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ABSTRACT

This thesis focused on the proanthocyanidins (PAs), a class of plant specialized metabolites that have a wide range of industrial applications. They play a vital role in the production of various food and feed products. Despite the abundance of natural PAs globally, many of them are still unexploited and could be used for various other industrial purposes. Further studies are needed to find ways to exploit these resources and make them useful for industrial purposes; this could include the extraction of PAs from plants and chemically modifying their structure to form other attractive products. The goal of this study was to investigate the possibility of modifying the natural PAs by studying their susceptibility to oxidation in alkaline conditions. This study investigated oxidized and non-oxidized PA-rich plant extracts from various plant kingdoms. It provides us to formulate a better understanding of PA oligomers and polymers in different PA-rich plants. The UHPLC-DAD-MS/MS method was used to provide these quantitative and qualitative data. The study revealed that the various structural features of PAs can be used to predict their susceptibility to oxidation. Overall, the results of the study revealed that the main reaction paths for procyanidins (PCs) and prodelphinidins (PDs) containing plant extracts were different under alkaline conditions. For instance, the former had an intramolecular route, while the latter had both intra and intermolecular reactions.

Besides, the selected plant extracts were analyzed using orbitrap high-resolution mass spectrometry, which revealed the complex structural information and diverse reactions within the PA classes. Plant extracts were found to contain natural PAs from both A- and B-type structural families, as well as galloylated PAs. The A- and B-type PCs were relatively stable following oxidation, but some plant extracts produced new ether linkages. Conversely, PAs containing plant extracts that consisted only of PDs or PC/PD mixtures were found to be more reactive under alkaline conditions. Extracts rich in galloylated PCs were generally stable after oxidation, but those containing galloylated PDs or PC/PD mixtures were found to be more reactive. However, in certain cases, the addition of two or more galloyl groups to galloylated PCs increased their reactivity compared to those with only a single galloyl group.

In addition, this study investigated the *in vitro* antiparasitic effects of various extracted PAs samples (original and oxidized) against *Ascaris suum* nematodes. The selected plant extracts were subjected to a detailed analysis of the antiparasitic properties by a highly reproducible *in vitro* larval migration inhibition assay. The modifications made to the PA-rich plant extracts significantly increased their antiparasitic activity. Some of the samples that showed no apparent antiparasitic activity prior to oxidation showed significant activity after the treatment and exhibited high levels of other polyphenols. Through the *in vitro* screening, we were able to gain a deeper understanding of how treating a plant with alkaline oxidation can improve its biological activity, which has the potential for novel anthelmintic drugs.

KEYWORDS: *Ascaris suum*, antiparasitic, bioactivity, high-resolution mass spectrometry, *in vitro*, modification, oxidation, plant extracts, proanthocyanidin, UHPLC-DAD-MS/MS

TURUN YLIOPISTO
Luonnontieteiden ja tekniikan tiedekunta
Kemian laitos
Luonnonyhdisteiden kemia
IQBAL BIN IMRAN: Luonnollisia ja muunnettuja proantosyanidiineja sisältävien kasviuutteiden karakterisointi ja *in vitro* antiloisvaikutukset
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TIIVISTELMÄ

Tässä väitöskirjassa keskityttiin proantosyanidiineihin (PA:hin), jotka ovat kasvien erikoistuneiden metaboliittien luokka, ja joita voidaan käyttää erilaisiin teollisiin sovelluksiin. PA:lla on esimerkiksi tärkeä rooli erilaisten elintarvikkeiden ja rehujen tuotannossa. Vaikka PA:ja on tutkittu maailmanlaajuisesti runsaasti, monet niistä ovat edelleen hyödyntämättä. Näiden PA-resurssien hyödyntämiseksi tarvitaan lisätutkimuksia ja uusia keinoja. Yksi potentiaalinen tapa olisi uuttaa PA:t kasveista ja muuntaa niiden rakenteita kemiallisesti uusien houkuttelevien tuotteiden aikaansaamiseksi. Tässä väitöskirjatyössä tutkittiin PA:en alttiutta hapettua emäksisissä olosuhteissa sekä mahdollisuutta muuttaa luontaiset proantosyanidiinit antiloisaktiivisimmiksi modifikaatioiksi hapettumisen kautta.

Tutkimuksessa analysoitiin ensimmäistä kertaa eri PA-rikkaiden kasviuutteiden hapettumista emäksisissä olosuhteissa. Vaikka monia PA:n ominaisuuksia voidaan verrata muiden oligomeerien ja polymeerien ominaisuuksiin, kuten sen rakenteeseen ja koostumukseen, tarvitaan lisätutkimuksia sen yleisominaisuuksien analysoimiseksi. Kvalitatiivisiin ja kvantitatiivisiin PA-analyyseihin käytettiin kahta erilaista UHPLC-DAD-MS/MS-menetelmää.

Tutkimuksessa selvisi, että PA:iden erilaisten rakenteellisten ominaisuuksien avulla voidaan ennustaa niiden hapettumisalttiutta. Tarkemmat rakenneanalyysit osoittivat, että luontaiset PA-rakenteet olivat hyvin erilaisia ja monimuotoisia sellaisenaan ja vielä monimuotoisempia hapettumisen jälkeen. Kasviuutteissa havaittiin erilaisia luontaisia PA:ja, kuten A- ja B-tyypin prosyanidiineja (PC), prodelfinidiineja (PD) sekä näiden PC/PD-seoksia ja galloyloituja PA:ja. Sekä A- että B-tyypin PC:t olivat suhteellisen pysyviä ja havaitut muutokset olivat vähäisiä. Jossain tapauksissa havaittiin uusien eetterisidosten muodostumista. PD-pohjaiset PA:t olivat reaktiivisempia ja muodostivat hyvin monimutkaisia polymeerirakenteita. Galloyloidut PA:t olivat suhteellisen pysyviä, jos ne olivat PC-pohjaisia, kun taas PD-pohjaiset galloyloidut PA:t olivat yhtä reaktiivisia kuin galloyloimattomat PD-rikkaat PA:t.

Luontaisten ja modifioitujen PA-rikkaiden kasviuutteiden antiloisvaikutuksia *Ascaris suum* -sukkulamatoja vastaan tutkittiin in vitro -kokeella. Uutteen PA-rakenteiseen tehdyt muutokset lisäsivät merkittävästi sen antiloisaktiivisuutta. Lisäksi havaittiin, että myös muita polyfenoleita sisältävien uutteiden antiloisaktiivisuus kasvoi merkittävästi hapettumisen myötä. In vitro -seulonnan avulla pystyttiin saamaan syvempi käsitys siitä, miten PA-rikkaan kasviuutteen hapettamisella voidaan parantaa sen biologista aktiivisuutta ja erityisesti antiloisvaikutuksia.

ASIASANAT: *Ascaris suum*, antiloisvaikutukset, bioaktiivisuus, hapettuminen, *in vitro*, kasviuutteet, korkean resoluution massaspektrometria, modifikaatio, proantosyanidiini, UHPLC-DAD-MS/MS

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Abbreviations

AGC	Automatic gain control
CID	Collision-induced dissociation
DAD	Diode array detector
ddMS2	Data dependant MS/MS
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
ESI	Electrospray ionization
GI	Gastrointestinal
HBSS	Hank's balanced salt solution
HCOOH	Formic acid
HESI	Heated Electrospray ionization
HHDP	Hexahydroxydiphenoyl
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HRF	Heterocyclic ring fission
HT	Hydrolysable tannin
LC-MS	Liquid chromatography-mass spectrometry
L3	Stage 3 larvae
mDP	Mean degree of polymerization
MIA	Migration inhibition assay
MIA%	Mean inhibition activity %
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NP	Normal phase
PA	Proanthocyanidin
PC	Procyanidin
PD	Prodelphinidin
PDA	Photodiode array detector
PPC	Protein precipitation capacity
PPO	Polyphenol oxidase
PTFE	Polytetrafluoroethylene

RDA	Retro-Diels–Alder
RP	Reversed phase
RPMI	Roswell Park Memorial Institute Medium
UHPLC	Ultrahigh-performance liquid chromatography
UHRMS	Ultrahigh-resolution mass spectrometry
QqQ-MS	Triple-quadrupole mass spectrometry
QM	Quinone-methide
Q-TOF	Quadrupole time of flight
UV	Ultra-violet

List of original publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Imran, I.B; Karonen, M.; Salminen, J.-P.; Engström, M.T. Modification of natural proanthocyanidin oligomers and polymers via chemical oxidation under alkaline conditions. *ACS Omega* 2021, 6, 4726–4739. https://pubs.acs.org/doi/10.1021/acsomega.0c05515

II Karonen, M.; Imran, I.B.; Engström, M.T.; Salminen, J.-P. Characterization of natural and alkaline-oxidized proanthocyanidins in plant Extracts by ultrahigh-resolution UHPLC-MS/MS. *Molecules* 2021, 26 (7), 1873. https://www.mdpi.com/1420-3049/26/7/1873

III Imran, I.B.; Engström, M.T.; Karonen, M.; Williams, A.R.; Salminen, J.-P. Alkaline oxidization can increase the *in vitro* antiparasitic activity of proanthocyanidin-rich plant extracts against *Ascaris suum*. *Experimental Parasitology* 2023, Volume 248, 108493, ISSN 0014-4894. https://doi.org/10.1016/j.exppara.2023.108493.

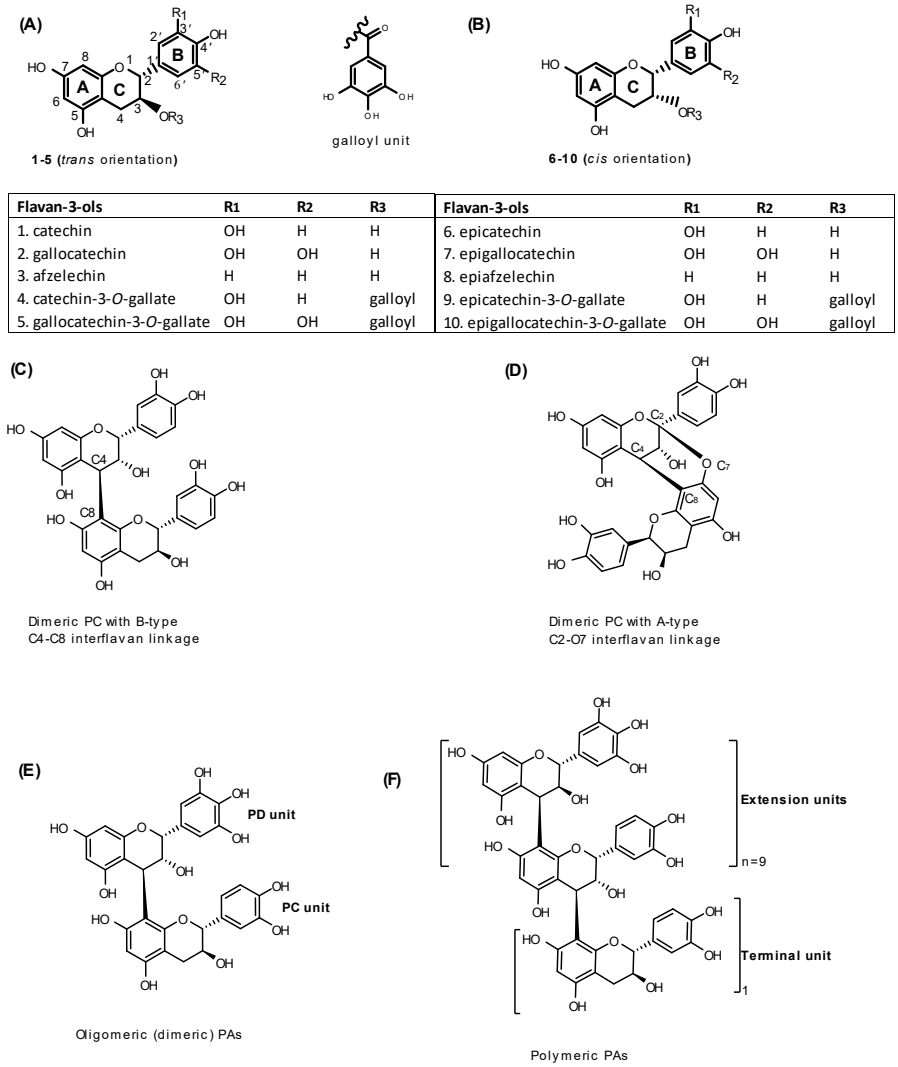
1 Introduction

1.1 Proanthocyanidins and their structures

Proanthocyanidins (PAs, also known as condensed tannins) are oligomers and polymers made from various flavan-3-ols subunits. They are commonly found in both woody and non-woody species in almost all plant families but are especially abundant in gymnosperms.[1] The term PA comes from the conversion of these types of tannins to coloured anthocyanidins via acid-catalyzed depolymerization reactions.[2] The complexity of the PAs depends on the various factors that affect their structure. Some of these include the degree of polymerization, the hydroxylation patterns, C2 and C3 stereochemistry and the linker types between the flavan-3-ol units.[2–5] Most commonly, the PAs are composed of two types: procyanidins (PCs) and prodelphinidins (PDs). The former comprises catechin or epicatechin units, while the latter includes gallocatechin or epigallocatechin units **(Figure 1)**. Usually, PAs are made up of mixtures of PCs and PDs, but they can also have rarer molecules like propelargonidins, which are made up of afzelechin or epiafzelechin units.[6] Because the subunits can be joined by bonds of either C-4/C-8 or C-4/C-6 (in the case of B-type PAs) or C-2/O-7 (in the case of A-type PAs), the possible number of oligomers and polymers can be increased to several hundred or perhaps a thousand.[4,6,7] This complexity is further enhanced by possible galloylation of the subunits.[6]

