, showed that variation in tolerance (the ability of the host to reduce the parasites' impact on the host's performance) is also, at least partly, genetically controlled, as genetically diverse lines of mice demonstrated difference in their tolerance to *H. bakeri*. We expected the C57BL/6 to have a lower tolerance to the infection compared to BALB/c, and that this would be improved by the bark extract treatment (Athanasiadou *et al.*, 2015).

The aim of our study was to quantify the impact of bark extract administration on the resistance, performance, and tolerance of genetically diverse mice. We hypothesised that infected slow responder mice would benefit more from the bark extract administration compared to those of the fast responder line. Our predictions were that infected bark extract

treated slow responder mice would experience a higher reduction in the parasitic burden, a higher increase in performance, and better tolerance to the parasitic infection compared to the fast responder mice. To test our hypothesis, we selected two genetically diverse lines of mice, a slow responder (C57BL/6) and a fast responder (BALB/c) line to *H. bakeri*.

## 4. Materials and methods

### 4.1. Experimental animals and housing

Female, five weeks old BALB/c (n=48; fast responders to *H. bakeri*) and C57BL/6 (n=48; slow responders to *H. bakeri*) mice (obtained from Envigo and Charles Rivers, respectively) were housed in pairs in standard transparent, solid bottom Home Office approved cages under standard environmental conditions (21±1 °C, relative humidity 45±5 %, 12 h light-dark cycle) with fresh sawdust bedding provided weekly. A plexiglass cylinder and shredded paper were provided as environmental enrichment. All animals were given a maintenance diet (14 % crude protein, Special Diet Services, Lillico Biotechnologies, UK) and water *ad libitum* throughout the experiment.

The experiment was approved by SRUC's ethical review committee (ROD062020) and carried out under Home Office authorisation (PP6868991).

### 4.2. Infective larvae (L3) of *H. bakeri*

*H. bakeri* infective 3$^{rd}$ stage larvae (L3) were cultured from mono-specifically infected donor mice and harvested seven months prior to infection. They were stored at 2-5 °C in distilled water ($dH_2O$) until use. Within one week before infecting the mice, the larvae were washed, counted, and the concentration was set at 1000 L3/mL $dH_2O$.

### 4.3. Bark extraction and CT determination

Bark from *Pinus sylvestris* (Scots pine) was ring debarked and collected in a sawmill in eastern Norway (Bergene Holm AS, Kirkenær) in March 2017 and stored at −20 °C until processing. The bark was milled to chips of 5-20 mm in a hammer mill (Schutte Mini Mill, Buffalo, NY, USA) and freeze-dried. The extract was prepared by adding 1200 mL aqueous acetone (70%) to 150 g ground bark, followed by incubation for 1 h in a water bath (40 °C) with slow stirring. The extract was filtered through a filter cloth and the bark was extracted a second time with 1.25 l acetone (70%) for 30 min. Finally, both extract volumes were

combined, and acetone was removed by evaporation (rotavapor, 40 °C) before freeze-drying. Total CT was quantified by the butanol-HCl assay. The freeze-dried extract was dissolved in methanol (80% in water) and analysed with cyanidin-HCl as standard, using the conventional reagent without acetone, 2.5 h, and absorbance reading at 545 nm (Grabber *et al.*, 2013).

As the bark extracts were not water soluble, dimethyl sulfoxide (DMSO) was used to reconstitute dry bark extract. Pure DMSO is toxic both to mice and the parasite, therefore, immediately prior to use, the dry bark extract was dissolved in 5% DMSO in $dH_2O$ and shaken on a shaker for 48 h (21 °C) to achieve 150 mg bark extract dry matter (DM)/mL in 5% DMSO (Worthley and Schott, 1969). This reconstituted extract was used as the high bark dose (see experimental design) and a 1:1 dilution of this was used for the low dose extract (to achieve 75 mg DM/mL). To quantify the exact amount of CT administered in each animal, CT content was quantified on the reconstituted extracts using the same method previously described (Grabber *et al.*, 2013).

### 4.4. Experimental design

Within genetic line, the animals were randomly allocated in treatment groups based on their arrival body weight, with ten animals allocated in each infected group and six animals in the non-infected control groups (Table 1). The animals were acclimatised in their respective cages for one week prior to infection (day 0).

