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Alkaline oxidization can increase the *in vitro* antiparasitic activity of proanthocyanidin-rich plant extracts against *Ascaris suum*

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ABSTRACT

Proanthocyanidins (PAs) are a class of plant specialized metabolites with well-documented bioactivities such as antiparasitic effects. However, little is known about how the modification of PAs influences their bioactivity. The objective of this study was to investigate a wide range of PA-containing plant samples to determine if extracts containing PAs modified by oxidation had altered antiparasitic activities, compared to the original extracts that had not been modified in alkaline conditions. We extracted and analyzed samples from 61 proanthocyanidin-rich plants. The extracts were then oxidized under alkaline conditions. We used these non-oxidized and oxidized proanthocyanidin-rich extracts to conduct a detailed analysis of direct antiparasitic effects against the intestinal parasite *Ascaris suum in vitro*. These tests showed that the proanthocyanidin-rich extracts had antiparasitic activity. Modification of these extracts significantly increased the antiparasitic activity for the majority the extracts, suggesting that the oxidation procedure enhanced the bioactivity of the samples. Some samples that showed no antiparasitic activity before oxidation showed very high activity after the oxidation. High levels of other polyphenols in the extracts, such as flavonoids, was found to be associated with increased antiparasitic activity following oxidation. Thus, our *in vitro* screening opens up the opportunity for future research to better understand the mechanism of action how alkaline treatment of PA-rich plant extracts increases their biological activity and potential as novel anthelmintics.

1. Introduction

Parasitic worms (helminths) are one of the most prevalent pathogens in humans as well as livestock production. Billions of people are directly or indirectly affected by these life-threatening parasites, which can be responsible for thousands of deaths annually, mostly in developing countries (Albonico et al., 2008; Hoste, 2001; Hoste et al., 2015a; Houdijk et al., 2017). One of the most prevalent parasites that infects humans is *Ascaris lumbricoides*, which can cause severe malnutrition in children (Albonico et al., 2008; Keiser and Utzinger, 2008). Moreover, the pig parasite *Ascaris suum* (Fig. 1), which has nearly identical characteristics, is another major gastrointestinal parasite that causes substantial economic losses in livestock industries (Kaplan et al., 2014; Williams et al., 2014). According to Danish surveys, the roundworm *Ascaris suum* thrives in nearly 80% of pig farms, with the prevalence estimated to be even higher in outdoor and organic production systems (Thamsborg et al., 2013). Moreover, related parasites cause substantial economic losses and animal health issues in ruminant livestock. Synthetic antimicrobial and anthelmintic drugs are routinely used to treat these parasitic worms. Possible resistance to anthelmintic drugs has recently been reported in *A. lumbricoides* (Charlier et al., 2022; Krücken et al., 2017), and anthelmintic resistance in ruminant nematodes is at critical levels (Mueller-Harvey et al., 2019; Thamsborg et al., 2013). Because of the rapid rise of drug-resistant pathogens, there has recently been a strong focus on reducing the use of these synthetic chemicals.

Thus, new treatment options for parasite infections are heavily focused on natural plant extracts (Kaplan et al., 2014; Thamsborg et al., 2013; Williams et al., 2014, 2016). However, scientific validation and identification of the active compounds from diverse plant species are still lacking. Over the past two decades, tannin-rich forage legumes and other plant sources have been extensively studied to understand the relationship between tannin structure and activity, and find an alternative source of synthetic drugs (Hoste et al., 2015b, 2022; Idris et al., 2019). So far, a few promising results of anthelmintic activity from

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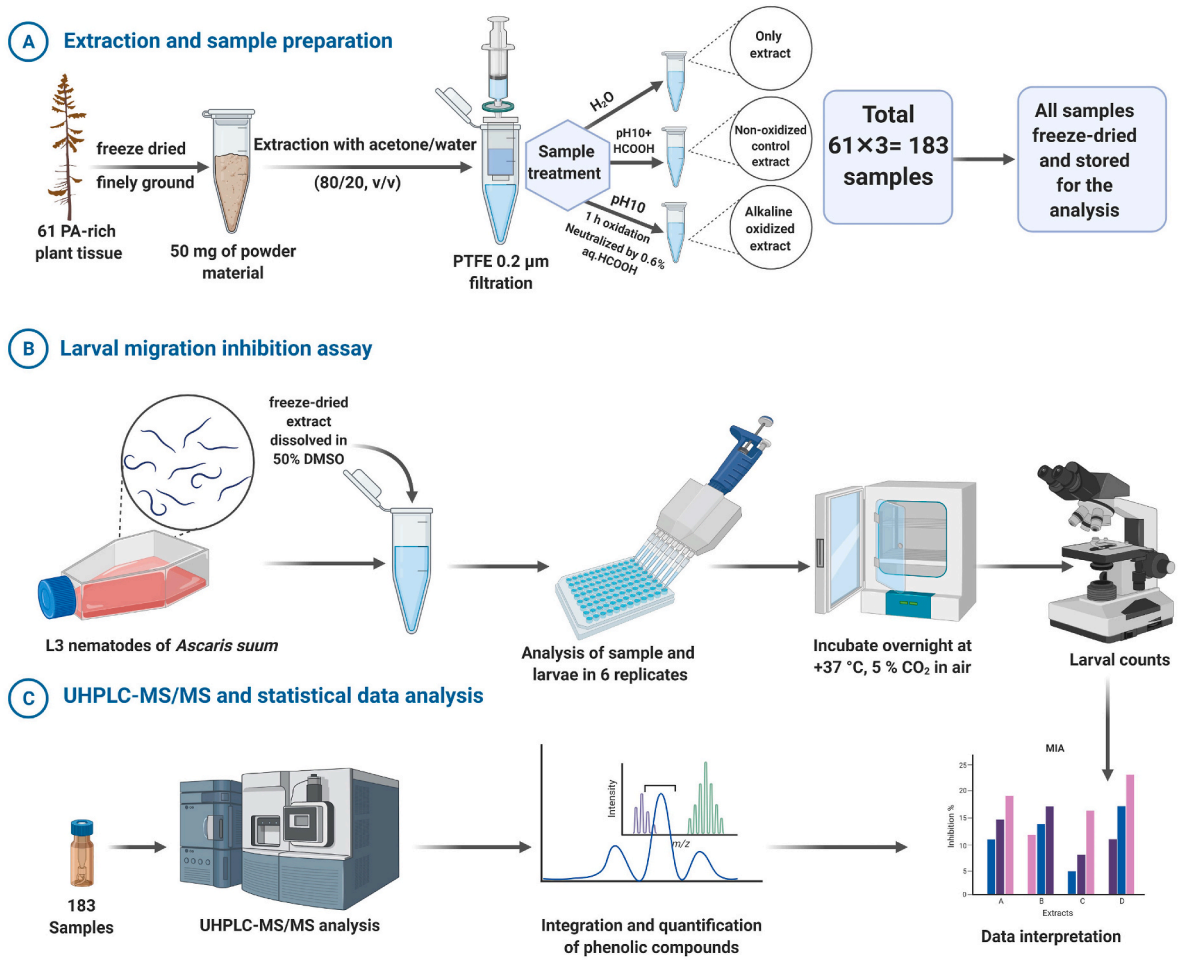