PAs are being used in many industrial applications. The demand for PAs in the global market is increasing rapidly in several diverse industries, and so far, the worldwide commercial utilization of PAs is estimated to be several hundred kilotons per year.[8–10] The majority of PAs were derived from various coniferous bark extracts, such as wattle barks, mimosa trees, quebracho, oaks, chestnut, mangroves, sumach, myrobalans, and tara, as well as various pine and fir tree species. Traditionally, tannin and PAs have been widely employed in leather tanning, wine and animal food production as well as in various industrial processes. They can also be used in the production of oil drilling fluids and mineral flotation.[11–13] Other underutilized PA sources are available, including as byproducts of the wood industry and other biomass sources, such as wood bark and leaves from the agricultural and forest industries. These tannins may not have the optimal structure and stability for the intended

bioactivities. However, their commercial value might be raised by chemically modifying their structures in order to create new molecules with better properties.[14–17] These newly modified PA-containing products could be used for various industrial applications, including sustainable food and feed, cosmetics, and pharmaceuticals.



Flavan-3-ols	R1	R2	R3	Flavan-3-ols	R1	R2	R3
1. catechin	OH	H	H	6. epicatechin	OH	H	H
2. gallocatechin	OH	OH	H	7. epigallocatechin	OH	OH	H
3. afzelechin	H	H	H	8. epiafzelechin	H	H	H
4. catechin-3-*O*-gallate	OH	H	galloyl	9. epicatechin-3-*O*-gallate	OH	H	galloyl
5. gallocatechin-3-*O*-gallate	OH	OH	galloyl	10. epigallocatechin-3-*O*-gallate	OH	OH	galloyl

Figure 1 Some examples of proanthocyanidin structures. (A) flavan-3-ol monomeric skeleton showing possible hydroxylation pattern in B ring, 1-5 monomeric subunits having *trans* configuration (B) flavan-3-ol monomeric skeleton showing possible hydroxylation pattern in B-ring, 6-10 having *cis* configuration, (C) a dimeric procyanidin linked with C4-C8 B-type interflavan bond, (D) a dimeric procyanidin joined by C4-C8 (B-type PAs) and C2-O7 (A-type PAs) interflavan bonds, (E) an example of oligomeric/dimeric proanthocyanidins composed of one terminal unit of a procyanidin (PC) and one extension unit of prodelphinidin (PD), (F) single terminal unit and ten extension units constitute polymeric proanthocyanidins.

1.2 Analyses of proanthocyanidins by UHPLC-QqQ-MS and ultra-high-resolution orbitrap-MS

Modern state-of-the-art liquid chromatography and mass spectrometry instruments (LC-MS) have made it possible to analyze the most complex macromolecules in plants. Nowadays, it is common to use high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC) to separate the targeted PA molecules using a variety of detectors, most commonly photodiode array detector (PDA), quadrupole and triple quadrupole MS, linear ion trap MS, Quadrupole time of flight (Q-TOF) MS, and ultra-high-field orbitrap MS etc.[18–29]

UHPLC is a technique that uses a stationary phase with small particle sizes (smaller than 2 μm) and high operating pressures (up to 1300 bars). The mobile phase is continuously moved over the stationary phase. Also, it features various advantages over its predecessor, HPLC. Some of these include fast analysis, increased sensitivity and improved resolution.[30–32] In PAs analysis, in addition to normal phase (NP) and reversed phase (RP), a lot of hydrophilic interaction liquid chromatography (HILIC) columns are used as stationary phase.[33] However, for better selectivity and separation of positional isomers of PAs molecules, the phenyl column has been commonly utilized as a stationary phase in modern PAs analysis. The phenyl ring provides a different selectivity than the typical C18 reverse phase column. Phenyl ring compounds mostly interact with the stationary phase's bonded hydrophobic moiety's phenyl ring via aromatic/π-π interactions.

Besides, mass spectrometry (MS) is commonly used to isolate compounds from a liquid chromatography (LC) column. The compounds enter the ion source using the elution profile generated by the LC column.[32] The LC-MS, in particular, electrospray ionization (ESI) techniques[34] is the most commonly used ionization technique. It is also known as a "soft" ionization technique because it provides intact molecular ions instead of multiple fragments. This means that the fragmentation of analytes remains subtle. It is a powerful ionization technique that can be coupled to almost any form of mass analyzer.[34–37] This technique is very important for studying highly polar, non-volatile, complex plant natural compounds. Over the recent two decades, PAs have been studied using these contemporary instruments, saving time and increasing the analysis method’s robustness and sensitivity.[4,26,32,38–43]

Several mass analyzers are used for structural characterization and quantification of PAs. Some instruments are more suited to qualitative analysis with high-resolution capabilities, while some are more suited to quantitative analysis with sensitive detection capacities. When used in PA analysis, it is sometimes connected to a quadrupole or triple quadrupole (QqQ) MS instrument.[23,26,44] As the chemical characterization of natural PAs is challenging; their structural details are best revealed using HPLC/UHPLC instruments coupled with high-resolution mass analyzers.[21,35,38,45,46] With the help of high-resolution MS analyzers, e.g., time-of-

flight (ToF), ion trap/quadrupole-ToF, Fourier-transform (FT)-ion cyclotron resonance mass spectrometry and orbirap ultra-high field mass spectrometers, it is possible to figure out the chemical formula of PA molecules in plant extracts by looking at their exact masses.[18,21,42,47–50] By fragmenting the PAs ions via collision-induced dissociation, more structural information can be obtained.[34,51]

In nature, PA oligomers and polymers typically yield a heterogeneous mixture of oligomeric and polymeric structure, resulting in multiple charged ions in ESI; these cannot be interpreted by other than high-resolution MS instruments.[47,52–54] PAs are fragmented by some well-recognized fragmentation reactions, including the quinone-methide (QM) cleavage, heterocyclic ring fission (HRF) and retro-Diels–Alder (RDA) fragmentation[21,47,53,55,56] shown in **Figure 2**. Although the B and A-type PAs are consistent in terms of their mass spectrum fragmentation, the differences between them can be distinguished from one another by their differing *m/z* values, which vary by 2 Da in mass spectra.[37,42,46,52,53,55,56]

In 2014, Engström et al. developed a novel PA quantification method using a highly sensitive triple quadrupole mass analyzer instrument (Water´s Xevo QqQ MS), which is so far one of the most potent and rapid tools in PA quantitative analysis,[57,58] This method quantifies the amount of PA content, the PC:PD, and the mean degree of polymerization (mDP) of the plant sample. It also provides a qualitative assessment of the presence of galloylated PAs in the plant sample.[57,58]

Moreover, the method can also be used to compare the changes in the fragmented ion sources produced by the quinone methide cleavage process. It can detect small oligomers (2-5 mDP) and polymers (≥10 mDP), as well as medium-sized oligomers (5-10 mDP). To achieve this, the cone voltages used in the process have to be optimized. While analysing the PAs, it is essential to consider that only ultra-violet (UV) or tandem mass spectrometry (MS/MS) information cannot provide a comprehensive picture of the samples. For instance, UHPLC-UV peak areas or the areas of chromatographic humps typical to polymeric PAs should be compared with MS/MS detection to know if it could detect all of the PAs present in the sample or not. The best tool for observing the detailed structural characterization of these complex PA oligomers and polymers in plant samples could be a high-resolution orbitrap-mass spectrometer.

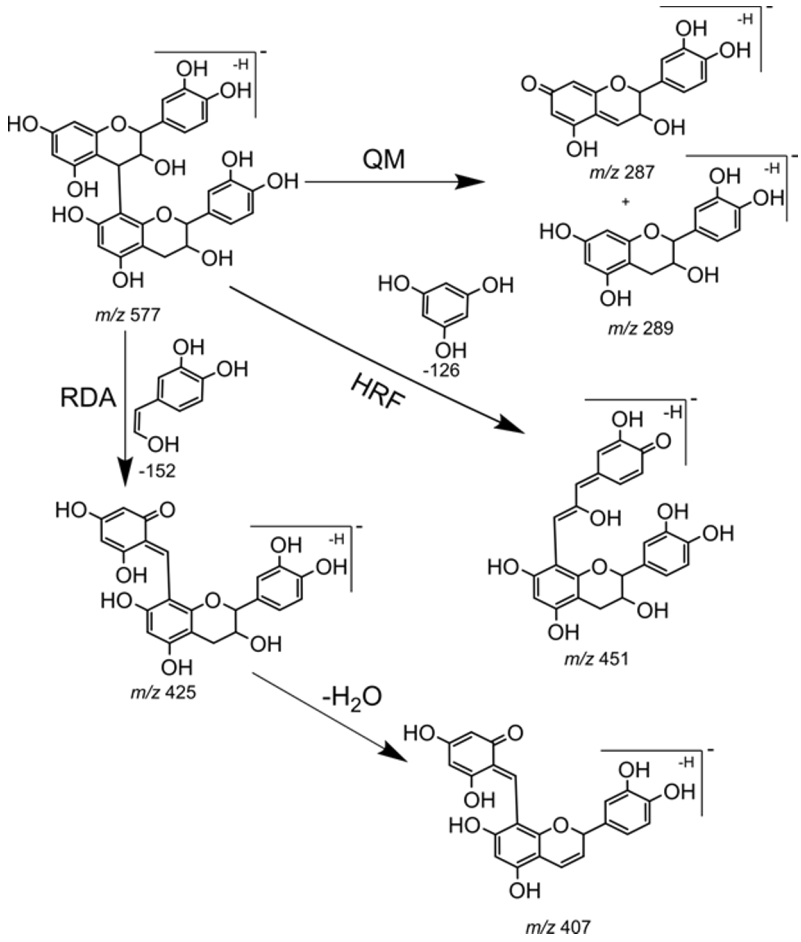


Figure 2 Conventional characteristic fragmentation reactions shown in the figure are known to lead to the formation of PAs (dimeric PC). These include the heterocyclic ring fission (HRF), retro-Diels-Alder (RDA) and quinone methide (QM) reactions.

1.3 General bioactivities linked to proanthocyanidins

The general bioactivities of PAs are influenced by their anti-prooxidant and protein precipitation capacity. The pH level of these compounds affects the effects they have on living organisms. In high pH conditions, however, the activity of the plant antioxidant enzyme polyphenol oxidase (PPO) is expressed.[59] PAs are considered bioactive plant specialized metabolites as they have shown numerous positive effects, e.g. on humans and animal health. Over the years, several molecular-level studies have been carried out to determine the bioactivities of PAs. The most prominent general bioactivities of PAs are antioxidant activity,[60,61] impacts on blood

sugar and cholesterol,[62,63] cancer-fighting activities,[64,65] cardiovascular disease prevention,[66,67] anti-inflammatory and bacteriostatic characteristics,[68,69] intestinal flora management,[63,70] and so on. Moreover, in the past couple of decades, PAs have been widely studied for their potential impact on ruminant nutrition and health.[70,71] There is significant evidence that PAs are effective natural anthelmintics against ruminant gastrointestinal nematodes, which reduces the need for synthetic drugs, improve the efficiency of the protein utilization process, and improve milk quality through better protein utilization (rumen-escape protein).[72–76] The mechanisms of action behind these positive effects are not entirely determined yet, but few particular PAs' features have been found to play a crucial role in their bioactivities.[49,73,77–80] In recent years, more in-depth research on general bioactivities, including protein precipitation capacity (PPC) and oxidative activity, has only been conducted in the case of a few chosen plant samples with purified fractions of PAs, a complete overview of the different positive bioactivities of the structurally diverse PA-rich plant extract is still lacking. The screening of several PA-rich plant extracts could be a vital tool to know the big picture, which could further give some hints for the accurate understanding of the PA bioactivities.