The experiment was executed in two blocks (balanced for group treatments) that differed by three days to facilitate post-mortem examinations. On day 0, mice were infected by oral gavage of 200 L3 (200 µL) with a bulb-tipped gastric gavage needle mounted to a 1 ml syringe. The non-infected mice were given 200 µl water.

Based upon our own experience and previous trials, we tested two levels of bark: high (150 mg/mL in 5% DMSO) or low (75 mg/mL in 5% DMSO) concentration of the bark extract, equivalent to 1.5 g DM/kg body weight (BW) and 0.75 g DM/kg BW (Morais-Costa *et al.*, 2016; Gutu, 2017). All mice received 200 µL of the bark extract (or 5% DMSO for the negative control animals) for three consecutive days, day 19-21 post infection.

### 4.5. Measurements and sample collection

#### 4.5.1. Parasitological measurements

All parasitological measurements were collected as indirect indicators of resistance (Ayres and Schneider, 2012). Individual faecal samples (n=10 for each mouse line) were collected at

four time points throughout the study from day 14 onwards (day 14, 17, 22, and 28); on each of these days mice were individually placed in a clean cage for a minimum of 20 min and faeces were collected, weighed, and processed for faecal egg count (FEC) within 48 h using a flotation method (Christie and Jackson, 1982). On day 28, mice were sedated through increasing $CO_2$ inhalation and euthanised by $CO_2$ asphyxiation. The colon contents were removed, weighed, and analysed for worm egg count as done for faeces, and this figure was multiplied by colon content to obtain the Eggs In Colon (EIC) readout. To recover the adult *H. bakeri*, we opened the small intestine longitudinally, placed it in PBS and let it incubate at 37 °C for 3 h to allow the worms to migrate out of the tissue. Small intestine and recovered worms were then fixed in 70% ethanol for sexing and counting. Per capita fecundity was calculated by dividing EIC over the total number of female parasites recovered.

#### 4.5.2. Performance measurements and tolerance calculation

BW, feed offered, and feed refused were measured regularly throughout the experiment, every 3-5 days. Feed refusals were weighed out and fresh feed was weighed in to calculate feed intake (FI) per cage per day. BW, carcass weight (CW), and FI were used as indicators of growth performance and were also used for tolerance calculations. FI:BW-ratio gives information about the efficiency of feed utilisation in the production. At post-mortem, the weight of spleen (as an indirect indicator of resistance), small intestines (SI), and carcass weight were recorded. We calculated the Spleen-CW and SI-CW ratio to account for the differences in the size of the animals, but since the effects of bark extract treatment was the same with and without the ratio and refrained from using the ratios in the further calculations. Tolerance was derived from Pearson's correlations between CW on the one hand, and total worm count (TWC), FEC (day 28), and EIC on the other hand for each treatment group (Athanasiadou *et al.*, 2015). A significant negative correlation between a parasitological indicator and CW would indicate low host tolerance towards the pathogen; a non-significant or significantly positive correlation would indicate high host tolerance.

### 4.6. Statistical analyses

For FI and FI:BW-ratio, the experimental unit was cage. For all other data, the experimental unit was animal. Data were analysed by using the MIXED procedure in SAS (SAS 9.3, 2014, SAS institute Inc., Cary NC, USA). As there was no difference between groups administered the two levels of extracts tested, all data from the two bark extract treated groups were pooled. For the analysis of the variables FEC, BW, FI, and FI:BW-ratio, the study was split into two

periods: days 0-19 were defined as post-infection, pre-treatment (P1) period, and days 20-28 were defined as post-treatment period (P2). The effects of line (BALB/c or C57BL/6), infection (infected or sham-infected with *H. bakeri*), bark dosing (with or without bark extract), and day in P2 (22 and 28) and their interactions were treated as fixed effects, and animal within cage as random effect. The average values in the P1 period were included as co-variate, and variation within animal was accounted for in an analysis of repeated measures. The same model was used for data obtained at post-mortem, but without "day in P2", with the effects of line (BALB/c or C57BL/6), infection (infected or sham-infected with *H. bakeri*), and dosing (with or without bark extract) and their interactions as fixed effects and animal within cage as random effect. The optimal covariance structure was assessed for each dependent variable with attention to Akaike's information criterion. Differences between least square means of response variables were estimated with Tukey's test. Results were considered significant at $P<0.05$.