Fig. 1. Experimental design and analytical procedure; (A) sample extraction and preparation for the analysis, (B) the procedure of larval migration inhibition activity measurement; and (C) UHPLC-DAD-QqQ-MS/MS analysis of the non-oxidized and oxidized samples and their data interpretation. L3 corresponds to third-stage larvae (L3) of *Ascaris suum* nematodes and MIA corresponds to migration inhibition activity.

natural tannins have been documented (Baert et al., 2016; Brunet et al., 2008; Desrues et al., 2016; Engström et al., 2016; Hoste et al., 2015a; Kaplan et al., 2014; Karonen et al., 2020; Kommuru et al., 2015; Mueller-Harvey et al., 2019; Ramsay et al., 2016; Williams et al., 2014, 2016). However, most of the studies have been carried out with tannin fractions and with a limited source of plant species. Therefore, we do not have enough knowledge of tannin structure-activity relationships and the most suitable plants for development of novel parasite control strategies.

Proanthocyanidin-rich plants are increasingly considered responsible for many positive effects related to ruminant nutrition and health (Copani et al., 2014; Hoste et al., 2010; Lüscher et al., 2014; Quijada et al., 2015). For instance, a novel feed supplement containing polymeric proanthocyanidins (PAs, syn. condensed tannins) could be a valuable tool to improve animal health in the face of gut parasite infections, thus reducing reliance on synthetic drugs (Engström et al., 2016; Hoste et al., 2015a; Mueller-Harvey et al., 2019; Williams et al., 2014, 2016). PAs are specialized plant metabolites that belong to the tannin subclass of polyphenols. They can be found in nearly all plant families, both in woody and non-woody plants (Dixon et al., 2005; Ferreira and Slade, 2002; Porter, 1989). PAs are oligomers or polymers of flavan-3-ol units. They are subclassified, e.g., into procyanidins (PCs) and prodelphinidins (PDs), if the monomeric units are catechins/epicatechins or gallocatechins/epigallocatechins, respectively. PAs are often mixtures of PCs and PDs, or they contain rarer monomers, such as afzelechin/epiafzelechin subunits in propelargonidins. The subunits can be linked via C-4/C-8 or C-4/C-6 bonds (B-type PAs) or additionally via C-2/O-7 (A-type PAs, thus increasing the potential number of oligomers (<10 subunits) and polymers (10 or more subunits) easily to several hundred and even thousands. Galloylation of the subunits increases this complexity even further (Ferreira and Slade, 2002).

Apart from plants as such, PAs could be obtained from the industrial waste materials such as the thousands of tons of pine and spruce bark waste annually produced by wood industries in temperate climates (Routa et al., 2017). According to the current view (Routa et al., 2017), these materials do not contain optimal PA structures. We hypothesized that their bioactivities could be enhanced via chemical modifications. Such attempts are gaining popularity in PA studies (Aires et al., 2016; Pizzi, 2019), but they are rarely accompanied by the knowledge of the accurate tannin structures present in the final products. For instance, the final products' actual structure is often missing in the large-scale processing of PA-rich products. The substance is most often not detailed but is only characterized by its average composition of PA. Thus, more rigorous and accurate studies are needed to gain a thorough understanding of modified PAs and their subsequent bioactivity. The PAs can be modified by changing their physicochemical characteristics in a chemical derivatization reaction, including *O*-acylation with acid or an-hydride reactions or alkylation with alkyl halides (Aires et al., 2016; García et al., 2016; Pizzi, 2006, 2019).

In our previous studies (Imran et al., 2021; Karonen et al., 2021), we have shown a PA modification process by a cost-effective and simple pH10 buffer oxidation process, mimicking the often-used alkaline

extraction process for the bark waste (Fradinho et al., 2002; Karonen et al., 2021; Kim et al., 2018; Vázquez et al., 1987; Vihakas et al., 2014). In those studies, we categorized all the studied samples into four different groups based on their UHPLC-UV area: The category (A) non-modified PAs without an evident loss (<20%) of PA concentration; (B) non-modified PAs with an evident loss (≥20%) of PA concentration; (C) modified PAs without an evident loss (<20%) of PA concentration; and (D) modified PAs with an evident loss (≥20%) of PA concentration, see for example Fig. 2 in Imran et al. (2021). In addition, the study results indicated different reactivities for the PA subunits of the 102 PA rich plant extracts and their structural modification pattern observed by the UHPLC-MS/MS fingerprint (Imran et al., 2021). These findings led us to conduct this current study to explore their antiparasitic activity using a well-characterized *in vitro* bioassay with *Ascaris suum* parasites. This assay assesses the ability of larvae to migrate out of a semi-solid medium after exposure to test compounds, and has proved a convenient and reproducible method for assessing the activity of putative anthelmintic compounds that may act against *Ascaris suum* and potentially also a range of other parasites of veterinary and medical importance (Valente et al., 2021; Williams et al., 2016).

In this study, we selected 61 PA-rich plant species based on our previous research (Imran et al., 2021; Karonen et al., 2021) and analyzed them before and after alkaline oxidation with Engström method (Engström et al., 2014, 2015; Salminen, 2018), our previously described method for understanding the oxidation reaction route with most of these samples (Imran et al., 2021). Moreover, we used oxidized and non-oxidized PA-rich plant extracts to conduct a detailed analysis of direct antiparasitic effects against *Ascaris suum* nematodes.

We aimed to address the following questions: 1) How many of 61 plant extracts show antiparasitic activity? 2) What happens with the oxidized extracts in the larval migration inhibition assay? 3) Is the activity of extracts increased or decreased after oxidation? 4) How do different structural features of PAs affect their bioactivity? 5) Is there any link with other phenolic compounds or polyphenol subgroups to increasing/decreasing the activity? This information will allow us to better understand how oxidation of PA-rich samples modulates their bioactivity and potential use as novel nutraceuticals to control intestinal pathogen infections.

2. Experimental section

2.1. Extraction and preparation of plant samples for the analysis

In total, 61 samples (mostly PA-rich plant materials) were collected for this study (Supplementary Table S1). For increasing the sample's heterogeneity from the PA point of view, the plant materials were selected from our larger species collection: from the botanical garden of the University of Turku, Finland, and Barro Colorado Island, Panama. In addition, commercial tannin-rich bark samples were purchased from "Xi'an Le Sen Bio-technology Co., Ltd" Xián, China. After the collection, plant samples were freeze-dried, finely ground in powder, and finally kept in the freezer for an additional process. Each plant tissue was extracted into three different Eppendorf tubes to prepare them for two further treatments along with the original one (for original extract, non-oxidized control extract and oxidized extract). Therefore, 61 species multiply with three treatments; altogether, 183 samples were prepared. In each tube, 50 mg of finely ground plant tissue was taken. The extraction process was the same as reported previously (Imran et al., 2021). However, a modified sample preparation protocol (25 times large scale than the previously reported) was used in this study for the oxidation by pH10 buffer since the same samples were used both for the larval migration inhibition assay and UHPLC-MS/MS analysis. The sample preparation was as follows: after successful extraction of each sample by acetone/water (80/20, v/v), the extracts were filtered with a 0.2 μm polytetrafluoroethylene (PTFE) filters (VWR International, Radnor, PA, USA). Then samples were freeze-dried and kept for further use.