1.4 Anthelmintic properties of proanthocyanidins

It has been established that parasitic worms (helminths) are among the most prevalent pathogens in humans and livestock processing. The gastrointestinal (GI) parasite that affects livestock is a big concern for the agricultural industry.[81,8283] To treat these parasitic worms, synthetic antimicrobial and anthelmintic medicines are routinely used. There has recently been a heavy emphasis on reducing the use of these synthetic chemicals because of the rapid growth of drug-resistant pathogens.[84,85]

PA-rich forage legumes and other plant sources have been extensively studied over the past decade to understand the relationship between PA's structure and function to find an alternative source of synthetic drugs to combat these infectious parasites worldwide.[30,85–87] A few positive findings of *in vitro* antiparasitic activities for natural tannins have been witnessed so far. However, most of the analysis was performed with tannin fractions and with a limited plant species origin.[36,71,72,81,88–91] As a result, it is challenging to build a more general view of PAs structure-activity patterns in ruminant related studies.

Previous research has shown that stage 3 larval (L3) assay is the most common option in anthelmintic/antiparasitic activity testing, as this stage is said to be the infective stage for the nematodes.[81,91–97] Moreover, recently, several studies have been conducted on the purified PAs against *Ascaris suum* nematodes; for instance Leppä et al. 2020 investigated *in vitro* antiparasitic activity on purified PA fractions

by measuring their migration inhibition and larval mortality.[49] Andersen-Civil et al. 2021 investigated the diets containing purified PAs modulated the inflammatory response of pigs to infection with the *Ascaris suum* parasite.[98,99] Their results showed that the presence of PCs attenuated the inflammatory response in the gut and caused increased T cell response. These findings suggest that the effects of these infections on the gut microbiota can be distinct.

Therefore, this thesis work utilized a highly reproducible third stage larval migration inhibition assay[81,92,100–106] for the *in vitro* antiparasitic activity test. In this study, the model parasite was used as *Ascaris suum* nematodes **(Figure 3)**, one of the destructive parasites that cause significant losses for the agricultural industry.[81,82,107–109]

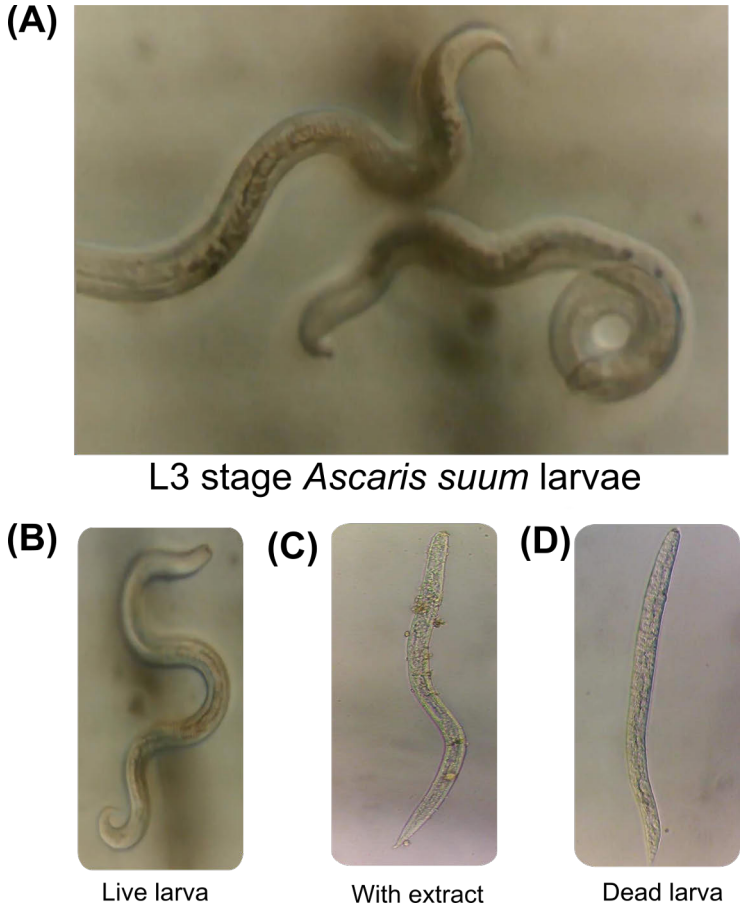


Figure 3 Inverse light microscopy of the stage 3 (L3) larvae of *Ascaris suum* nematodes was observed in the study.

1.5 Ways to modify proanthocyanidins

The structure of PAs can be modified through derivatization reactions, which can involve the addition of functional groups.[106,110,111] These reactions can be performed by reacting an acid with a group of alkyl halides or by anhydride or an acid chloride. However, they require to be controlled in order to get the most out of the reactions.[110]

One of the most promising ways to modify the structure of PAs is by oxidizing them.[111–113] This process can produce new molecules with improved bioactivities. It can be done through the oxidation of dimeric or flavan-3-ols. According to the literature, the various oxidation reactions that occur in these compounds are known i.e oxidation of *o*-dihydroxy polyphenols and conversion of B-type procyanidin dimer to A-type.[112–114] Mass spectrometric analyses can also be performed on the effects of oxidation. In most cases, the resulting signals are tiny, which is 2 Da smaller than those produced by PCs. In contrast, in alkaline oxidation, the formation of *o*-quinones by *o*-dihydroxy polyphenols is initiated. The formation of *o*-quinone forms is unstable and can lead to further reactions. By oxidizing the hydride ion at the C-ring, which is one of the most prevalent mechanisms, it is possible to transform B-type PCs into A-type ones. This process results in the B-type procyanidin dimer oxidising and ultimately converting into A-type structure.[38,45,115–121] The mechanism is known as the quinone methide mechanism, and it has been investigated at various controlled temperatures, pH levels, and catalytic conditions.[117,118,122,123]

However, the information about the structural and oxidation reactions of large oligomeric and polymeric PAs is still relatively limited.[10,124–127] This is because of the challenges inherent in analyzing complex PAs, which get even more complex during oxidation.[128] Most research on PA oxidation has focused on chemical depolymerization[128–130] and/or identification of oxidation markers.[37,38,129,131,132] Based on the chemical properties of PAs and sample concentration, the findings suggest that there are two main types of oxidation reactions: intramolecular and intermolecular.[38,117,128,129,131,133,134] These studies were conducted on unique sources of PAs, such as wine-making and apples.[38,129,131,133,134] Nevertheless, it is essential to define the reactivity of numerous PA sources; much further research is required to develop a solid understanding of how the different PA types are chemically reactive and the mechanisms through which they can cause structural modification.

Moreover, The study on the reactions of various dimeric and monomeric flavan-3-ols at high pH revealed that the alkaline conditions are mainly responsible for forming B-ring quinone-methides and the oxidation of B-type to A-type PAs.[115,117] However, the exact oxidation behaviour of plant extracts is still not fully understood. This is probably because analyzing the structurally diverse PAs in plants is challenging and even tougher to study after they have been oxidized.

1.6 Aims of the study

The primary goal of this PhD thesis was to extensively investigate a wide range of PA-containing plant extracts with distinct PA fingerprints to identify their differences in both reactivity and modification processes under alkaline conditions. Furthermore, to see if plant extracts containing modified PAs have better antiparasitic properties than the original extracts that were not modified in alkaline circumstances.

Thus, the main objective of the study was:

1. To determine the various modifications that occur in the plant PAs under alkaline conditions.
2. To characterize the non-oxidized and oxidized plant extracts by high-resolution mass spectrometry.
3. To study the *in vitro* antiparasitic properties of the extracted PAs (non-oxidized and oxidized).

The following tasks have been completed in order to achieve these objectives:

1. A study of the consequences of non-specific alkaline oxidation on PAs found in 102 plant samples were analyzed (both the non-oxidized and oxidized extracts) by the rapid UHPLC-DAD-MS/MS quantitative method[44,58] to classify the natural PAs into different categories depending on their structural details and susceptibility towards oxidation at high pH. In addition, it has attempted to clarify the possible reaction routes for the oxidative modification reactions (Articles I-II).
2. The most noteworthy PA-rich samples were analyzed using UHPLC and ultrahigh-field orbitrap mass spectrometry to characterize the natural PA structures in more detail and better understand the tiny structural modification that took place at high pH. (Article II).
3. The original (non-oxidized) and oxidized PA-rich plant extracts were analyzed for their *in vitro* antiparasitic activity against *Ascaris suum* nematodes. The study was thus able to show whether PA modification at high pH typically increases the activity of the plant extracts towards *Ascaris. suum* nematodes or if the processes are more complex and species-specific (Article III).

2 Materials and Methods

A complete summary of the experimental design, analytical methods and instruments used in this study has been illustrated in **Figure 4**.

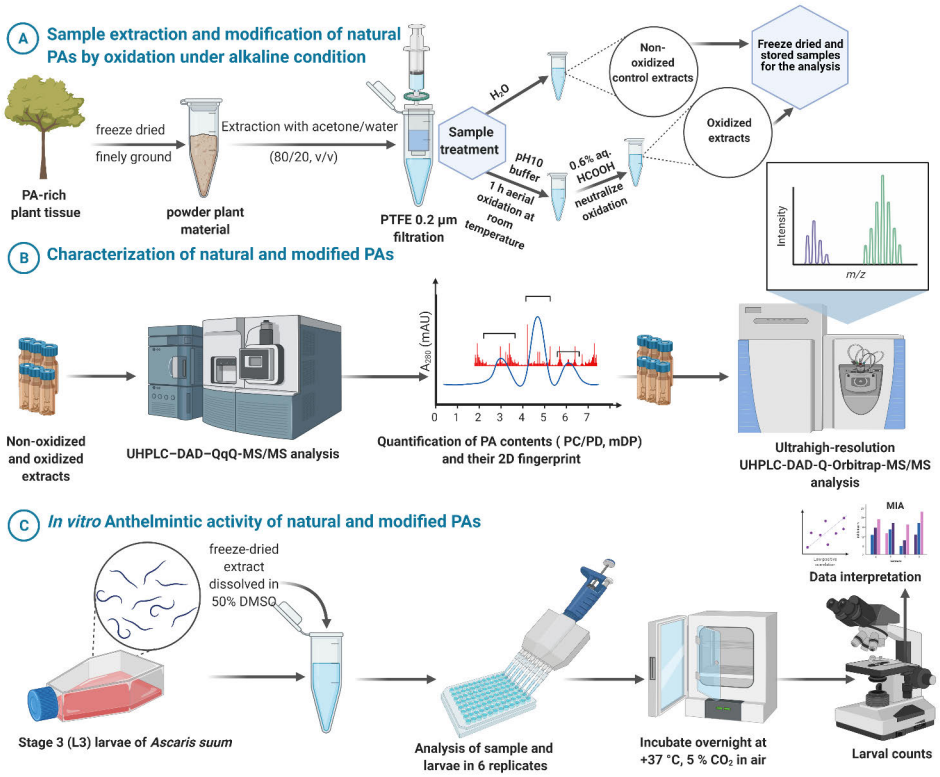


Figure 4 Summary of the experimental design and analytical methods applied in this thesis. In the illustration, (A) sample extraction and their modification process of natural proanthocyanidins (PAs) by rapid aerobic oxidation under alkaline condition (Article I, II and little modified protocol in Article III), (B) The analytical process of the quantification of PA contents, PC/PD fingerprint by UHPLC-DAD-QqQ-MS/MS (Article I) and characterization of non-oxidized and oxidized PAs by UHPLC-DAD-Q-Orbitrap-MS/MS (Article II), and (C) *In vitro* larval migration inhibition assay (MIA) to know the antiparasitic activities of natural and modified PAs against *Ascaris suum* nematodes (Article III). Illustration was created by BioRender.com.

2.1 Plant samples and extraction

Three hundred plant samples were collected from the botanical garden (60° 26'0 " N, 22° 10 '19' 'E) of Turku University, Finland. All the plant samples (i.e. leaves, flower, bark, leaflets, needles, branches) were correctly identified by the help of botanical garden herbarium (articles I and II).[135,136] Additional samples were also taken from Barro Colorado Island in Panama to add to the diversity from the PA's point of view (seed extracts, article III).[137] Commercial tannin-rich bark samples were purchased from "Xi'an Le Sen Bio-technology Co., Ltd" Xián, China (article III). A phylogenetic tree **(Figure 5)** was constructed for PA-rich plant families' selected plant species. The phylogenetic tree was constructed by transforming the list of plant species into a format used by phylomatic based on the method of Slik et al. 2018,[138] further processed by the online software iTOL (interactive tree of life). After the plants were collected, they were freeze-dried, ground into a fine powder, and placed in the fridge at -20°C. For the extraction, 20 mg (articles I and II) and 50 mg of finely ground dried plant tissue (article III) were mixed with 1.4 mL of acetone/water (80/20, v/v) and left to rest overnight at 4 °C. The samples were extracted twice for 3 hours before it is combined. Under vacuum, acetone was removed from the extracts, and the aqueous phases were finally freeze-dried.