If the residual variance of the data was not constant, the variables were transformed, either via Box Cox transformation (FEC: lambda=0.2, EIC: lambda=0.25), or logarithmically (log10). Tests of significance were made on the transformed scale and then back transformed to the original scale for presentation. Predicted means on the transformed scale, when back transformed, give predicted medians on the original scale. Because standard errors are not constant for comparison on the original scale, the results for the transformed variables are presented as least squares means with pooled standard errors based on the original values.

Tolerance data was normalised and tested in R (v. 4.1.0) and RStudio (v. 1.4.1717).

## 5. Results

### 5.1. Characterisation of bark extracts

The CT concentration in the acetone pine bark extract was 80.3 mg/g DM extract (±4.2). The calculated dose of the high and low bark extract dose was 12 and 6 mg CT/mL. The analyses of the reconstituted extracts showed that the mean CT concentration given the animals was 4.2 and 2.0 mg CT/mL (SEM 0.450 and 0.038) for the high and low dose.

### 5.2. Parasitological measurements

Nematode eggs were firstly observed on day 14 and peaked on day 17 in both mouse lines. There was no significant effect of extract administration in any of the parasitological

parameters measured ($P$>0.1). There was a significant effect of line on FEC, EIC, and TWC; during P2, the slow responder line had a greater FEC, EIC, and TWC than the fast responder line ($P$<0.05 Table 2). There was a significant line by day interaction in FEC, where the rate of reduction in the fast responder line was greater compared to the slow responder line ($P$<0.05), with 2159 epg and 757 epg day 22 and 28, respectively, for the fast responder line and 2370 epg and 2016 epg for day 22 and 28, respectively, for the slow responder line. There was a tendency of a greater female worm fecundity in the slow responder line compared to the fast responder line ($P$=0.07).

### 5.3. Performance measurements

Animals in all experimental groups continued to grow throughout the whole experiment period. The average growth was greater for the pre-infection period (0.45 g/d) compared to the post-infection period across all groups (0.11 g/d). During P1, infection had no impact on BW, FI, or FI:BW-ratio on any of the mouse lines ($P$>0.1). The mean BW of the fast responder mice during this period was 18.3 g, and of the slow responder mice 18.5. FI during P1 was greater ($P$<0.05) for the fast responder (3.25 g/d) compared to slow responder (3.07 g/d) mice

During P2, a significant three-way line by infection by extract interaction was evident on mean BW and CW ($P$<0.05). Bark extract administration had a negative impact on the BW of infected, fast responder mice whereas it had a positive impact on the BW and CW of infected mice in the slow responder line compared to their respective untreated controls (Figure 1). Furthermore, the administration of bark extract did not have any impact on the BW and CW of the sham-infected, fast responder mice whereas it had a negative impact on the sham-infected, slow responder mice. There was no difference in BW-P2 between the lines ($P$>0.1), and infection alone had no impact on BW-P2, CW, and FI-P2 ($P$>0.1).

During P2, fast responder mice had larger FI compared to slow responder mice ($P$<0.05). There was a line by infection interaction, where infection resulted in a reduction in FI in slow responder mice ($P$<0.05). This was not observed in the fast responder mice (Table 3). There was no effect of extract on FI ($P$>0.1).

In a similar way as for BW, there was a three-way factorial interaction between line, infection, and extract on the FI:BW-ratio ($P$<0.05). The administration of the extract reduced the FI:BW-ratio in sham infected fast responder mice and increased the ratio in sham infected slow responder mice (Table 3). Furthermore, the bark extract administration gave an

increased FI:BW-ratio in infected fast responder mice and a reduced FI:BW-ratio in infected slow responder mice.

### 5.4. Spleen weight, SI weight, spleen-CW ratio, and SI-CW ratio

Spleen weight, SI weight, spleen-CW ratio, and SI-CW ratio were greater in the fast responder line compared to the slow responder line ($P<0.05$, Table 3). In addition, these were all greater in infected animals compared to sham-infected ($P<0.05$). We observed no effect of bark extract treatment or any interaction between line, infection, and bark extract treatment on spleen weight, spleen-CW ratio, and SI-CW ratio. There was a tendency of a line by infection by extract interaction on SI ($P=0.077$). The pattern observed was similar to that of BW-P2: the administration of bark extract resulted in a reduction in SI weight in infected, fast responder mice and in the sham-infected, slow responder mice but an increase in the SI weight of the sham-infected fast responder mice.