Three replicate samples included the original extract, the non-oxidized control sample, and the oxidized sample in alkaline conditions (see Fig. 1A). In briefly, the freeze-dried extracts were dissolved in 500 μL of Milli-Q water and vortexed for adequately mixing the sample for each tube. The original extract sample was prepared by adding 7 mL of Milli-Q water so that for each sample, the final concentration was the same for every analysis. The non-oxidized control sample was prepared by adding 4.5 mL of the pH10 buffer (NaOH) and 2.5 mL of the 0.6% aqueous HCOOH mixture. The oxidized sample was prepared by adding 4.5 mL of pH10 buffer and let the test tube for 1 h at room temperature for the aerobic oxidation process. The oxidation was stopped by adding 2.5 mL of 0.6% HCOOH. After that, 200 μL of each sample were transferred into an Eppendorf tube and preserved for further UHPLC-MS/MS analysis. Then, the rest of the samples were freeze-dried and transported for the larval migration inhibition assay.

2.2. In vitro antiparasitic activity tests

2.2.1. Preparation of Ascaris suum larvae

The embryonated *Ascaris suum* eggs were collected from fresh pig intestines at a local slaughterhouse (Danish Crown, Ringsted, Denmark). The third-stage larvae (L3) stage hatching of *Ascaris suum* nematodes was performed as described in Williams et al., (2014) & 2016, previously modified from Han et al., (2000) method (Han et al., 2000; Williams et al., 2014, 2016). Briefly, *Ascaris suum* eggs were decoated in sodium hydroxide and stored in 1 M H_2SO_4 solution at 4 °C temperature. The eggs were hatched by washing 3–4 times with Hank's Balanced Salt Solution (HBSS) and grinding them gently with glass beads at 37 °C, 5% CO_2 in the air for 40–60 min. The larvae were then incubated overnight in a Baerman funnel, dissolved in sterile HBSS at 37 °C, 5% CO_2 in the air condition. L3 stage larvae then separated from the debris of unhatched eggs. The larvae were washed and collected in larval culture media (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) to use them in the antiparasitic activity measurement.

2.2.2. Larval migration inhibition assay

The migration inhibition assay was conducted, as described previously (Williams et al., 2014, 2016) with modification protocol (Leppä, 2020). Briefly, the L3 stage larval concentration was set to 0.69/mL, and 145 μL of larval suspension was added in a 96-well on a tissue culture plate so that on average, 100 larvae would be present in each well (see Fig. 1B). The freeze-dried plant extracts were first dissolved in 200 μL of 50% DMSO (DMSO/water, (50/50, v/v)) to mix the tannin samples completely soluble. This solution was then made with 10-fold dilution to conduct the migration inhibition activity. For the assay, 5 μL of sample solution was added into the culture plate and mixed properly with the larval suspension. Sample dissolving solvent 50% DMSO and pH10 buffer were used as negative controls and 200 μg/mL levamisole drug as a positive control. An internal standard Oenoethin B (5 mg/663.87 μL) was used to observe the control stability and optimize each 96-well plate's larval assay. The plates were then incubated overnight at 37 °C, 5% CO_2 in the air.

On the next day, an inverted light microscope was used to count the total larvae from each well of the plate. Then, an equal amount of 150 μL of 1.6% agar solution was added at 45 °C temperature to each well and mixed thoroughly. The agar was allowed to solidify for 10–12 min before adding a new culture media (100 μL of RPMI) on top of each well by a multichannel pipette. Lastly, the plate was then kept in an incubator overnight at 37 °C, 5% CO_2 in the air, and allowed the larvae to migrate.

The next day, larval migration was counted by a direct light microscope from the agar's top. For the larval migration inhibition analysis, the well plate was kept at room temperature for a maximum of 15 min so that larvae did not expire before the measurement. Both plate's larval count was combined before the result calculation. Larval migration

Table 1
Summary of the quantified phenolic compounds found in three different samples of 61 studied plant species.

Type of compound	Non-oxidized initial extract					Non-oxidized control extract					Alkaline oxidized extract				
	N	max	min	mean	median	N	max	min	mean	median	N	max	min	mean	median
		(mg/g DW)					(mg/g DW)					(mg/g DW)			
Tannin subgroups															
Total tannin	56	143.6	0.5	34.0	24.4	56	140.1	0.6	33.1	24.6	54	114.8	0.3	20.7	13.6
Proanthocyanidins (PA)	56	143.6	0.1	31.1	20.5	56	140.0	0.6	30.7	19.8	53	114.7	0.3	18.0	11.5
Procyanidins (PC)	55	142.9	0.5	24.0	15.6	55	138.6	0.4	23.7	15.2	53	113.3	0.2	15.2	8.0
Prodelphinidin (PD)	48	114.7	0.1	8.8	0.9	47	117.2	0.1	8.2	1.4	44	32.7	0.1	3.3	1.1
Hydrolysable tannins (HT)	28	37.2	0.1	5.7	4.4	26	37.7	0.1	6.4	4.3	31	55.0	0.1	5.3	2.9
Galloyl derivative	23	9.2	0.1	3.6	3.5	23	9.6	0.2	3.7	4.0	23	9.9	0.2	3.5	2.3
HHDP derivative	17	32.8	0.1	4.5	0.2	15	33.3	0.1	5.9	0.3	23	46.9	0.1	3.6	0.3
Other polyphenol subgroups															
Flavonol glycosides	33	8.1	0.1	2.1	1.3	32	8.4	0.1	2.2	1.2	33	7.8	0.1	1.9	1.2
Kaempferol derivatives	25	1.9	0.1	0.6	0.4	25	1.8	0.1	0.6	0.4	25	1.9	0.1	0.6	0.4
Quercetin derivatives	29	6.5	0.1	1.5	0.7	28	6.8	0.1	1.5	0.8	29	6.5	0.1	1.5	0.7
Myricetin derivatives	9	4.4	0.1	1.3	1.2	9	4.3	0.1	1.3	1.2	7	3.5	0.1	1.2	0.7
Quinic acid derivatives	27	10.3	0.1	2.5	1.0	27	10.0	0.1	2.5	0.9	25	7.4	0.1	2.2	1.2
Total polyphenol	58	145.3	0.5	35.8	25.3	57	142.9	0.5	34.9	26.5	56	116.0	0.3	22.1	15.7

*N indicates the number of plant species in which each compound or their subgroups were detected. DW indicates the content (mg) obtained of the measured compounds in per dry weight mass of the plant material (g). The limit of quantification was 0.1 mg/g. Mean concentrations are reported by excluding the samples where the compound or their subgroups were not detected by the method. The structures of all compounds are shown in Supplementary Fig. S1.

mean inhibition activity percentages (MIA%) were calculated using the minimal and maximal migration percentage obtained from both the positive and negative control samples by using the following equation:

$$\text{MIA } \% = 100 - \left(\frac{\text{Larvae migrated in sample} - \text{Larvae migrated in positive control}}{\text{Larvae migrated in negative control} - \text{Larvae migrated in positive control}} \right) \times 100$$

2.3. Chemical analysis

2.3.1. Chemicals and reagents

Analytical grade acetone (VWR International S.A.S., France) was used in the extraction. For the oxidation, a pH 10 carbonate buffer (50 mM; sodium carbonate/sodium hydrogen carbonate, J.T. Baker, Deventer, Netherlands) and formic acid (J.T. Baker, Deventer, Netherlands) were used. For the UHPLC analysis, LC-MS grade acetonitrile was purchased from VWR International S.A.S. (USA) and LC-MS grade formic acid from Sigma Aldrich (Seelze, Germany). The Milli-Q water used was purified with Millipore Synergy UV (Merck KGaA, Darmstadt, Germany) system.