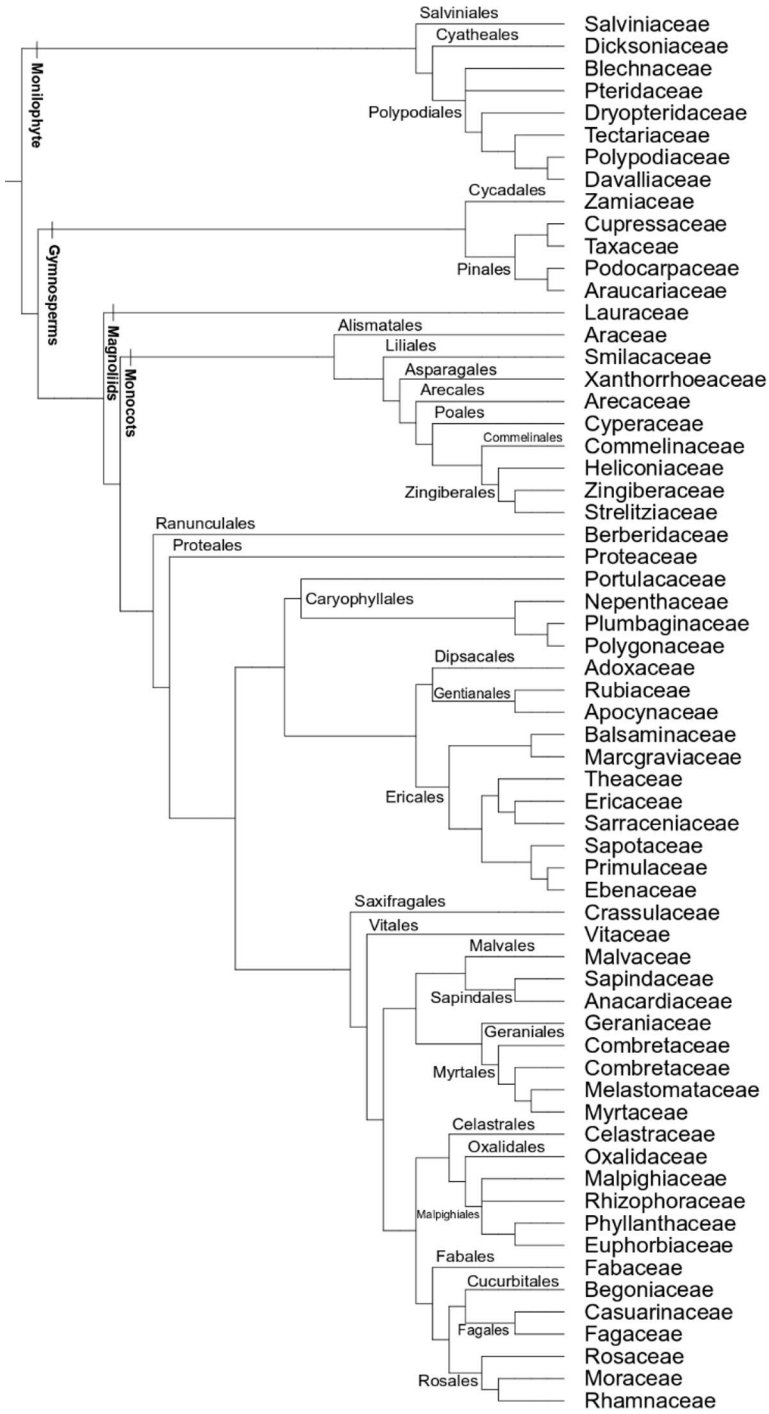


Figure 5 Phylogenetic tree of proanthocyanidin-rich plant families of the studied plant species constructed by using phylomatic and iTOL online tools.

2.2 Sample preparation

For the PAs screening phase in articles I and II, the freeze-dried extracts were dissolved in 1 mL of Milli-Q water and vortexed for 15 minutes. The extracts were filtered using polytetrafluoroethylene (PTFE) filters with 0.2 µm pore sizes (VWR International, Radnor, PA, USA) to get rid of the sample's non-watersoluble components. The samples were analyzed by the UHPLC-DAD-QqQ-mass spectrometer for article I and UHPLC-DAD-Q-Exactive-orbitrap mass spectrometer for article II. The short protocol is described below: after fivefold dilution with water, 20 µL of each sample was dissolved with 180 µL of pH 10 buffer and left for 1 hour for the pH10 oxidation stage of the PA-containing extracts. By adding 100 µL of 0.6% aq. HCOOH, the oxidation was stopped. The control extracts were made by taking 20 µL of each extract and mixing it with 280 µL of the 180/100 (v/v) mixture of the pH 10 buffer and 0.6% aq. HCOOH. After further vortexing for 5 minutes, 100 µL of each sample (non-oxidized and oxidized) was inserted into a separate UHPLC vial for the both UHPLC-DAD-QqQ-MS/MS and UHPLC-DAD-Orbitrap-MS/MS analyses.

Even though the extraction method was the same as Articles I and II, a bit changed sample preparation technique (25 times larger scale than the previous sample preparation) was used in Article III, since the same samples were used both for the larval migration inhibition assay and UHPLC-MS/MS analyses. The sample preparation for Article III is as follows: After dissolving the freeze-dried extracts in 0.5 mL of ultra-pure LC-MS grade water, the mixture was vortexed to ensure that the contents of each tube's sample were properly homogenized. The original control extract was prepared by adding 7 mL of ultra-pure LC-MS grade water so that for each sample, concentration and dilution profile were the same for every analyses. The non-oxidized control sample was prepared by adding 7 mL of the 9/5 (v/v) mixture of the pH10 buffer and 0.6% aqueous HCOOH solution. The oxidized sample was prepared by adding 4.5 mL of pH10 buffer and let the test tube for one hour 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 samples were transferred into an eppendorf tube and preserved for further UHPLC-MS/MS analyses. Then, the rest of the samples were freeze-dried and transported for the larval migration inhibition assay.

2.3 UHPLC–DAD-MS/MS Analyses

In Articles I and III, the UHPLC-DAD-MS/MS analyses were carried out using a Xevo TQ triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) combined with an Acquity UPLC system (Waters Corp., Milford, MA, USA). The UPLC apparatus includes a sample manager, a binary solvent manager, a column,

and a diode array detector. The UPLC column used for the analysis was a 1.7 µm, 2.1 × 100 mm ACQUITY UPLC® BEH Phenyl column (Waters Corp., Wexford, Ireland). Oligomeric and polymeric PC and PD, as well as their mean degree of polymerization (mDP), were detected and quantified using the group-specific multiple reaction monitoring (MRM) method described in Engström et al. 2014.[139,140]

The same elution profile, ion source settings, UV and MS/MS detection techniques were utilized as in Engström et al. (2014). From 0 to 7 minutes, UV and MS data were continually collected. The stability of UHPLC retention times and *m/z* values of the MS detector were observed in a flavonoid mix stock solution, including 4 µgmL^{-1} in each of kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside in acetonitrile with 0.1% HCOOH (1:4 v/v). Before and after each batch of 10 samples, the MS/MS response stability was monitored five times by injecting a 1 µg mL^{-1} catechin solution (in acetonitrile 0.1% HCOOH (1:4 v/v)). Within each analysis set and between different sets, quantitative results were corrected for possible variations in the quantitative efficiency of the system. The Engström method was used to detect and measure the PC and PD subunits and the mean degree of polymerization (mDP), these were achieved by altering the cone voltages used in the method, i.e., 75 V, 85 V and 140 V for PC units and 55 V, 80 V and 130 V for PD units, respectively. The reported PC and PD traces were smoothed (Window size 30 scans × 5 smoothing iterations) and integrated with the TargetLynx software (*V4.1 SCN876 SCN 917 © 2012* Waters Inc.). Quantitative data were compiled from individual calibration curves generated for PC, PD, and mDP. The PC and PD calibration curves were obtained using two sephadex LH-20 fractions: the PC standard from *Tilia* flowers and the PD standard from *Ribes nigrum* leaves. The concentration range of the calibration curves for the PC standard was 1.50-0.1875 mg mL^{-1}, and for the PD standard was 2.00-0.25 mg mL^{-1}. The standard calibration curve for mDP was obtained from six sephadex LH-20 fractions of *Vaccinium vitis-idaea* leaves, *Calluna vulgaris* flowers and *Tilia* flowers (to fine-tune the mDP calculation equation). All calibration curve samples were prepared in a solution of 1/4 acetonitrile/0.1% HCOOH (v/v). From the MRM raw data, the PA fingerprints were created by calculating the PA concentration with PC and PD calibration curves at each time point, as explained in Salminen (2018).[140]

2.4 The ultrahigh-resolution orbitrap-mass spectrometric analyses

A UPLC-DAD-Q-Exactive-Orbitrap-MS instrument was used to conduct the ultrahigh-resolution mass spectrometric analyses. The device included an Acquity

UPLC system (Waters Corp., Milford, MA, USA) and a quadrupole-Orbitrap hybrid mass spectrometer (QExactive™, Thermo Fisher Scientific GmbH, Bremen, Germany). An Acquity UPLC BEH Phenyl column (2.1 × 100 mm, 1.7 µm, Waters Corp., Wexford, Ireland) was used as the stationary phase. For the mobile phase, UPLC-MS grade acetonitrile and 0.1%formic acid were used as eluent for A and B, respectively. The following elution profile was generated with a flow rate of 0.5 mL min^{-1}: from 0 to 0.5 minutes, pump 0.1% A in B (isocratic); from 0.5 to 5 minutes, pump 0.1 to 30% A in B (linear gradient); from 5 to 6 minutes, pump 35% to 90% A in B (linear gradient); from 6.1 to 9.5 minutes, wash the column and re-equilibrate the system. The sample amount injected from the vial was 5 µL. UV (at 190–500 nm) chromatogram and MS data were collected throughout the experiment. Ionization parameters were as follows: Negative ionization was applied in the HESI source with a spray voltage of -3.0 kV, sheath gas (nitrogen) flow rate of 60, auxiliary gas (nitrogen) flow rate of 20, sweep gas flow rate of 0, ion transfer tube temperature of +380 °C, and 30 eV in-source collision-induced dissociation (CID). For full scan MS, the orbitrap mass range was *m/z* 150-2250, the resolution was 35,000, and the automatic gain control (AGC) was 3×10^6. For MS/MS analysis, dd-MS2 (TopN) acquisition technique was used. The parameters were as follows: TopN 3, Normalized stepped collision energy 20, 50 and 80 eV, resolution 17,500 and AGC 1×10^5. Pierce ESI negative ion calibration solution (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used for calibration. Thermo Xcalibur Qual browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to analyze the MS data. Since the oxidized samples were collected in a sodium carbonate buffer (pH10), sodium generated formic acid cluster ions during the UHPLC-MS/MS analyses. For this reason, a prominent peak of sodium formate clusters was always seen at the starting of each total ion chromatogram of oxidized samples (Article II, Table S9). The peak was rapidly identified and did not influence the PAs analyses and interpretation.

2.5 *In vitro* antiparasitic activity tests

2.5.1 Preparation of *Ascaris suum* larvae

Embryonated *Ascaris suum* eggs were obtained from fresh pig intestines (Danish Crown, Ringsted, Denmark). The third-stage hatching of *Ascaris suum* was conducted according to Williams et al. 2014 & 2016. Sodium hydroxide was used to decoat *Ascaris suum* shells, deposited in a 1 M H_2SO_4 solution at +4 °C. The eggs were hatched by washing them 3-4 times with Hank's Balanced Salt Solution (HBSS) and gently grinding them at +37 °C with glass beds, 5 % CO2 for 40-60 minutes in the air. The larvae were then incubated in a Baerman funnel overnight, dissolved at

37 °C in sterile HBSS, 5 % CO2 in air conditioning. L3 stage larvae were then removed from the unhatched egg waste. The larvae were then rinsed and placed in larval culture media (RPMI 1640 with 2 mM L-glutamine, 100 U/mL penicillin, and 100 g/mL streptomycin) for the *in vitro* antiparasitic activity test.