### 5.5. Tolerance measurements

A significant negative correlation between TWC and CW ($r=-0.5$, $P=0.03$), FEC and CW ($r=-0.48$, $P=0.03$), and between EIC and CW ($r=-0.55$, $P=0.01$) was evident in the fast responder line treated with bark extract (Table 4). There was no significant correlation between the named parameters in the untreated fast responder mice.

There was no significant correlation between TWC, FEC, or EIC, and CW for the slow responder line ($r=-0.24$--$0.16$, $P>0.1$), irrespective of bark treatment.

We also identified a positive correlation between TWC and spleen weight for both fast and slow responder lines ($r=0.58$ and 0.46, respectively, $P<0.05$). There was a significant positive correlation between TWC and the spleen weight for the treated, fast responder line and for the untreated, slow responder line. There was no such correlation for the untreated fast responder line, but for the treated slow responder line we could see a positive correlation close to significant ($r=0.4$, $P=0.08$).

## 6. Discussion

The objective of the current study was to test the impact of bark extract administration on the resistance, performance, and tolerance of genetically diverse mice to infection with *H. bakeri* L3 larvae. We hypothesised that slow responder mice would benefit more from the bark

extract administration compared to those of the fast responder line. Although bark extract administration had a positive impact on the performance of infected, slow responder mice as shown by a greater BW, CW, and a lower FI:BW-ratio in the post-treatment period (P2), it did not result in any benefits on the resistance of parasitised mice as measured by FEC, EIC, TWC, and female worm fecundity. Furthermore, bark administration penalised the tolerance of fast responder mice whereas it had no impact on the tolerance of slow responder mice.

To the best of our knowledge this is the first study where the antiparasitic activity of bark extract from *Pinus sylvestris*, and more specifically CT in pine bark extract, is assessed against GIN *in vivo*. Here, we found no reduction of the parasitic burden, and the first prediction was void. Previous studies on the impact of plant extracts on the resistance of *H. bakeri* infected mice have reported variable results. For example, some studies have shown a reduction in the faecal egg counts and/or total worm counts when treating *H. bakeri* infected mice with various plant extracts in dosages of 250-500 mg DM/kg BW (Enejoh *et al.*, 2015; Gutu, 2017), when others have found no antiparasitic effect when drenching mice with plant extracts at doses of 125-500 mg DM/kg BW (Githiori *et al.*, 2003a; b). In a study where *Nippostrongylus*-infected rats were treated with CT-rich quebracho extract in the feed (40 g DM extract/kg feed, equivalent to 6.8 g DM extract/kg BW), a reduction in the adult worm population in the intestines was evident compared to untreated controls (Butter *et al.*, 2001). Blomstrand *et al.*, 2022, observed antiparasitic effect (expressed as reduced oocyst count) of water extracted spruce bark when drenching *Eimeria* infected lambs with a CT dose ten times higher than the dose used in the present trial. Githiori *et al.*, 2003a; Enejoh *et al.*, 2015; Gutu, 2017, used plant extracts of various origin with similar dry matter dosages as used in the present study. Githiori et al., 2003a, achieved no antiparasitic effect on the plant extracts while Enejoh et al., 2015, and Gutu, 2017, saw a reduction in FEC and TWC by approximately 70%. None of these studies have suggested a possible component responsible for the antiparasitic effect. As different plants vary in their content of PSM, several of which may have antiparasitic properties, and different solvents are used to make the extracts, it is challenging to make reasonable comparisons across studies. Factors within the host affect the bioavailability of the active compounds. For instance, the bioavailability of the active compounds at different compartments of the gastrointestinal tract, interaction between the host and the PSM, and the parasite specificity could affect the antiparasitic efficacy of the bark extract. Furthermore, the way of administration may affect the bioavailability: by administering the plant extract with a gastric tube, CTs will be directly available in the

stomach, whereas CTs administered as feed are possibly bound to salivary proteins, rendering CTs unavailable in the stomach (Mueller-Harvey, 2006).