2.3.2. UPLC-DAD-QqQ-MS/MS analyses

The extracts were then filtered again with a 0.2 μm PTFE filter to eliminate the probable precipitation in refrigeration. After vortexing for 5 min, 100 μL of each sample (original extract, non-oxidized control extract, and oxidized alkaline extract) was inserted into a separate UHPLC vial (see Fig. 1C). The samples were then analyzed by a group-specific MS/MS method (Engström et al., 2014, 2015; Salminen, 2018) to obtain the quantitative measurements of PAs, procyanidins (PCs), prodelfinidins (PDs), mean degree of polymerization (mDP), and other phenolic compounds such as gallic acid derivatives, hexahydroxydiphenoyl (HHDP) group, kaempferol-, quercetin- and myricetin based flavonol glycosides, as well as quinic acid derivatives. The UHPLC-MS/MS system and the conditions and parameters used, such as the column selection, the ionization, the elution profile, the standards, and compound identification, were the same as the previously reported in Imran et al. (2021) (Imran et al., 2021). The recorded traces and peaks were integrated by TargetLynx software (MassLynx V4.2 SCN982 © 2017 Waters Inc.).

2.4. Statistical analyses

A two-way analysis of variance on the data using sample and oxidation status as factors was conducted. Multiple comparison testing (Fisher's LSD) and multivariate data were analyzed with both oxidized and non-oxidized samples. Principal component analysis was carried out to observed clustering between samples with increased or decreased bioactivity following oxidation (Metsalu and Vilo, 2015). Differences in chemical composition (e.g., PA%) between samples with increased or decreased bioactivity were determined by Mann-Whitney test. All statistical data analyses were performed in GraphPad Prism (v9.00, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

3. Results and discussion

3.1. Polyphenol compositions by UHPLC-DAD-MS/MS analyses

The polyphenol composition of non-oxidized and oxidized plant extracts were analyzed UHPLC-DAD-MS/MS methods. By comparing the UV-DAD profiles at 280 nm and MS/MS data, we quantified the phenolic composition of altogether 183 samples (Table 1). Most of the samples were determined to be tannin-rich, dominated mostly by PAs (56 out of 61 species), with lower levels of other tannin subgroups and flavonol and quinic acid derivatives. The highest total polyphenol content 145.3 mg/g was found in *Prioria copaifera* seed extract. The polyphenol content was the lowest 0.5 mg/g in *Tara spinose* bark extract. In the original, i.e. non-oxidized, extracts the PA contents varied from 0.1 mg/g (limit of quantification) up to 143.6 mg/g. In the non-oxidized control extracts, the contents were in a similar level as expected. In the oxidized extracts, the polyphenols were modified due to the oxidation in alkaline conditions. These modification reactions of PAs have been previously described in detail (Imran et al., 2021; Karonen et al., 2021). Under the

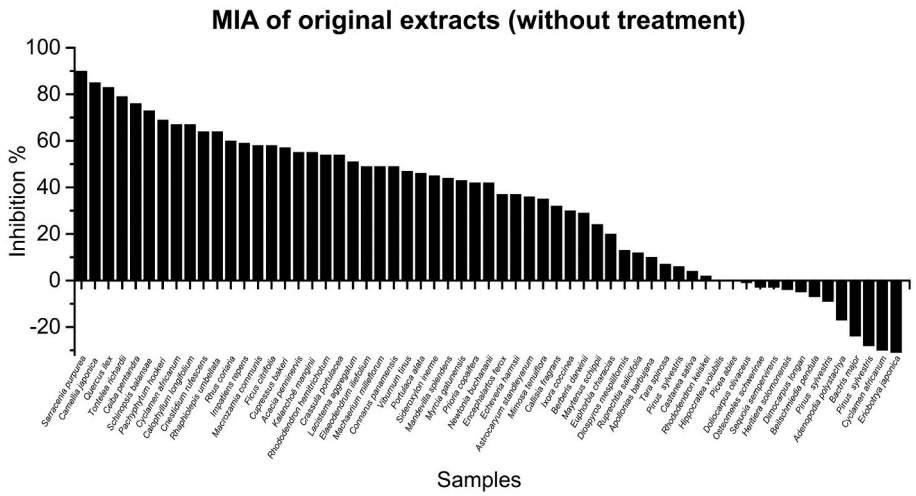

Fig. 2. Mean inhibition activity (MIA) of 61 studied plant species (without alkaline treatment).

aerial alkaline oxidation, different PAs in different plant matrices can undergo no, minor or clear modifications and these reactions can results in no, minor or clear loss in their contents (Imran et al., 2021). These modification reactions can be intramolecular, such as formation of new or additional A-type ether linkages, or intermolecular producing new types of PAs, such as linear or branched high polymers which are not detected under standard ESI-MS conditions typically used for the analysis of PAs (Karonen et al., 2021). Typically, PD-rich PAs are more reactive than the PC-based ones (Imran et al., 2021; Karonen et al., 2021). The modification of PAs and the PA compositions and contents determined in this study were consistent with the previous results (Imran et al., 2021; Karonen et al., 2021). The PAs were modified so that their contents in oxidized extracts seemed to be lower (Table 1) but in reality, the contents are underestimated due to the fact the MS/MS method used is not capable to detect the modified units nor the units with the A-type ether linkages.

The detailed polyphenol composition of each plant species including the PA content, mean degree of polymerization (mDP) of PAs, PC/PD ratio and the contents of other phenolics are presented in Supplementary material in Table S2. The mDP of the PAs in non-oxidized extracts varied between 1.1 and 34.7. It was the lowest in the bark extract of *Rhus coriaria* and the highest in the leaf extract of *Crassula portulacea*. The average mDP of PAs in the studied samples was 8.4. Altogether, 15 samples contained polymeric PAs with the mDP higher than 10 and other 41 samples contained oligomeric PAs with the mDP of 2–10 on an average. The PC/PD ratio varied from 1:99 (*Elaeodendrum iliefolium*) to 100:0 (*Callisia fragrans*). A majority, 37 of the studied 61 samples, belonged to PC-rich (90%–100% PC share). Six of the studied samples belonged to PD-rich (90%–100% PD share) samples, and thirteen samples contained equal levels of PC and PD units in their PA structures. The corresponding detailed information for non-oxidized control extracts and for oxidized extracts is presented in Supplementary material in Table S3. In general, the contents of modified and detectable PAs in individual oxidized extracts were lower than the contents of initial PAs in non-oxidized extracts as discussed above, expect for *Hippocratea volubilis* (Table S3). Similar observations were made for the mDP as the mDP of modified and detectable PAs in individual oxidized extracts was lower in than the mDP of initial PAs in non-oxidized extracts (Table 1). The only exceptions were *Adenopodia polystachya*, *Pinus sylvestris*, *Rhododendron keiskei* and Rhus coriaria, where the mDP was found to be slightly higher in the oxidized extracts.