2.5.2 Larval migration inhibition assay

The larval inhibition assay was conducted as previously described.[49,81,137,141] Briefly, the L3 stage larval concentration was set at 0.69 U/mL, and 145 µL of larval suspension was applied to each well of a 96-well tissue culture plate, taking an average of 100 larvae per well. In order to mix the PA rich plant extracts fully soluble, freeze-dried extracts were first dissolved in 200 µL of 50% DMSO (DMSO/water, (50/50, v/v)). The migration inhibition activity was then tested using a 10 fold dilution of this solution. In order to conduct the assay, 5 µL of sample solution were added to the culture plate and thoroughly mixed with the larval suspension. The 50 % DMSO and pH10 buffer sample dissolving solvent were used as negative controls and the 200 µg/mL levamisole drug as a positive control. To observe the control stability and optimize each 96-well plate's larval assay, an internal standard oenoethin B (from 5 mg/663.87 µL stock solution) was used. The plates were then left in an incubator at 37 °C with 5% CO2 for the next day. A direct light microscope was used to count larval migration from the agar's top on the next day. For the larval migration analysis and larval count, the well plate was stored at room temperature for a maximum of 15 minutes so that the larvae would not die before the calculation. Larval migration inhibition activity (MIA %) was obtained for both positive and negative control by comparing the total number of larvae with the percentage of migrated larvae.

The migration inhibition activity (MIA %) was determined using the control sample's minimal and maximal migration calculations. Using the following equation in brief:

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

2.6 Statistical analyses

All the correlations and R2 values shown in Article I were obtained using a linear regression model created in Microsoft Excel. The statistical analyses were conducted in Article III by a two-way analysis of variance with sample and oxidation status as variables. Both oxidized and non-oxidized samples were processed for multiple

comparison testing and multivariate data analysis. The clustered and variable correlations were also tested using principal component analysis. GraphPad Prism was used to conduct all statistical data analyses (v9.00, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

3 Results and discussion

3.1 Modification of natural proanthocyanidins as observed by UHPLC-DAD fingerprints

The chromatographic hump of various oligomeric and polymeric PAs were identified by the UHPLC-DAD analyses using a photo diode array ultra-violet (UV) detector at λ=280 nm. Due to the complexity of plant extract´s PA oligomers and polymers, UV absorbance at 280 nm showed a large hump between retention times of 2-6 minutes rather than a sharp peak. Preliminary analyses on 300 plant species revealed that only 102 extracts contained high levels of procyanidin and prodelphinidin oligomers and polymers. These extracts were then tested for high-pH oxidation experiments. Different plant species contained varying quantitative characteristics of total PA, PC/PD ratio, and mDP in both original and oxidized extract. **Figure 6** is an example of two different types of UV chromatograms before and after oxidation at high pH. It shows that in the non-modified sample, UV fingerprint of PA polymers is stable with no changes in PA concentration **(Figure 6 A)**. However, in the modified sample **(Figure 6 B)**, the UV fingerprint of PA polymers is shifted to a higher retention time with no changes in PA concentration. The details describing before and after the samples' oxidation, the PAs content, mDP, and possible galloylations found in each sample were presented in Table 1 in Article I. In general, oxidation reduced the concentration of PAs, or the hump disappeared after the incubation in alkaline conditions. However, the UHPLC-DAD measurements after the oxidation at pH 10 showed that some specific PAs were not modified at all. In contrast, other types of PAs were modified in multiple different ways.

After the initial determination and integration of the total chromatographic PA hump area from the UHPLC-DAD profile before and after oxidation, the examined plant extracts were separated into four different categories based on their UV absorbance area and modification pattern. These categories are (A) non-modified PAs without a clear loss of PA concentration; (B) non-modified PAs with a clear loss of PA concentration; (C) modified PAs without a clear loss of PA concentration;

and (D) modified PAs with a clear loss of PA concentration. Examples of the chromatograms of PAs in each category are shown in **Figure 7**.

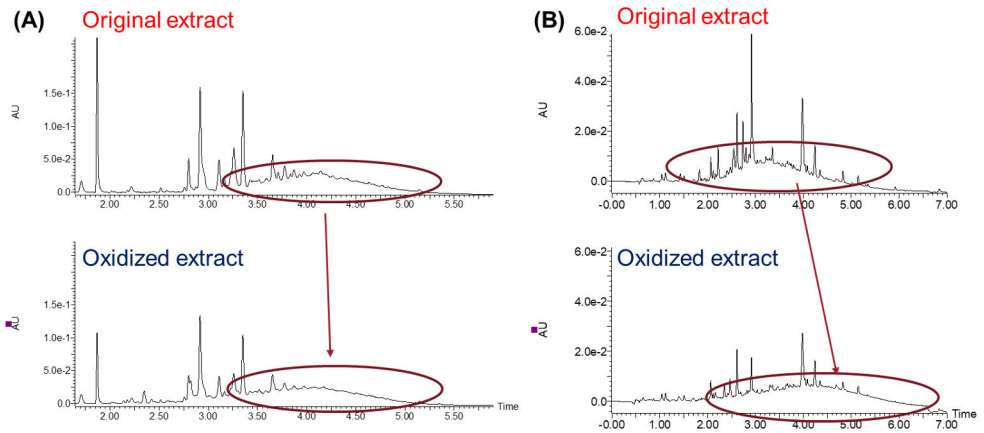


Figure 6 Effect of oxidation of PA oligomers and polymers at high pH. (A) Examples of the UV chromatogram of non-modified sample, where UV fingerprint of PA polymers is stable, no changes in PA concentration. (B) Examples of the UV chromatogram of modified sample, where UV fingerprint of PA polymers is shifted to higher retention time, but not changes in PA concentration.

In category (A), it was clearly seen that the area of the PA hump of *Heritiera solomonensis* leaflets was the same before and after oxidation, indicating no structural changes or modification after oxidation. The total areas of the PA humps of the non-oxidized and oxidized samples were identical, and PAs elute in the same retention time scale in both cases. In category (B), the area of the PA hump of *Coffea arabica* flowers was decreased after the oxidation. However, their PA hump was not changing retention time-wise, suggesting no changes after oxidation for the PA oligomer and polymer structures.

On the other hand, in category (C), the area of PA hump of *Newtonia buchananii* leaves was identical before and after oxidation, but the hump was shifted to the right in the chromatogram; the top of the hump shifted from 4.0 min to 4.5 min, suggesting structural changes in the PAs. Finally, in category (D), the PA hump of *Aeonium arboreum* flowers was reduced after the oxidation. All oxidized samples are shown in Table 1 in Article I with the categories A-D together with the quantitative PA data.

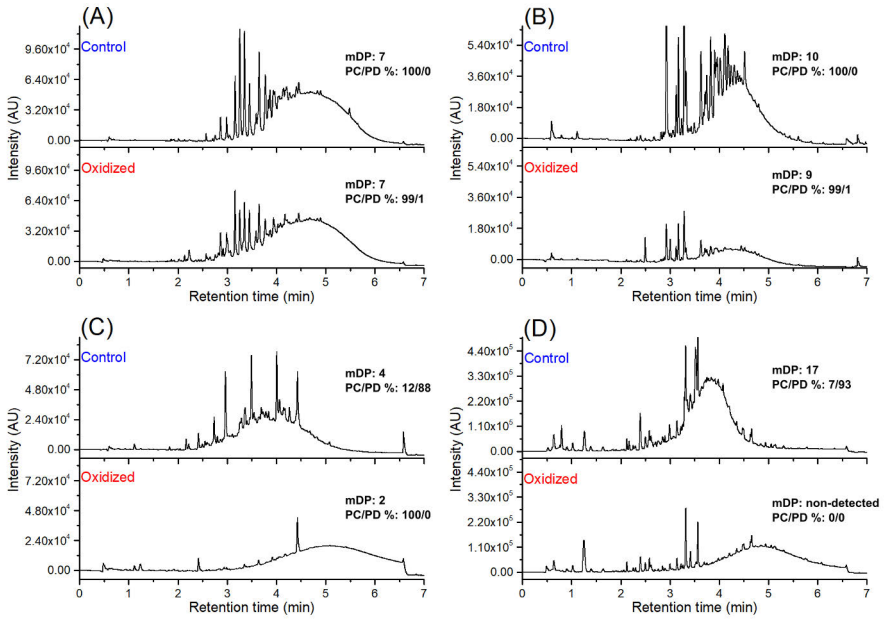


Figure 7 The UHPLC-DAD chromatograms (λ = 280 nm) of PA-rich plant extracts showing four different categories depending on their modification pattern where (A) non-modified PAs without a clear loss of PA concentration; (B) non-modified PAs with a clear loss of PA concentration; (C) modified PAs without a clear loss of PA concentration; and (D) modified PAs with a clear loss of PA concentration. The upper panel representing the non-oxidized control samples and lower panel represent the oxidized samples under alkaline condition. In each chromatogram, they are also showing the corresponding quantitative PC/PD ratio and mDP values. The figure is adapted and modified from Imran et al. 2021 (Article I, Figure 2).

3.2 Oxidation mediated modifications in the four categories revealed by UHPLC-DAD-MS/MS analyses

The results of the UV chromatograms and the MS/MS quantitative analyses revealed that the reduction in the PA concentration was significant after the oxidation of all studied samples. At the same time, the UV peak area changes were diverse. The MS/MS data also indicated that most of the samples were reactive when exposed to alkaline conditions. The reactivity of certain PAs was higher when the PD percentage was higher in the extracts. In the previous study,[119,142,143] results indicated that PD-rich samples were more reactive than those with PC-rich samples when exposed to high pH. The findings of current study also support the idea that the oxidation reaction was regulated by the amount or percentage of PD present in some extracts.

This oxidative modification revealed that the effects of various structural modifications on the UV absorbance of PAs were less evident than those of quinone methide fragmentation. It has also been observed that the fragmentation of the PD and PC units from different oxidation sites was more uniformly affected by the oxidative structural modifications. Eventually, the MS/MS fingerprints and UV data were compared across four categories and within individual categories to determine how reactive and stable the samples were.

The results of the study revealed that every sample analyzed for the A category had a high proportion of PC and a low proportion of PD, with a PD percentage of only 2% for some samples. Also, the increase or decrease in the mDP in a few samples was not more than two units. The data also showed varying levels of reactivity and resistance to certain PC and PD units. For example, after oxidation, the number of unmodified units in the high mDP samples was slightly higher than in the other samples. These results support the idea that the various types of UHPLC-MS/MS fingerprints exhibited high stability when subjected to alkaline conditions. However, in some samples, the shape or intensity of the MS/MS fingerprints shifted. **Figure 8A-C** shows few MS/MS fingerprints of A category samples observed in the analysis.

On the other hand, the stability of the mDP remained the same. This phenomenon can also happen in two ways: either the number of terminal and extension units decreases completely, or the formation of longer and shorter polymers and oligomers does not change. To determine the exact cause of this phenomenon, samples were compared with the MRM detections of these two units individually. The results indicated that the rate of reduction in the MRM's detection in the terminal and extension unit was similar in the majority of the samples. In most cases, the difference in the mDP of an extension and a terminal unit is just one or two units.

Most of the samples in category B had high percentages of PC, but the decreases in MS/MS data and UV areas were different from those found in the category A samples. The variable in the total decrease in the UV areas ranged from 21 to 96 percent, although the reduction in the PA content (MS/MS) differed significantly. Compared to category A, where the changes in the mDP were more consistent, the changes in the category B samples' mDPs were less apparent. They also exhibited a significant increase in the mDP after oxidation. However, all samples with detectable PDs showed substantial decreases in their mDPs. This suggests that the presence of PDs increases the reactivity of the samples. **Figure 8D-F** shows a few MS/MS fingerprints of some B category samples observed in the analysis.