Although the level of the active compounds in the extract may have been too low for any clear antiparasitic activity, supported by the observation that achieved levels of CT offered was about ⅓ of planned, it had an impact on the performance and the tolerance of mice. In the current study we clearly demonstrated that bark extract administration had a positive impact on the performance of the infected, slow responder animals as indicated by a higher BW and CW compared to the untreated control, and the prediction saying that bark extract treated slow responder mice would display a better performance compared to the fast responder mice confirmed our hypothesis. This is consistent with Athanasiadou *et al.*, 2001a, who found that weaned lambs fed a high protein diet supplemented with CT rich quebracho extract at 6% of the diet had a greater bodyweight gain compared to the control non-supplemented animals. Athanasiadou et al., 2001a, suggested that increased feed intake is a mechanism to compensate for the CT-generated loss of endogenous proteins. In contrast, when the CT-rich quebracho extract was added at 8% of FI it resulted in lower FI and BW (Athanasiadou *et al.*, 2003). Blomstrand *et al.*, 2022, also demonstrated a significant reduction in FI and BW in parasitised animals treated with pine bark extracts. This discrepancy between the different studies may be explained by the bark extract dose, which was equivalent to a CT dose six times higher in the lamb study of Blomstrand et al., 2022, compared to the dose in the present mouse trial.

CT rich extract consumption has been associated with reduced feed intake and growth (Joslyn and Glick, 1969; Barry and McNabb, 1999; Athanasiadou *et al.*, 2001a; b; Blomstrand *et al.*, 2022), and in some cases increased mortality has been observed after administering plant extracts to animals (Githiori *et al.*, 2003a; Gutu, 2017). Despite these potentially negative consequences, it is possible to observe a positive impact from CT-consumption if the positive (antiparasitic) effects outweigh the negative (anti-nutritional) effects (Athanasiadou and Kyriazakis, 2004). In our study, it was only the slow responding infected mice, the most susceptible of all hosts to the negative consequences of parasitism, that appeared to have benefited from the trade-off, whereas all the other mice experienced a penalty from the CT-rich extract administration (Athanasiadou and Kyriazakis, 2004).

In addition to the positive impact of the bark extract on the performance of the infected, slow responder mice, we also observed a significant negative impact on the tolerance of the infected fast responder mice. In the slow responder line, on the other hand, bark

administration did not have any impact on the tolerance The third prediction saying that bark extract treated slow responder mice would have a higher tolerance to the parasitic infection compared to the fast responder mice, was confirmed.

We observed that infected slow responder mice treated with bark extract had a higher BW and CW compared to infected and treated fast responder mice, despite no change in FI, and it seems the infected fast responding line utilised the feed better. This observed effect might have had a microbiological basis. The intestinal microbiome is known to differ between the lines, and it could have been affected by CT (Turnbaugh *et al.*, 2006; Zhao *et al.*, 2019; Somayajulu *et al.*, 2021).

In this trial we found that two out of three predictions supported our hypothesis saying that slow responder mice would benefit more from the bark extract administration compared to fast responder mice. There was no change in parasitic burden in any of the animals, regardless of line and treatment. Nevertheless, the infected slow responder mice experienced a benefit of the bark extract treatment compared to the fast responder mice, with regards to performance and tolerance. Nevertheless, since only two out of the three predictions in the hypothesis were met, the hypothesis had to be rejected. Antiparasitic treatment may have various goals: i) to reduce the parasitic burden in the animals, the shedding of infective stages, hence reduce the infection pressure in the environment, or ii) to improve the host animals' tolerance and performance to the parasitism, accepting the presence of parasites. In mixed groups with animals with different susceptibility and tolerance to the parasite, it may be of importance to reduce the infection pressure. However, a high infection pressure might be tolerable if all the host animals have a high tolerance to the infection.

When analysing the CT content in the reconstituted bark extracts, we found a difference between the calculated CT doses and the measured CT doses of the reconstituted extracts, showing that the reconstituted extracts contained approximately ⅓ of the calculated dose. The same method was used to analyse the extracts in both cases (butanol-HCl-assay), but the solvent used to reconstitute the extract was different (methanol when analysing the dry extracts *versus* 5% DMSO when analysing the reconstituted extracts). The difference in results indicates that methanol is a better solvent and that the realistic amount of CT given the animals is likely the latter of the two (⅓ of the calculated dose).

## 7. Conclusion

After administering acetone pine bark extract to *H. bakeri* infected slow and fast responder mice, we found no effect on the nematode load of parasitised animals, but a significant improvement was observed in the performance of slow responder mice. In addition, fast responder mice experienced penalties in their tolerance to the pathogen, unlike the slow responder mice. The inherent tolerance of the slow responder mice against the parasite was not altered when treated, but the tolerance of the fast responder mice was reduced by the bark extract. This shows that the bark extract had a beneficial impact on the infected slow responder mice (a positive trade-off), and that the fast responder mice was penalised by the bark extract (a negative trade-off). Our results imply that mice resistant to the parasitic infection may not benefit from treatment with the pine bark extract, and that less resistant mouse lines benefit more from the treatment, hence it might be better to avoid treatment of more resistant animal lines. To confirm this, it would be necessary to perform similar studies on other target host species, e.g., sheep or cattle, with a breed known to have a higher resistance to certain parasites compared to other, more susceptible sheep breeds. It would also be of interest to treat *H. bakeri* infected mice with the bark extract at a higher dose to assess if it is possible to achieve an antiparasitic effect and still achieve a positive trade-off.