Furthermore, 28 species were found to contain hydrolyzable tannins (HTs) with the highest concentration (37.2 mg/g) in *Myrcia gatunensis* seed species. The same species also contained the highest amount of HHDP derivatives (32.8 mg/g). The mean contents of HTs and HHDP derivatives were 5.7 mg/g and 4.5 mg/g, respectively. In addition, 23 samples were found to contain galloyl derivatives, 25 samples kaempferol-based flavonol glycosides, 29 samples quercetin-based flavonol glycosides, and 9 samples myricetin-based flavonol glycosides, and 27 samples as quinic acid derivatives. The more detailed species-by-species quantification of all compounds is presented in Supplementary Material in Table S2. After the alkaline oxidation, the contents of other phenolics than PAs were, in general, at the same level (Table S1). However, a decrease was detected in the contents of quinic acid derivatives and an increase in the contents of HHDP derivatives. The change in HHDP derivatives in Table S1 was related to one sample with a clearly higher content as the median values were in the same level (Table S2). This sample was the seed extract of *Myrcia gatunensis,* where the content of HHDP derivatives was 33.3 mg/g in the non-oxidized extract and 46.9 mg/g in the oxidized extract. This phenomenon might be related to unusual ET structures. Previously, we have detected *Myrcia splendens* to contain ETs with cyclic and acyclic glucose cores but also an unresolved ET hump likely consisting of a complex mixture of ET oligomers or even polymers (Kim et al., 2021).

3.2. *In vitro antiparasitic activity of initial non-oxidized plant extracts*

We evaluated the antiparasitic activity of all extracts using the migration inhibition assay. In this assay, we used both DMSO and pH10 buffer as a negative control in the test; however, only the pH 10 buffer was used for the final calculations to compare the larval migration with positive control. We also used pure oenothein B as an internal positive control to monitor the performance of the assay during the whole experiment. We found that our negative control, oenothein B, and commercially available anthelmintic drug, levamisole, both showed a stable activity during the whole experiment that confirmed the repeatability and reliability of the method used. Most of the PA-rich plant extracts showed potential antiparasitic activity (Fig. 2). Forty-seven plant extracts showed positive antiparasitic activity ranging from 2% to 90%. However, at the same time, plant extracts from 14 species had either no activity or tended to enhance the larval migration.

Almost 21 plant extracts showed more than 50% inhibition activity, and in general, these samples were found to contain high amount of tannins. However, when closely looking at the MS/MS quantitative data (Supplementary material, Table S2), we found that the PA contents in these samples varied from 0.1 mg/g (in the bark extract of *Rhus coriaria*) up to 140.2 mg/g (in the bark extract of *Schinopsis balansae*) meaning that all extracts showing the highest antiparasitic activity were not necessarily rich in PAs. Altogether, five samples had PAs less than 10 mg/g, i.e., the petal extract of *Camellia japonica*, bark extract of *Rhus*

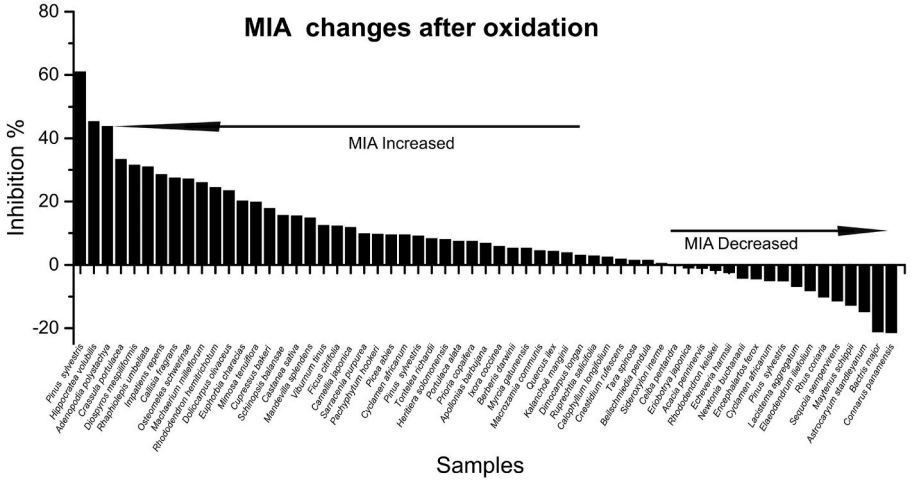

Fig. 3. After oxidation, all the samples were grouped by the difference in inhibition value of non-oxidized and oxidized samples. Here, MIA represents mean inhibition activity. Left side samples show the increasing activity, and the right side shows the decreasing activity.

coriaria, leaf extract of *Impatiens repens*, and bark extract of *Acacia penninervis*, and leaf extract of *Crassula portulacea*. In addition, it was noticed that the PA contents of the extracts were not directly proportional to the level of the antiparasitic activity of the extracts. The mDP of PAs in these most active samples ranged from 1 (in the bark extract of *Rhus coriaria*) to 34.7 (in the leaf extract of *Crassula portulacea*) (Supplementary material, Table S2). Correspondingly to the PA content, it was noticed that the mDP of PAs in the extracts was not directly proportional to the level of the antiparasitic activity of the extracts. Similar observations were made for the monomeric units of PAs. The most active plant extracts could be PC-rich, such as the leaf extract of *Sarracenia purpurea* and the petal extract of *Camellia japonica*, or PD-rich, such as the bark extract of *Rhus coriaria* having pure PDs or the flower extract of *Cyclamen africanum* having PC/PD ratio of 9/91, or equal PC/PD mixtures, such as the seed extract of *Tontelea richardii* having PC/PD ratio of 44/56 or the branch extract of *Cupressus bakeri* having PC/PD ratio of 50/50 (Supplementary material, Table S2). Previously reported findings have shown that the high mDP and high PD share of PAs in the samples increase their anthelmintic activity (Brunet et al., 2008; Desrues et al., 2016; Mueller-Harvey et al., 2019; Quijada et al., 2015; Ramsay et al., 2016). We could not make similar conclusions. However, this is perhaps explained by the fact that these other studies utilized purified PA fractions in different kind of *in vitro* assays and with different nematodes, i.e. the adult motility assays with *Ostertagia ostertagi* (Desrues et al., 2016) and *Cooperia oncophora* (Desrues et al., 2016), and larval exsheathment inhibition assay with *Haemonchus contortus* (Engström et al., 2016) (Kommuru et al., 2015) (Karonen et al., 2020) and *Trichostrongylus colubriformis* (Karonen et al., 2020) of third stage larvae.