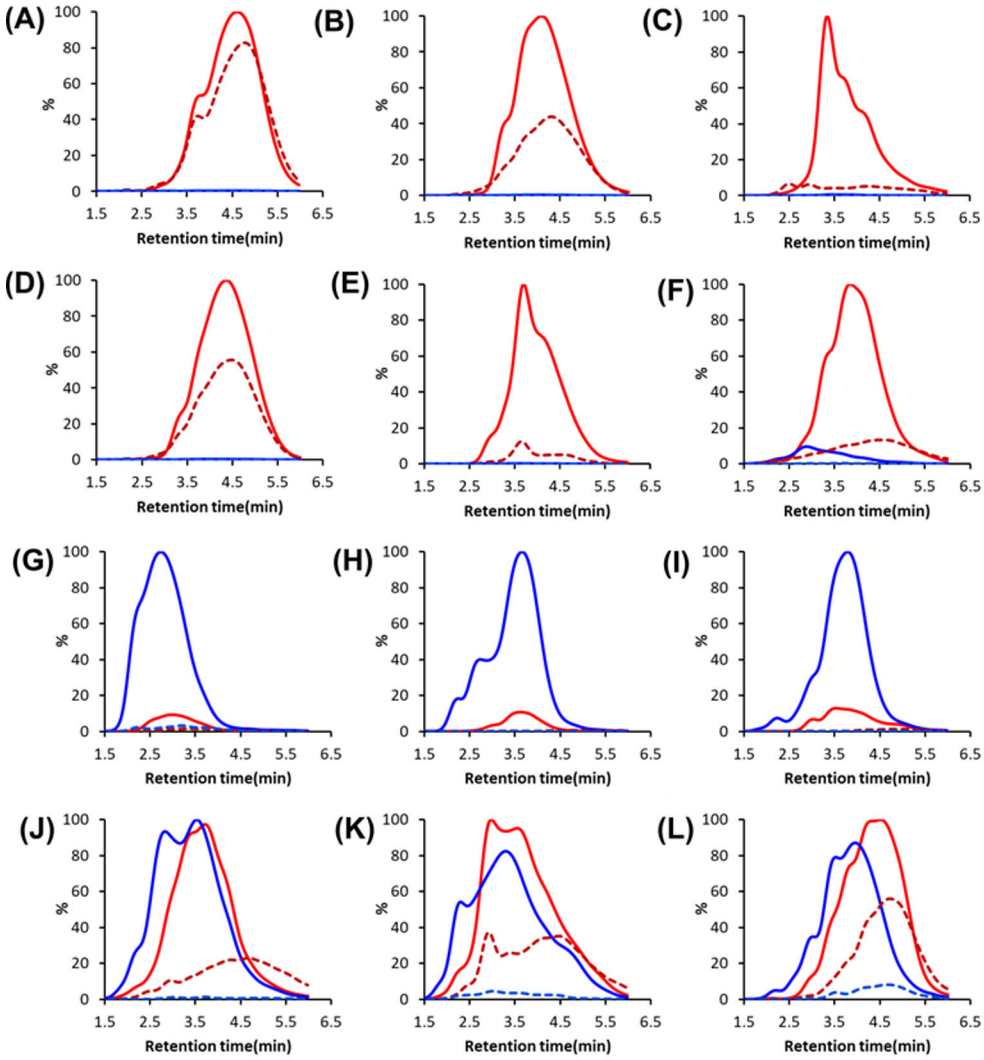


Figure 8 A few illustrations of the UHPLC-DAD-MS/MS fingerprints of various plant extracts are presented before and after oxidation. Solid red lines are PC units, blue lines are PD units, and dashed red and blue lines represent PC and PD units following oxidation. Showed plant species are: (A) *Mandevilla splendens* leaves, (B) *Alpinia purpurata* leaves, (C) *Camellia japonica* leaves, (D) *Encephalartos ferox* leaflets, (E) *Aglaonema crispum* leaves, (F) *Acca sellowiana* leaves, (G) *Callisia gentlei* leaves, (H) *Aeonium arboretum* flowers, (I) *Kalanchoë manginii* flowers, (J) *Acacia melanoxylon* leaflets, (K) *Tetraclinis articulate* pieces, (L) *Leea guineense* leaves. The figure is adapted and modified from Imran et al. 2021 (Article I, Figure 3 and 4).

On the other hand, the sizes and shapes of UHPLC-DAD chromatographic PA humps were different in category C and D samples due to their elution time shifting and concentration decreased. These samples had high concentration of oxidation,

which led to a decrease in MS/MS quantitative data. In some of the samples, it was observed that the relative decrease was not detected in the UV peak areas in the current method. The presence of controlled oxidation reactions supported the MS/MS data in this category of samples. In addition, the number of mDP units decreased by two and seven, indicating that numerous structural units were involved in the reactions. When the mDP was high enough for non-oxidized samples with large oligomers and polymers, the large molecules were delayed in their fragmentation and ionization. This is because the high degree of polymerization contributes to the late retention times. **Figure 8G-L** shows some of the MS/MS fingerprint results on various samples from C and D categories, wherein the high PD share of the samples, both PDs and PCs, decrease considerably. All of the observations mentioned above, correlation data, and quantitative measures are presented in detail in Article I Table 1, Figure 4-5, and the supplemental information Figure S3-S5.

3.3 Possible oxidative modification reaction routes observed by UHPLC-DAD-MS/MS

Previous research suggests that there are two types of PAs oxidation reactions: intramolecular and intermolecular.[38,117,129,134] The intramolecular interactions between different PAs are involved in rearrangement reactions,[144] including the formation of new connections between the monomeric units. The effects of epimerization and A-type linkage formation on UHPLC-DAD absorbance are not as apparent as they would be if the PAs were oxidized.[144] Nevertheless, depending on the types of PAs in the sample, the consequences of these variations on retention times can be substantial.[144,145]

On the other hand, the Engström method used in Article I relies on detected MS/MS data to identify the ions formed by quinone methide cleavage. In this method, epimerization would have minimal effect on PA detection since the detection effectiveness of single PAs is averaged over multiple cone voltages. However, other rearrangement processes and link creation, such as A-type PA converted from B-type PAs, might affect PA detection and hence quantification. The mDP decreases if the extension unit gets damaged and increases if the terminal units are affected. The effect of the damage on mDP is constant if both units decline at about the same rate. According to previous studies,[38,117,129,134] the intermolecular reactions of PAs can affect the structure of the compounds. When this reaction takes place, a linear structure is formed in the terminal-to-terminal linkage, and a branching structure is formed in the terminal-to-extended linkage. On the other hand, if the PA units are not detected by MS/MS analysis, the resulting mDP is likely to

exhibit very minor alterations. This phenomenon occurs when the interflavanyl bonds are sensitive to pH 10. Eventually, it also adds to the complexity of the intermolecular reactions. Table 1 in Article I, shows some examples of these kinds of samples found in the current investigation.

In article I, it was discussed how different data were obtained from various analyses used to characterize PAs, including the DAD, the UHPLC-MS/MS, total ion chromatogram, and full scan data. The PC dimer for A and B category samples was the most common product, with fragments at *m/z* 575, *m/z* 449, and *m/z* 423. Contrarily, identified PAs in categories C and D showed evidence of modifications to the UV humps. Due to the decreased efficiency, several novel compounds were formed with higher mDP levels. The intermolecular and intramolecular oxidation processes were also performed in multiple ways in these samples. The low concentration and insufficient ion efficiency of individual PAs prevented them from being adequately characterized during the data processing in Article I. Therefore, in article II, an ultrahigh-resolution Mass Spectrometer was used to identify the potential reaction pathways of oxidized and non-oxidized PAs.

3.4 Modified and non-modified PA structural details revealed by high-resolution MS/MS data

The 55 plant extracts (Article II) were selected based on their complex natural PAs identified from the article I. They were then similarly oxidized in alkaline condition. The complexity of the PA compounds (i.e. PC/PD structural combination and their degree of polymerization) in the oxidized extract was much higher than those in the original samples. In order to determine the detailed changes to the PA structures, a high-resolution orbitrap mass spectrometer was used with a UHPLC-DAD apparatus. The studied method was able to detect the smallest details of the structures by looking at exact mass, chemical formulas and singly or multiply charged ions. Since other phenolic compounds were more strongly ionized than PAs, the PA hump was less noticeable in the total ion chromatogram. However, as orbitrap-UHRMS is so accurate, it was easier to determine the MS findings by precisely confining the isotopic patterns of multiple charged *m/z* for the oligomeric and polymeric PAs. Article II, figure 2, shows an example of this identification phenomenon.

The comprehensive description of the identified PAs in each plant gave so much mass spectral information. In article II, supplementary Table S8 contains LCMS profiles, i.e. the UV (280 nm) and total ion chromatograms of non-oxidized and oxidized extracts of each plant species. In addition, a brief discussion of each PA composition, as well as the variations between natural and alkaline-oxidized PAs were documented. The MS/MS fragmentation of all the PAs with a single molecular

ion that have been characterized by the TopN technique are shown in article II supplementary table S10.

Both B-type and A-type PCs were identified in the original (non-modified) plant extract. These were identified by their distinctive fragmentation patterns and single or multiple charged molecular ions. For instance, in PC-rich samples, a sequence of $[M–H]^-$ ions at *m/z* 289 to *m/z* 1729 and $[M–2H]^{2-}$ ions at *m/z* 1008 to *m/z* 1296 was observed. These ions were separated by 288 Da and 144 Da, respectively, and which were referring to B-type PCs monomer to hexamer and B-type PCs heptamer to monomer. In addition, the mass spectra of epicatechin and catechin showed a distinct fragmentation ion at *m/z* 245.[53,55,118] The MS/MS analyses showed that the most common QM fragmentation patterns were at *m/z* 287 and *m/z* 289, and the most common RDA fragmentation patterns were at *m/z* 425 and *m/z* 407. This form of fragmentation is likely to assist the extension unit.[53,55,56] The fragmentation pathways of these B-type PC dimers of original plant extracts are shown and discussed details in article II, Figure 3. A-type PCs were also elucidated by their fragmentation pattern similarly to B-type PCs. The most common A-type fragmentation pathway of PC dimer and trimer are shown and discussed in details in article II, Figure 4 and 5, also compare the interpretation with previous studies done with the pure PC compounds.[37,42,46]

On the other hand, in modified samples, different B-type PC extracts were found to be changed differentially after oxidation. Some of the samples had no or minimal changes. In the case of small PC oligomers, a minor modification was seen. In **Figure 9 A**, show that after the extract was oxidized, the B-type PC oligomers were not modified. The total ion chromatograms of the original and oxidized plant extracts showed no noticeable difference. When checking the spectra by zooming, the PC dimer at *m/z* 577 was found to be paired with *m/z* 575, and the PC trimer at *m/z* 865 was paired with *m/z* 863. However, these non-modified structural features were only detected by the high-resolution mass spectra. Although, the modification was clearly visible in the mass spectra with 2 Da changes in some other oxidised samples. For example, in **Figure 9 B**, the mass spectra show that the shorter B-type oligomers had a 2 Da mass change compared to the original PCs.

In other cases, the signals from B-type PC oligomers disappeared after they had been oxidized. This suggests that they have become unidentified or degraded. This phenomenon was also observed in article I while using the low resolution UHPLC-DAD-MS/MS analyses. The effect may be related to the experimental conditions utilized in the analyses. For instance, the low starting PA levels may have led to intense activity.

Moreover, many samples also included the PC/PD combinations, which have a distinct destiny following alkaline oxidation. Unlike PCs, these combinations have a distinct fate. For example, UV and MS analysis of *Podocarpus macrophyllus* leaf

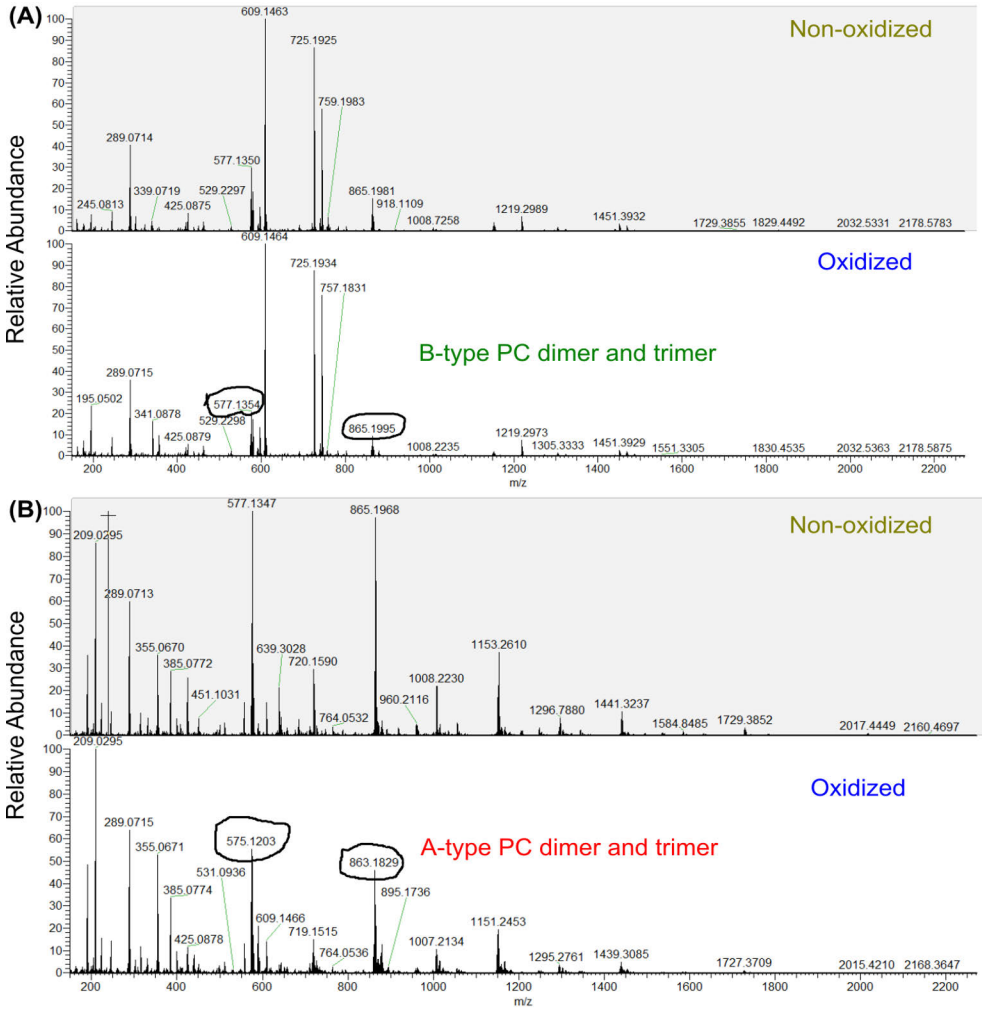


Figure 9 The full scan total mass spectra of non-modified category samples. In the figure, (A) *Begonia bowerae 'Nigra'* leaf extract and (B) *Alpinia purpurata* leaves extract exhibit the B-type and A-type PC oligomer formation after oxidation.