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## 9. Declaration of interests

There are no conflicts of interest.

## 10. References


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## 11. Figures and tables

**Table 1** The experimental setup in the *in vivo* trial where *H. bakeri* infected mice of two mouse lines with different susceptibility to the parasite were infected with 200 third stage larvae (L3) and treated with 200 µL of acetone extracted pine bark extract at 0, 75, and 150 mg DM/mL dissolved in 5% DMSO.

| Fast responder line, n=48 | | | | | | Slow responder line, n=48 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sham-infected, n=18 | | | Infected, n= 30 | | | Sham-infected, n=18 | | | Infected, n= 30 | | |
| 0 mg/mL n=6 | 75 mg/mL n=6 | 150 mg/mL n=6 | 0 mg/mL n=10 | 75 mg/mL n=10 | 150 mg/mL n=10 | 0 mg/mL n=6 | 75 mg/mL n=6 | 150 mg/mL n=6 | 0 mg/mL n=10 | 75 mg/mL n=10 | 150 mg/mL n=10 |

Abbreviations: DMSO: dimethyl sulfoxide, DM: dry matter bark extract.

**Table 2** Effect of bark extract administration to fast and slow responder mice (n=10) infected with *H. bakeri* larvae on eggs per gram faeces in the post-treatment period (FEC), total worm count in the small intestines (TWC), egg count in colon (EIC), and egg count per female nematode (Fecundity). $P<0.05$ indicates a significant impact of line, extract (Ext), day, and/or combinations thereof on the parasitological parameters (in bold).

| **Parameter** | **Fast responder line** | | **Slow responder line** | | **SEM** | | ***P*-value** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T | C | T | C | T | Line | Ext | Line*Ext | day | Line*day |
| FEC | 4095 | 3548 | 6035 | 5544 | 1.2 | 0.9 | **0.045** | 0.568 | 0.900 | **<.0001** | **0.004** |
| TWC | 14 | 14 | 33 | 41 | 0.2 | 0.1 | **0.016** | 0.815 | 0.759 | n.a. | n.a. |
| EIC | 332 | 329 | 865 | 1521 | 2.3 | 1.6 | **0.004** | 0.422 | 0.412 | n.a. | n.a. |
| Fecundity | 28.6 | 28.0 | 39.2 | 42.2 | 0.10 | 0.07 | 0.070 | 0.894 | 0.804 | n.a. | n.a. |

Abbreviations: C: negative (infected, untreated) control; T: infected, extract treated groups. Ext: extract treatment.

**Table 3** Effect of bark extract administration on feed intake (FI), body weight (BW), FI:BW-ratio, carcass weight (CW), spleen weight, weight of small intestines (SI), the spleen-CW ratio (Spleen:CW), and SI-CW ratio (SI:CW) in fast and slow responder mice sham infected (n=6) and infected (n=10) with *H. bakeri* larvae. *P*<0.05 indicates a significant impact of line, infection (Inf), extract (Ext), day, and/or combinations thereof on the performance parameters (in bold).