In some samples, the antiparasitic activity can be clearly linked with the presence of PAs, for example, in the seed extract of *Tontelea richardii* and *Calophyllum longifolium*, where they are the only polyphenols detected (99.8% and 100% of all polyphenols, respectively), but in other samples, it is also evident that the other compounds present can affect. For example, in the bark extract of *Rhus coriaria*, only 2% of all polyphenols are PAs, and these are mainly monomeric PDs, and the rest 98% are HTs. In the leaf extract of *Impatiens repens*, 60% of all polyphenols were quinic acid derivatives, but otherwise, quinic acid derivatives seemed to have a minor role. Four samples, i.e., the leaf extract of *Sarracenia purpurea*, the petal extract of *Camellia japonica*, the flower extract of *Cyclamen africanum* and the leaf extract of *Rhododendron hemitrichotum* contained flavonoids ≥10% of all polyphenols (Supplementary material, Table S2), but 11 of these 21 active extracts did not contain any of these flavonoids detected by MS/MS.

We also looked in detail those samples that showed no antiparasitic activity (*Hippocratea volubilis*, *Picea abies*, and *Doliocarpus olivaceus*) or even increased the larval migration moderately, i.e. ≤ 10% (*Osteomeles schwerinae*, *Sequoia sempervirens*, *Heritiera solomonensis*, *Dimocarpus longan*, *Beilschmiedia pendula*, and *Pinus sylvestris* inner and outer bark) or substantially, i.e. ≥ 10% (*Adenopodia polystachya*, *Bactris major*, *Pinus sylvestris* outer bark, *Cyclamen africanum* leaves and *Eriobotrya japonica)*. Even in these cases, there was no connection between the total content, mDP or PC/PD ratio of PAs and the activity test result. The PA contents varied from 1.4 mg/g to 76.4 mg/g, mDP values varied from 2.4 up to 12.7, and PC/PD ratios from PC-pure ones to 82% PD-share. Twelve samples contained PAs more than 80% of all polyphenols, but in *Dimocarpus longan* and *Doliocarpus olivaceus,* only 30% and 64% of all polyphenols were PAs, respectively. *Doliocarpus olivaceus* is known to contain PCs and galloylated PCs in addition to galloylglucoses (Kim et al., 2021). The inner and outer bark extract of *Pinus sylvestris* contained no detectable PAs, which is surprising as pine bark is known to contain a lot of PCs (Karonen et al., 2004; Matthews et al., 1997). Most probably, this is due to the fact that something has happened to the commercial pine park material during the manufacturing process and the sample is not representative for the species completely. So the MIA changes must be due to other substances in the extract. Other phenolics did not seem to play a role either: flavonoids accounted for 0–12% of all phenolics and quinic acid derivatives for 0–7%, and no patterns were observed.

3.3. *In vitro* antiparasitic activity of oxidized extracts

We next tested the antiparasitic activity of oxidized PAs to explore if activity was altered compared to the non-oxidized extracts. In addition, we tested that the buffer used in the oxidation did not have an effect on the antiparasitic activity. In Supplementary figure Fig. S22., we show and compare the inhibition activity of non-oxidized and oxidized extracts. In most of the cases, the modification of PAs via oxidation increased the antiparasitic activity of the extracts. By conducting a two-way analysis of variance on all studied species, using sample (plant species) and oxidation treatment (non-oxidized vs oxidized) as factors, the results showed that both contribute to the variation in the data set (data shown in Supplementary Material in Table S3). Moreover, multiple comparison testing showed that for the samples, which had a significant difference (e.g., *Machaerium milleflorum*, p = 0,0249; *Rhododendron hemitrichotum*, p = 0,0345; *Hippocratea volubilis*, p = <0,0001; *Diospyros mespiliformis*, p = 0,0066; *Impatiens repens*, p = 0,0140; *Pinus sylvestris*, p = <0,0001; *Crassula portulacea*, p = 0,0041; *Rhaphiolepis umbellate*, p = 0,0075, *Doliocarpus olivaceus*, p = 0,0426;