extract revealed the presence of small PA oligomers **(Figure 10)**. However, MS was unable to detect these tiny oligomers after oxidation, although they were still present in UV data at 280 nm **(Figure 10 A)**. No additional signals from the changed PC/PD combinations or their breakdown products were detected in MS data. These modified PAs were not identified in this study **(Figure 10)** by existing orbitrap-ESI-HRMS settings for PAs analyses.[135] Since the composition of the PAs subunits is modified after alkaline oxidation, the group-specific MRM method in QqQ-ESI-MS conditions also could not reveal these small structural details.[135]

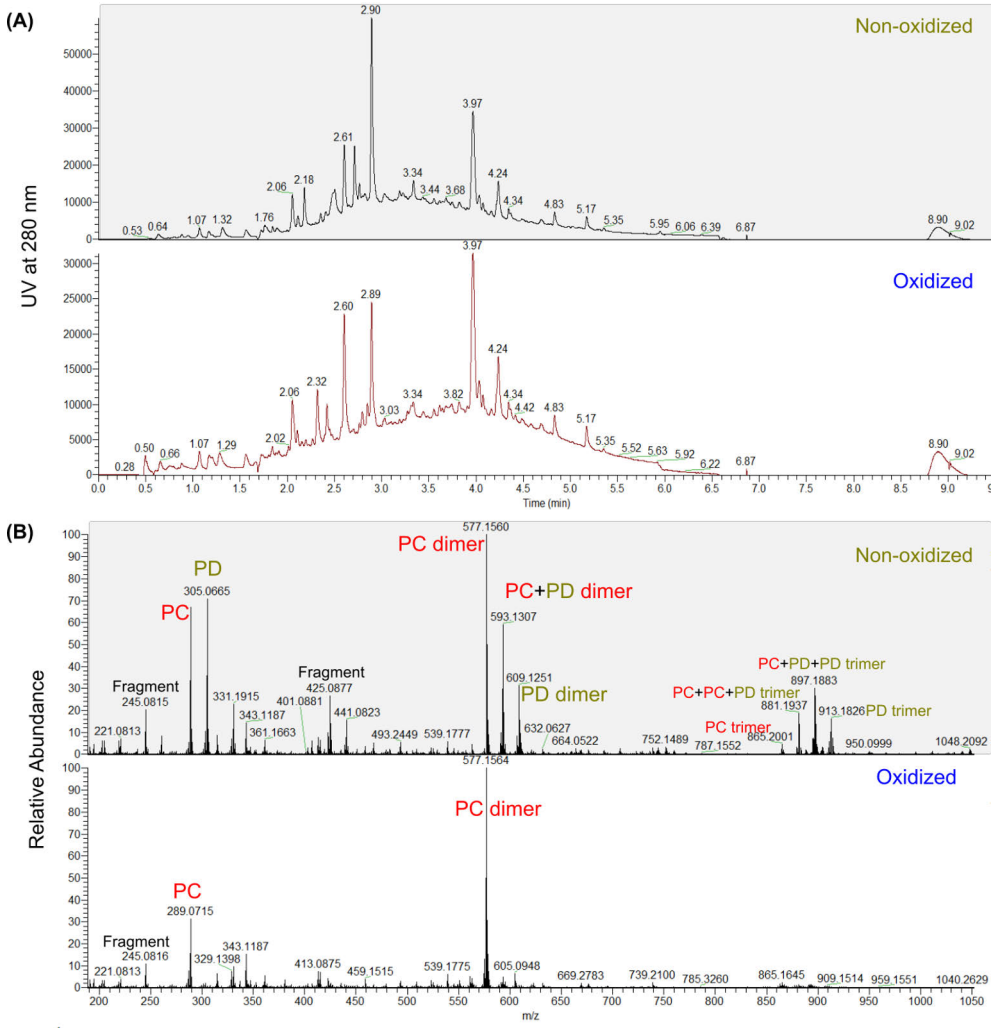


Figure 10 UV and MS data of *Podocarpus macrophyllus* leaf extract revealed the presence of small PA oligomers. UV and MS data of Podocarpus macrophyllus leaf extract revealed the presence of small PA oligomers. In the figure (A) UV fingerprint of both non-oxidized and oxidized sample observed at 280 nm, (B) Mass spectra of non-oxidized and oxidezed sample shows the B-type PC, PC/PD oligomers in the non-oxidezed sample and after oxidation only PC stable and PD disappear.

Intermolecular interactions between PAs could explain why visible PC/PD oligomers disappear in ESI-MS.[45,115,117,129,134,145] Intermolecular interactions between two or more oligomers can lead to the production of modified PAs. This process can enhance the overall mean degree of polymerization.[38,128,128,130,134] However, the techniques utilized in the literature for the oxidation process of PAs differ significantly from this studied method, the findings may not be directly comparable. Previous studies have used water acidified with trifluoroacetic acid to approximate

wine pH and stirred the sample in air for many days to produce oxidation.[129,131,134] On the other hand, in this study a carbonate buffer of pH 10 was utilized for one hour.[135,136]

3.5 Structural modification of B-type and A-type PAs in plant extracts due to alkaline oxidation

In mass spectrometric analyses, oxidation can also occur. The orbitrap high-resolution mass spectrometric analysis technique makes it easier to observe this phenomenon. During ionization, PCs can oxidize or produce quinone forms, resulting in little signals in the mass spectra that are 2 Da smaller than PCs. In this study, the fragment ions of modified PCs were compared with those of A-type PCs in B-type PC dimers, which revealed strong similarities. Article II, Figure 8 shows that modified PCs exhibit distinct fragmentation patterns that can result in the formation of ions at *m/z* 285 and *m/z* 289 (QM cleavage), *m/z* 423 (RDA fragmentation), and *m/z* 449 (HRF fragmentation). The data collected during the study were qualitative, so they cannot be directly compared with the ion abundances of other A-type and B-type PC oligomers. However, rough estimates indicated that the relative abundances of ions in both types of oligomers were similar. It was proposed that combining B- and A-ring quinone-methides at high pH might be used to produce various flavan-3-ols, such as dimeric and monomeric molecules.[117] These compounds then induce the oxidation of B-type molecules and lead to the conversion of these to A-type PAs. **Figure 11** shows the mechanism by which this happens.

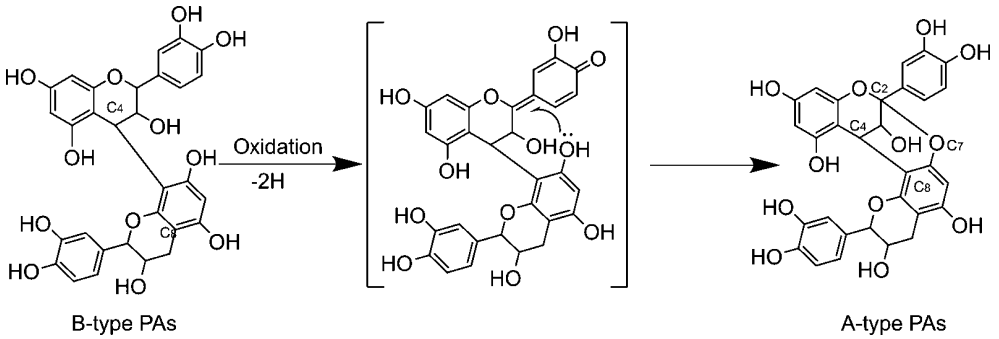


Figure 11 The suggested way to modify proanthocyanidins by oxidation. The mechanism indicates the oxidative conversion of B-type to A-type procyanidin dimer (Ferreira et al. 1992).

In addition, the effects of alkalizing A-type PCs on the mass spectrum varied. For instance, some samples exhibited minimal changes while others had significant differences (for example, Article II, Supplementary Table S3). After alkaline oxidation, the total mass spectrum of some plant extracts showed no notable changes.

However, in the data of some samples, mass differences of 2 Da were detected. This phenomenon, which was explained in detail in article II, Figure 9 A-D, was caused by the formation of additional A-type ether linkages.

3.6 Effect of galloylation to the alkaline oxidation in plant extracts

A galloyl hump was detected in a few of the plant extracts using galloyl-specific MS/MS analyses. The Galloylated PAs extracts showed different characteristics before and after oxidation at high pH. Due to the structural modifications, the fragmentation pathway of the quinone methide was hindered. The initial galloyl compounds were modified to allow for the detection of the galloyl groups, but the PC and PD units could not split them. Previous investigations[146–149] it has been shown that the high levels of galloyl PAs in the extracts can lead to higher oxidation rates than those produced by non-galloylated PAs. This phenomenon is supported by the reduction in the MS/MS levels and the UV peak areas. This phenomenon was shown and explained in detail in Article I, Figure 5.

In article II, the existence of galloylated PCs, galloylated PDs, and galloylated PC/PDs in plant extracts was verified by observing the fragment ions of these compounds. The detailed identification and fragmentation patterns are explained in Article II, Figure 11 and supplementary Table S7. In general, the various types of galloylated PAs exhibited similar fragmentation patterns (RDA, HRF, and QM processes) in MS analysis. Under alkaline conditions, various galloylated PAs reacted differently. For example, galloylated PCs-rich extracts were relatively stable after oxidation, but the galloylated PDs or PC/PD mix extracts were found to be more reactive. However, in some cases, adding two or more galloyl groups to galloylated PCs increased reactivity compared to those single containing galloyl groups. On the other hand, galloylated PDs showed similar reactivity as non-galloylated PDs. These PAs were not detected by MS/MS after oxidation, although being detectable at UV at 280 nm. There were no other signals that may have been associated with these modified PAs or their potential degradation products.

3.7 *In vitro* antiparasitic activity of natural PA-rich plant extract

The selected 61 plant extracts (Article III) were analysed by the migration inhibition assay to evaluate the *in vitro* antiparasitic activity. To compare the larval migration with the positive control, both DMSO and pH10 buffer were utilized as negative controls. Levamisole was used as a positive control (widely available anthelmintic drug). In addition, oenothein B was employed as an internal positive

control throughout the experiment. All the negative and positive controls demonstrated steady action throughout the investigation, confirming the method's reproducibility and accuracy. The results show that most PA-rich plant extracts had an antiparasitic effect **(Figure 12A)**; among them, 47 plant extracts had antiparasitic activity ranging from 2% to 90%. Interestingly, plant extracts from 14 species either had no effect or increased larval movement. On average, these samples included a significant quantity of tannins, with over 21 plant extracts showing 50% antiparasitic activity. In fact, the PA concentration in these samples ranged from 0.1 mg/g (*Rhus coriaria bark extract*) to 140.2 mg/g (*Schinopsis balansae* bark extract), indicating that the strongest antiparasitic activity was not always associated with the highest PA level.

More detailed quantitative results of all studied plant species could be found in Article III, Supplementary materials, Table S2. Five samples showed PAs less than 10 mg/g: *Camellia japonica* petal extract, *Rhus coriaria* bark extract, *Impatiens repens* leaf extract, *Acacia penninervis* bark extract, and *Crassula portulacea* leaf extract (Article III, Supplementary materials, Table S1). Also, the PA content of the extracts did not immediately correlate with their anti-parasitic activity. The mDP of PAs in the extracts was shown to be uncorrelated to their antiparasitic potency.