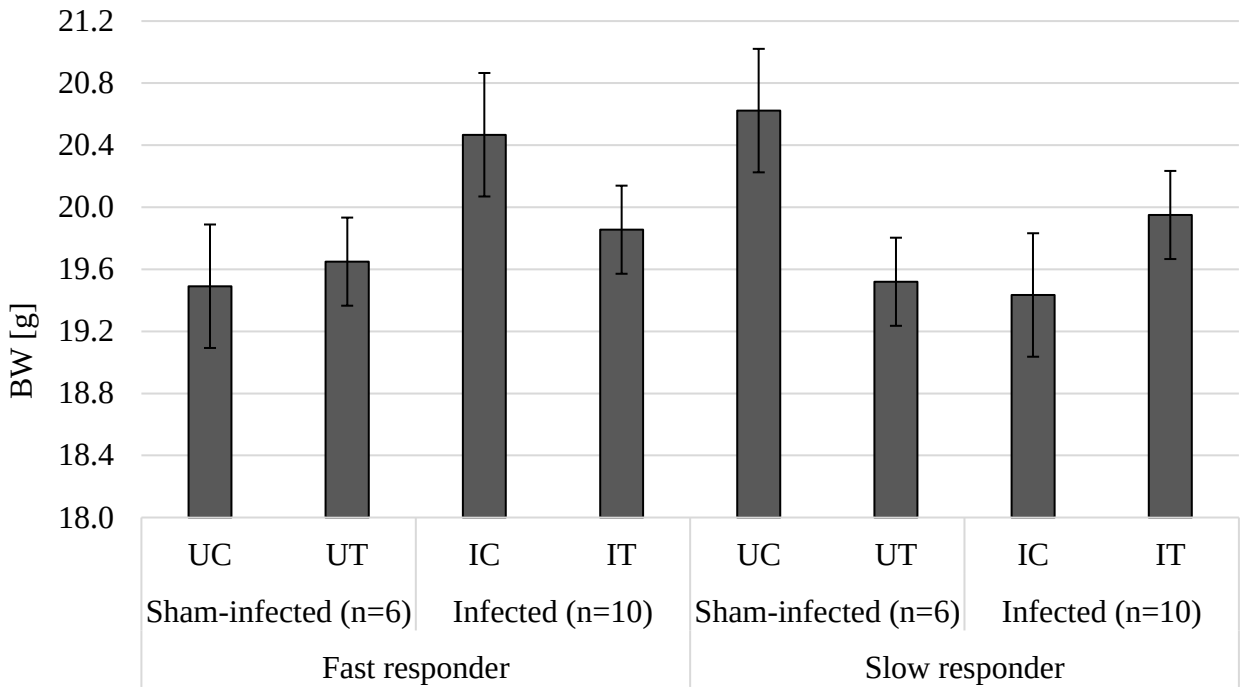
| **Para-meters** | **Unit** | **Fast responder mice** | | | | **Slow responder mice** | | | | **SEM** | | ***P*-value** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Sham** | | **Inf** | | **Sham** | | **Inf** | | C | T | Cov | Line | Inf | Line *Inf | Ext | Line *Ext | Inf *Ext | Line* Inf*Ext | day | day* line |
| | | C | T | C | T | C | T | C | T | | | | | | | | | | | | |
| FI-P2 | g | 3.4 | 3.2 | 3.4 | 3.4 | 3.1 | 3.0 | 2.9 | 2.9 | 0.10 | 0.07 | 0.701 | **<.001** | 0.484 | **0.027** | 0.345 | 0.466 | 0.377 | 0.660 | 0.137 | 0.996 |
| BW-P2 | g | 19.5 | 19.6 | 20.5 | 19.9 | 20.6 | 19.5 | 19.4 | 20.0 | 0.40 | 0.28 | **0.004** | 0.952 | 0.666 | 0.057 | 0.288 | 0.890 | 0.387 | **0.019** | **<.001** | **0.035** |
| FI:BW-P2 | g/g | 0.17 | 0.16 | 0.16 | 0.17 | 0.15 | 0.16 | 0.15 | 0.15 | 0.004 | 0.003 | 0.135 | **<.001** | 0.576 | 0.540 | 0.723 | 0.414 | 0.817 | **<.001** | 0.361 | 0.665 |
| CW | g | 14.2 | 14.3 | 14.4 | 14.0 | 15.4 | 14.8 | 14.5 | 14.7 | 0.24 | 0.17 | **<.001** | **<.001** | **0.046** | 0.134 | 0.274 | 0.942 | 0.496 | **0.030** | n.a. | n.a. |
| Spleen | g | 0.09 | 0.10 | 0.15 | 0.15 | 0.07 | 0.07 | 0.08 | 0.09 | 0.035 | 0.025 | 0.481 | **<.001** | **<.001** | **0.021** | 0.634 | 0.857 | 0.206 | 0.226 | n.a. | n.a. |
| SI | g | 1.48 | 1.60 | 2.26 | 2.10 | 1.30 | 1.22 | 1.62 | 1.62 | 0.022 | 0.015 | 0.889 | **<.001** | **<.001** | 0.114 | 0.644 | 0.599 | 0.477 | 0.077 | n.a. | n.a. |
| Spleen:CW | g/g | 0.007 | 0.007 | 0.010 | 0.011 | 0.005 | 0.005 | 0.006 | 0.006 | 0.033 | 0.024 | **0.008** | **<.001** | **<.001** | **0.032** | 0.461 | 0.852 | 0.222 | 0.424 | n.a. | n.a. |
| SI:CW | g/g | 0.10 | 0.11 | 0.16 | 0.15 | 0.08 | 0.08 | 0.11 | 0.11 | 0.022 | 0.015 | **0.006** | **<.001** | **<.001** | 0.264 | 0.919 | 0.602 | 0.373 | 0.293 | n.a. | n.a. |