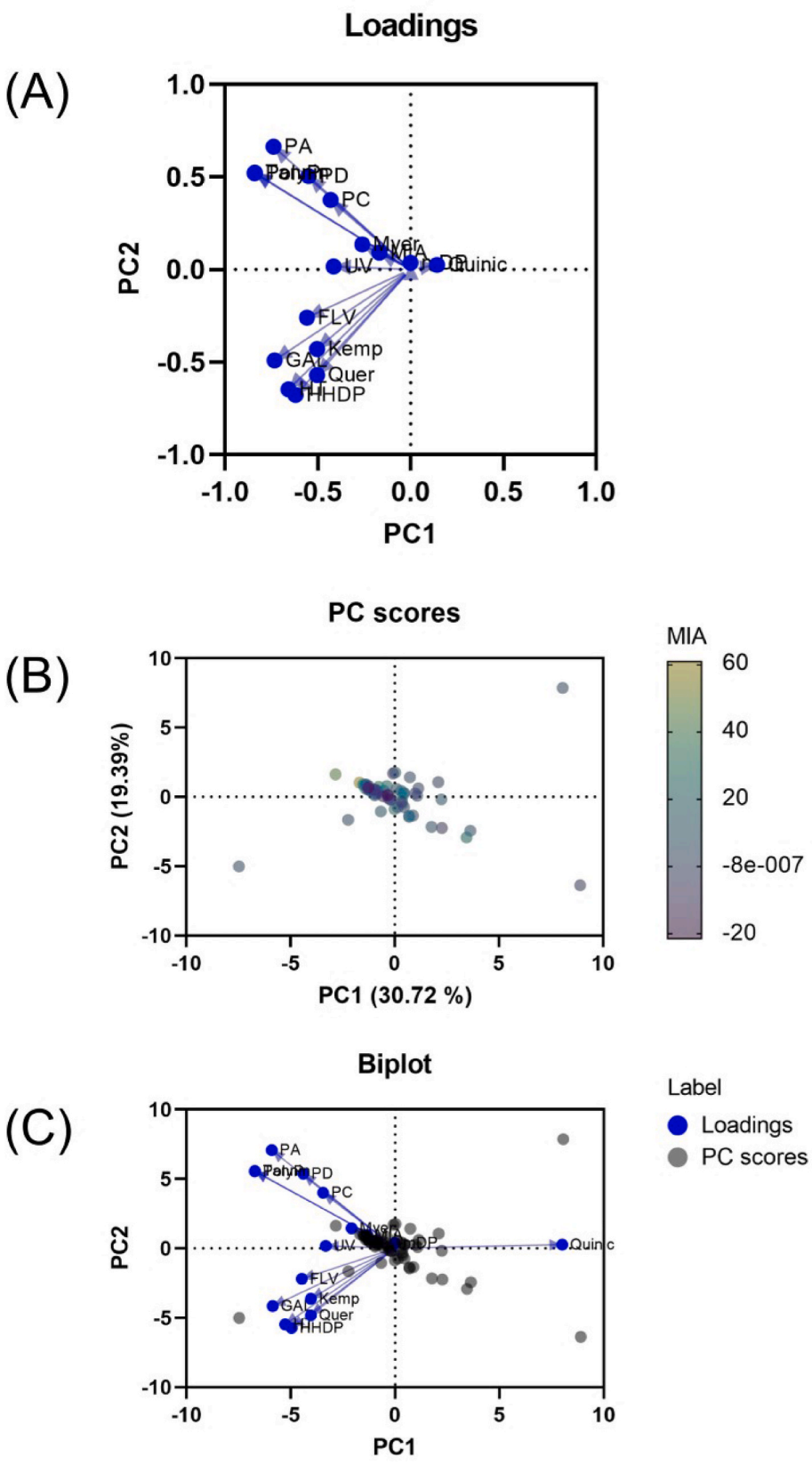

Fig. 4. Principal component analysis (PCA), where (A) shows the loadings of all dependent variables on PC1 and PC2, the (B) score of the difference in inhibition value of non-oxidized and oxidized samples on PC1 and PC2, and (C) showing the biplot of each dependent variable. PA = proanthocyanidin, PC = procyanidin, PD = prodelphinidin, mDP = mean degree of polymerization, GA = galloyl derivatives, HHDP = hexahydroxydiphenoyl esters, HT = hydrolysable tannin, QA = quinic acid derivatives, KAE = kaempferol-based flavonol glycosides, QUE = quercetin-based flavonol glycosides, MYR = myricetin-based flavonol glycosides, FLV = flavonoids.

Adenopodia polystachya, p = 0,0002; *Osteomeles schwerinae*, p = 0,0191) between the oxidized and the non-oxidized, for every case, the oxidized sample had a higher inhibition value (Supplementary Fig. S22.). Based on this observation, we concluded that, in general, the oxidation of PAs in the extract increases the antiparasitic activity, although not for every sample.

For the better visualization of the data, the samples were grouped by the difference in inhibition between the oxidized and non-oxidized

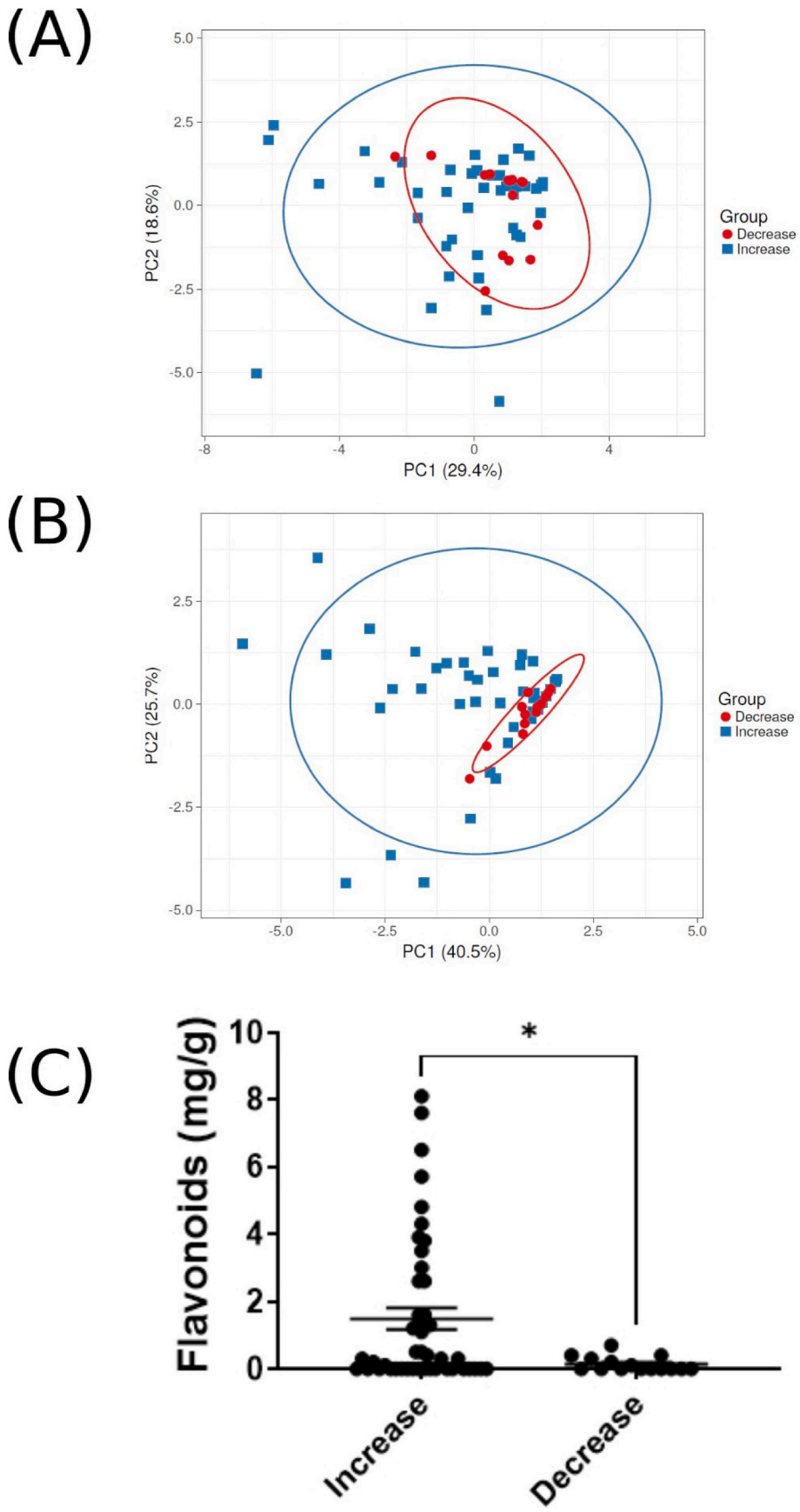

Fig. 5. Principal component analysis of two groups those had increased inhibition activity after oxidation (increase) and those that did not (decrease). Both Figure (A) and (B) shows decreased samples clustered together. In Figure (C), increased samples have high levels of other phenolic compounds than the tannins.

samples (see Fig. 3 and the MIA increase/decreases in Supplementary Table S1). These clearly shows that 72% of the samples had a positive difference (i.e., the mean inhibition activity is higher in the oxidized sample). In addition, we plotted similar graphs for all of the variables found in this study (Supplementary Material Figs. S3–S21). For example, we looked at the differences in the UV peaks area at 280 nm after the alkaline oxidation (Fig. S3). The changes in UV peak area did not show any trend. In addition, we looked at the effects of different tannin-relates factors, such as the effects of the mDP of PAs (Fig. S4), of the PA contents (Fig. S5), of PC-unit contents (Fig. S6), of PD-unit contents (Fig. S7), of the HT contents (Fig. S8), of contents of galloyl derivatives (Fig. S9), of contents of HHDP derivatives (Fig. S10), of the total tannin contents (Fig. S11), of HT share of all tannins (Fig. S12), of PA share of all tannins (Fig. S13). These tannin-related factors did not show any clear trend. We also check the effects of other phenolic compounds on the changes in MIA before and after oxidation. There were no clear patterns detected for quinic acid derivatives (Fig. S14), for flavonoids (Figs. S15–S18), for total polyphenol contents (Fig. S19), for flavonoid share of all polyphenols (Fig. S20) or for the share of quinic acid derivatives in relation to all polyphenols (Fig. S21) after the alkaline oxidation. Detailed quantitative MS/MS data showing the changes due to the oxidation are presented in Supplementary Material in Table S3.