The most active plant extracts found to be PC-rich (i.e., *Sarracenia purpurea* leaf extract) or PD-rich (i.e., *Rhus coriaria* bark extract with only PDs or *Cyclamen africanum* flower extract with PC/PD ratio of 9/91), or PC/PD mixtures (i.e., *Tontelea richardii seed extract* with 44/56 PC/PD ratio or *Cupressus bakeri* branch extract with 50/50 PC/PD ratio) (Article III, Supplementary material, Table S2). PAs with high mDP and high PD-share have been demonstrated in the previous study to have increased *in vitro* antiparasitic activity.[71,72,88,92,150] However, in this study, it does not tell the same story as other studies that used purified PA fractions in various *in vitro* experiments and different nematodes, such as adult motility tests with *Ostertagia ostertagi*[150] and *Cooperophora oncophora*[150] and third stage larval exsheathment inhibition tests with *Haemonchus contortus*[89,91,151] and *Trichostrongylus colubriformis.*[91] When looking for the samples that had no antiparasitic activity or increased larval migration moderately, there was no correlation found between PA total content, mDP, or PC/PD ratio and activity test result. In terms of PA content, it ranged from 1.4 mg/g to 76.4 mg/g, with PC/PD ratios ranging from 100% PC to 82% PD share. In 12 samples, PAs made for over 80% of all polyphenols, while in *Dimocarpus longan* and *Doliocarpus olivaceus*, only 30% and 64% were PAs.

3.8 *In vitro* antiparasitic activity of the alkaline oxidized extract

The antiparasitic activity of oxidized PAs was evaluated to see if it differed from non-oxidized extracts. The antiparasitic activity of the oxidation buffer was also investigated. In Article III, Supplementary materials, Figure S22 shows the comparison of the mean inhibition activity of non-oxidized and oxidized extracts. The oxidation of PAs enhanced the antiparasitic activity of the extracts in most cases. The findings of a two-way analysis of variance on all investigated species revealed that both sample (original plant extract) and oxidation treatment (non-oxidized vs oxidized) contribute to the data set's variation (data shown in Article III, Supplementary materials in Table S3). The results indicated that the modification of the PAs through oxidation increased their antiparasitic activity. In order to make the results easier to understand, the samples were divided into oxidized and non-oxidized groups (see **Figure 12B**) and for MIA increase/decrease in Article III, Supplementary materials, Table S1). For instance, 72% of the samples exhibited a substantial difference; the inhibition activity is higher in the oxidized sample. Similar graphs were made for all of the variables revealed in this study (see Article III, supplementary materials Figures S3-S21).

3.9 Link of other phenolics to increase or decrease the antiparasitic activity after oxidation

In Article III, the effects of various tannin-related factors, such as the mDP of PAs, PA contents, PC-unit contents, PD-unit contents, HT contents, galloyl derivative contents, HHDP derivative contents, total tannin contents and HT share of all tannins were investigated (See Article III, supplementary materials, Figure S4-S13). There was no discernible pattern found in these tannin-related variables. Moreover, other phenolic compounds' effects on MIA variations before and after oxidation were also investigated. Neither the flavonoid share of all polyphenols nor the amount of quinic acid derivatives in relation to all polyphenols showed significant trends following alkaline oxidation (see Article III, Supplementary materials Figure S14-S21). Table S3 in the supplementary material of Article III contains detailed quantitative MS/MS data showing the modifications caused by oxidation.

The MIA changes after oxidation **(Figure 12 B)** were the greatest for *Pinus sylvestris* inner and outer bark extracts that had no detectable PAs. However, it has been identified that *Pinus sylvestris* contains a high concentration of PCs oligomers and polymers.[55] Since the bark sample was purchased commercially, it is likely that something happened to the commercial pine park material, and the sample is not representative of the species. So the changes must be due to other substances in the extract.

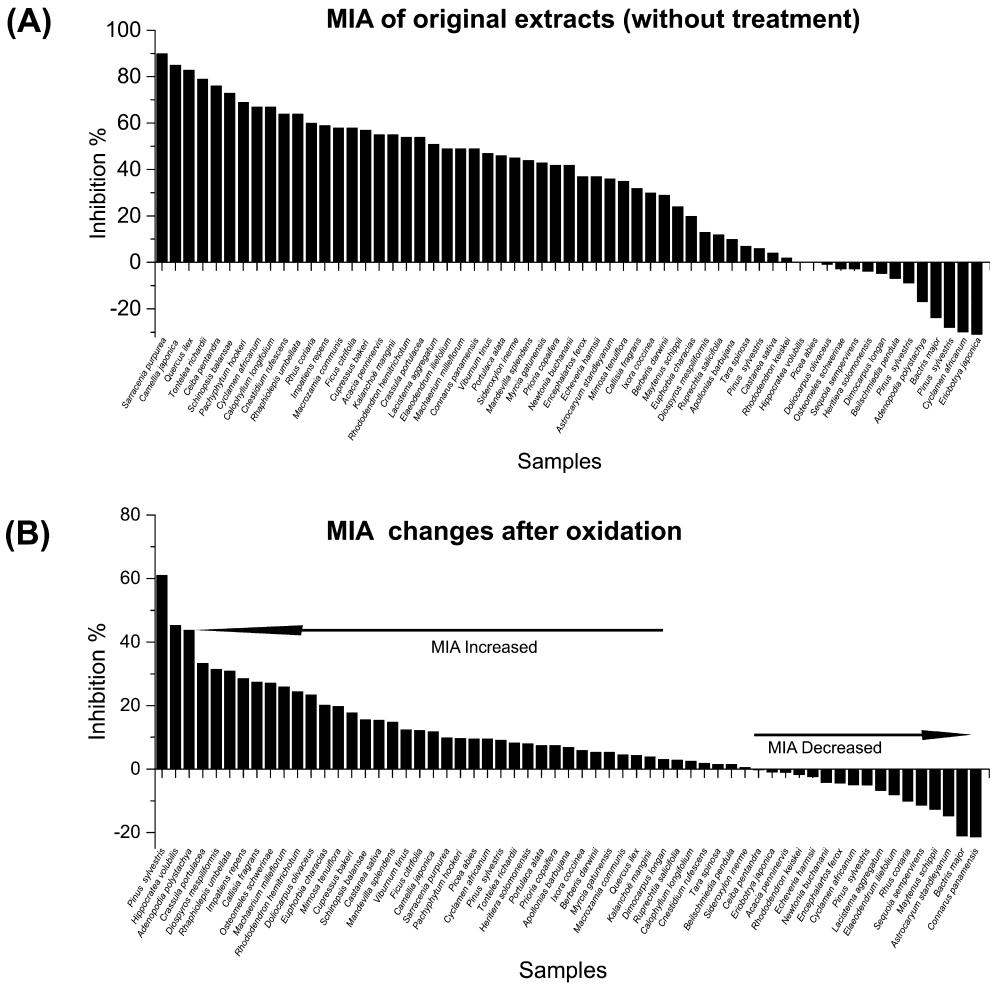


Figure 12 Mean inhibition activity of 61 studied plant species. (A) original plant extract without alkaline treatment and (B) categorized samples after oxidation based on the difference in inhibition percentage between non-oxidized and oxidized samples. The figure is adapted and modified from Imran et al. 2023 (Article III, Figure 2 & 3).

Another major change was seen in *Hippocratea volubilis*, which had little antiparasitic effect prior to oxidation. Increasing antiparasitic activity is surprising. The first *Hippocratea volubilis* extract has modest levels of PAs (PC and PD) (Article III supplementary materials Table S2). The composition of detectable PAs remained the same after alkaline oxidation, although the PA concentration increased (Article III supplementary materials Table S3). Alkaline oxidation increased the concentration of HHDP compounds in the plant extract *Hippocratea volubilis*, which had less HHDP before oxidation. As a result, these increased HHDP derivatives may explain the observed increase in inhibition activity. Moreover, under alkaline

conditions, PDs were more sensitive to modification, as previously observed in articles I and II. In fact, PDs were generally modified in article III investigation (See Article III, supplementary materials Table S3), for example, in the case of *Crassula portulacea*, the modification improved the extract's antiparasitic activity. Although oxidation boosts the antiparasitic activity of PA-rich extracts in general, the change is always dependent on the other compounds present and their transformation.

4 Summary/Conclusions

The main objective of this study was to screen various PA-rich plant extracts to get an overall understanding of how different types of PAs respond to alkaline conditions. The samples were then divided into various categories based on their total PA contents and PC/PD ratio and mDP by using UHPLC-DAD-MS/MS method. The first part of the study serves as a screening step to investigate the various types of plant-based PAs that can tolerate the harsh conditions. It also helps in our understanding of the various compositions and properties of these PAs. The concentrations of PAs were not measured individually but instead were compared to the percentage decrease of terminal units versus extension units of the same category samples. As for the reactivity of the compounds, focus has been given by looking at the drop in UV peak areas and changes in the retention time by comparing before and after oxidation of the sample. After oxidation, the main reaction route in most PC-rich samples was that the detected PAs hump retention time by UHPLC-DAD was unchanged. The reduction in the UV peak area was generally moderate (not that significant amount of reduction, on an average 9%). The change in the quantitative MS levels varied significantly depending on the type of reactions involved. For instance, the galloylated and PD-rich plant extracts exhibited different reactions depending on their retention time. In addition, because of the oxidation of the plant extracts, the decrease in the quantitative MS/MS levels was high (on average, more than 90% decrease) among these samples with a high change of UV peak area. Moreover, based on the UV peak area observations before and after oxidation, all studied samples were categorized into four different categories. Although intramolecular reactions predominated in samples categorized as A and B, both intra- and inter-molecular reactions were observed in samples categorized as C and D. However, this study was not able to provide us with the necessary information to study the effects of different isomers and epimers on the reactivity of PAs.

After this initial screening, certain plant species were selected for a more detailed study with high-resolution orbitrap mass spectrometry to verify the findings and determine the predominant reaction pathways in the samples. The ultra-high-resolution MS revealed the complex molecular structures of the various PAs that were studied, for example, the natural PA structures included various A- and B-type

PCs, as well as various PDs and PC/PD mixtures. With no or modest modifications, both A and B-type PCs were relatively stable, whereas certain plants exhibited the formation of new ether linkages after oxidation. On the other hand, in plant extracts containing only PDs or mixed PC/PD units, the reactivity was higher. After alkaline oxidation, many PAs were no longer detected by MS due to their modified molecular structures. However, UV was still able to detect their delayed PA humps at 280 nm. Thus, a new analytical method could resolve this issue for better identification and detailed oxidized PA structure characterization in future.

Although, galloylated PCs-rich extracts were relatively stable after oxidation, but the galloylated PDs or PC/PD mix extracts were more reactive. However, in some cases, adding two or more galloyl groups to galloylated PCs increased reactivity compared to those single containing galloyl groups. The galloylated PDs showed similar reactivity to that of non- galloylated PDs. Moreover, the conversion from B-type galloylated PAs to A-type galloylated PAs was supported by additional ether linkages.

Finally, the *in vitro* results indicate different antiparasitic activities of the PA containing plant extracts based on their reactivities in alkaline conditions. The results also supported the hypothesis that some oxidized samples may result in increased antiparasitic activities and some in decreased antiparasitic activities based on their reactivity in alkaline conditions. Although, a diverse and complex structure-activity relationship between the MIA of *Ascaris suum* and its various plant extract constituents is often challenging to obtain. This is mainly due to the complexity of the extracted compounds and their various derivatives. Even though the results indicated that PAs have potent antiparasitic activity against *Ascaris suum* larvae, it is still important to study the molecular level of these compounds as well as the functional properties of the plant extracts.

Overall, the oxidation processes of PAs are significant in a wide variety of plant-related research fields, ranging from their fundamental roles in plant ecology and physiology as well as their applications in the food, feed and pharmaceutical industries. Hence, understanding the above issues enriches all of these study disciplines and gives valuable insights for future investigations. Also, the rapid and straightforward oxidation process makes it possible to generate large-scale production of PA rich plant extract. This process opens the door to study how beneficially PA rich plant extract can be used to increase anthelmintic activity for the potential use of novel drugs.

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Helsinki, 02.04.2023

Iqbal Bin Imran

"If you work on something a little bit every day, you end up with something that is massive"
—Kenneth Goldsmith

"I can accept failure. Everyone fails at something. But I can't accept not trying"

— Michael Jordan

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