Abbreviations: Sham: non-infected group, Inf: infected group, C: untreated control group, T: treated group, SEM: standard error of the mean, Cov: covariate, P2: post treatment period (day 20-28). n.a.: not applicable.

**Table 4** Pearson's correlation between total worm counts (TWC), faecal egg count (FEC), eggs in colon (EIC) and mean carcass weight (CW) for the two selected mouse lines, untreated or treated with bark extract (n=10). Results in bold show significant negative correlation between the parasitological parameter and CW ($P<0.05$), which means the treated fast responder group was penalised with regards to tolerance

| **Correlation** | | **TWC*CW** | | **FEC*CW** | | **EIC*CW** | |
|---|---|---|---|---|---|---|---|
| **Line** | **Treatment[1]** | ***r*** | ***P*** | ***r*** | ***P*** | ***r*** | ***P*** |
| Fast responder | C | -0.42 | 0.230 | -0.15 | 0.670 | -0.29 | 0.420 |
| | T | **-0.50** | **0.030** | **-0.48** | **0.030** | **-0.55** | **0.010** |
| Slow responder | C | 0.00 | 0.980 | -0.39 | 0.260 | -0.28 | 0.440 |
| | T | -0.16 | 0.510 | -0.18 | 0.440 | -0.24 | 0.299 |

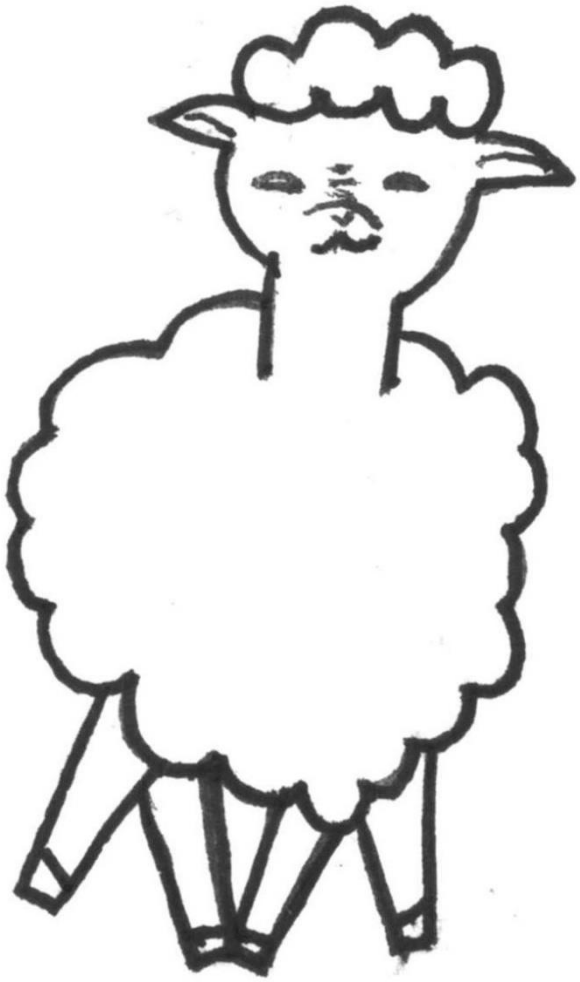
[1] C: untreated control groups, T: treated groups




581

582 **Figure 1** Mean body weight (BW; back-transformed) for the post treatment period (P2, day
583 20-28) for sham-infected and infected, fast, and slow responder mouse lines not treated and
584 treated with bark extracts. Abbreviations: UC: uninfected, untreated control group; UT:
585 uninfected, treated control group; IC: infected, untreated control group; IT: infected, treated
586 group; error bars: standard error of the mean.

587





"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."
Marie Curie (1867-1934)

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