MIA changes after oxidation (Fig. 3) showed that the changes was the strongest for *Pinus sylvestris* inner and outer bark extracts which did not contain detectable PAs at all. Therefore, this changes must be related to other compounds present in the extract. The second-largest change was detected for *Hippocratea volubilis* which did not have any antiparasitic activity before the oxidation. The clear increase in the antiparasitic activity is interesting. The initial *Hippocratea volubilis* extract contained moderate amounts of PAs consisting of both PC and PD units (Table S2). After the alkaline oxidation, the composition of detectable PAs seemed to be the same, but the PA content was observed to be higher (Table S3). In addition, HHDP derivatives may play a role in *Hippocratea volubilis* extract as discussed in Chapter 3.1. The third-largest change was detected for *Adenopodia polystachya* which even increased the larval migration up to 17% before the oxidation. Initial *Adenopodia polystachya* extract also contained moderate amounts of PAs consisting of both PC and PD units (Table S2). After the alkaline oxidation, the composition of detectable PAs seemed to be the same, but the PA content was observed to be lower (Table S3). The fourth-largest change was detected for *Crassula portulacea*. This plant species contained the low amounts of PAs in the non-oxidized extract, but they were PD-rich exhibiting the highest mDP detected. As previously reported, PDs are more susceptible to modification under alkaline conditions (Karonen et al., 2021). Also, in this study, PDs were typically modified (Table S3) and in the case of *Crassula portulacea*, the modification also increased the antiparasitic activity of this extract. The fifth strongest change for observed for *Diospyros mespiliformis* which contained more PAs than *Crassula portulacea* with a smaller PD share and lower mDP. These PD-rich PAs were also modified, and the modification increased the antiparasitic activity of the extract. Even though the modification of PDs increased the antiparasitic activity of these two extracts, that did not happen in all extracts. For example, in *Sequoia sempervirens* PD-rich PAs were modified but this modification did not increase the antiparasitic activity of the extract, instead it actually decreased MIA (Fig. 3). These individual examples support the observations made based on the plotted graphs of all of the variables found in this study (Figs. S3–S21) and highlight the role of all compounds present in the plant matrices. Even though, in general, the oxidation increases the antiparasitic activity of the PA-rich extracts, the change is always dependable on the other compounds present and their modification.

Principal component analysis of the data is shown in Fig. 4. In this analysis, the variables were MIA, UV area at 280 nm, mDP of PAs and all the polyphenol subgroups quantified in this study. Altogether, 16 variables were considered for the PCA analysis. The analysis showed that the data were highly variable to each sample. The eigenvalue of the PCA analysis found to be 4.9. The proportion of variance was 30.72% in PC1 and 19.39% in PC2 (Fig. 4). The loading vectors clustered with the highly correlated variables. The entire chemical feature that seemed to be correlated with MIA was a weak association between total tannins and the percentage of inhibition activity.

Furthermore, we split our data set into two different groups: those that had MIA increased after oxidation ('increase') and those that did not ('decrease'). PCA analysis of these two groups showed that when all the chemical characteristics were included as variables in the PCA, the two groups did not form distinct clusters (Fig. 5A). However, when only the levels of flavonoids (kaempferol, quercetin, myricetin, total flavonoids) and quinic acid were included, the 'decrease' group formed a highly distinct cluster (Fig. 5B), suggesting that flavonoid content was a major driver of the propensity of the samples to display altered antiparasitic activity following oxidation. Consistent with this, we noted that the total flavonoid content was significantly higher in those samples that did increase their activity following oxidation (Fig. 5C).

4. Conclusion

To obtain a clear structure-activity related correlation against the MIA of *A. suum* tend to be a highly challenging task with these types of diverse crude plant extract consisting of different polyphenols and other compounds. Moreover, most of the studied plant extracts contained large oligomeric and polymeric PAs, and oxidation further increased their complexity. However, it is important to understand the combined effects of PAs, and other compounds present if initial or modified PA-rich feed are, and therefore more research on the functional properties of plant samples as well as the molecular level study with *Ascaris suum* parasites are needed. In this study, most of the selected 61 PA-rich plant extracts showed potent (low to high) antiparasitic activity against *Ascaris suum* parasites. When these extracts were treated with alkaline oxidation at high pH, the antiparasitic activity was found to increase even further in 72% of the studied samples. Some samples that did not show antiparasitic activity before oxidation, they showed very high antiparasitic activity after the oxidation. Therefore, in general, our results showed a way for the woodside stream leftover raw materials processing industry to increase their bioactivity by oxidation in alkaline condition. The oxidation process is very rapid and straightforward, which will be of a strong interest industrially for large-scale production. PA-rich feed supplements are an increasingly popular dietary additive in livestock production due to their health benefits, which include anthelmintic properties. Our results suggest that if large-scale and cheap oxidation procedures can be applied during the production of these supplements then the anthelmintic activity could be significantly enhanced. This may have substantial benefits, in an era of reduced anthelmintic drug usage. Thus far, our *in vitro* screening opens the door for future study to understand better the mechanism of action in increasing or decreasing antiparasitic activity under alkaline treated PA rich plant extract against *Ascaris suum* parasites and helps other researchers as well as the industry in selecting the right plant samples.

Author contributions

Conceptualization, J.-P.S., M.T.E, A.R.W.; M.K. and I.B.I; methodology, M.T.E, A.R.W, I.B.I; software, I.B.I, A.R.W.; validation, I.B.I, A.R.W.; formal analysis, I.B.I, investigation, I.B.I. and A.R.W.; data curation, I.B.I. A.R.W, M.K.; writing—original draft preparation, I.B.I.; writing—review and editing, I.B.I., M.K, A.R.W, M.T.E. and J.-P.S.; visualization, I.B.I and A.R.W; supervision, J.-P.S, A.R.W, M.T.E and M.K.; project administration, J.-P.S.; funding acquisition, J.-P.S, A.R.W and M.K. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2023.108493.

Abbreviations

DMSO	dimethyl sulfoxide
ESI	electrospray ionization
HBSS	hank's balanced salt solution
HHDP	Hexa hydroxyl diphenoyl
HT	hydrolysable tannin
mDP	mean degree of polymerization
MIA	mean inhibition activity
MRM	multiple reaction monitoring
PA	proanthocyanidin
PC	procyanidin
PD	prodelphinidin
PTFE	polytetrafluoroethylene
QqQ-MS	triple-quadrupole mass spectrometer
UHPLC	ultrahigh-performance liquid chromatography

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