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**Phenolics and condensed tannins from
sulla (*Hedysarum Coronarium*) leaves
and their biological significance**

This thesis was presented in partial fulfilment of the requirements for the
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Palmerston North, New Zealand

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ABSTRACT

The objective of this study was to isolate and characterise condensed tannins (CT) and phenolic compounds from the leaves of the forage legume sulla (*Hedysarum Coronarium*) and evaluate their structure-activity relationships with *in vitro* parasite assays. The study was performed on samples which were collected over different seasons (spring-23/09/02, spring-05/08/02, and summer-21/12/01) from the same site. The effects of processing in different manners, for both freeze-dried and fresh frozen plant material was examined. CT extracts were purified using step and gradient Sephadex LH-20 chromatography methods. The CT fractions obtained were analysed using thiolytic degradation, electrospray ionisation mass spectrometry (ESI-MS), matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS) and ¹³C-nuclear magnetic resonance (NMR) spectroscopy.

Acid catalysed degradation of the CT polymer with benzyl mercaptan afforded catechin, epicatechin, galocatechin and epigallocatechin in both the terminal and extender units. Epigallocatechin was the major extender unit (69%) while galocatechin was the major terminal unit (54%) with the overall characteristic of a predominantly prodelphinidin-type CT. Sulla CT oligomers and polymers had variable chemical composition with procyanidin:prodelphinidin ratios ranging from 27:73 to 11:89 and *cis:trans* ratios ranging from 56:44 to 82:18. The CT oligomers of gradient LH-20 fractions obtained from fresh frozen material (spring-05/08/02) ranged between 2.9 and 6.9 mean degree of polymerisation (mDP), while CT fractions from the freeze-dried material (summer-21/12/01) varied between 3.1 and 9.1 mDP as determined by thiolysis. The CT polymer from step LH-20 fractions had CTs ranging from 12 to 26 mDP as determined by thiolysis. A medium molecular weight CT with mDP of 46 was identified. No high molecular weight CT (mDP > 50) was obtained. Screening the LH-20 fractions collected in spring (23/09/02) by HPLC-PDA indicated that there was no extractable CT. No seasonal or freeze-drying effects were observed on the chemical composition of CT. ¹³C NMR provided information on the stereochemistry of the heterocyclic C-ring and the existence of procyanidin and prodelphinidin units in the B-ring. The ¹³C NMR spectrum confirmed sulla CTs to be predominantly of the *cis*-stereochemistry composed of prodelphinidin units.

Analysis of the CT oligomers from the 100% MeOH fractions from the gradient LH-20 with ESI-MS provided information on the molecular weight distribution and the procyanidin and prodelphinidin unit composition. Singly charged species from dimers to trimers, doubly charged species from tetramers to octamers, and triply charged species from nonamers to undecamers were detected. MALDI-TOF-MS verified the ESI-MS data and fractions were found to contain singly charged ions up to hexamers. The ions consisted of homogenous and heterogeneous CT oligomers, with the overall characteristic of a PD-type CT. This technique demonstrated that the gradient LH-20 method improved separation with fractionation of CT oligomers from polymers.

Investigation of the low molecular weight phenolics (flavonoids) was performed using ESI-MS and atmospheric pressure chemical ionisation mass spectrometry (APCI-MS). Chlorogenic acid, quercetin-7-O- α -L-rhamnosyl-3-O-glucosylrhamnoside, rutin, quercetin-3-O- α -L-rhamnosyl-7-O-glucoside, kaempferol, kaempferol-3-O- β -D-glucoside-dirhamnoside, genistein-7-O- β -D-glucosyl-6''-O-malonate, formononetin-7-O- β -D-glucoside-6''-O-malonate and afrormosin were isolated for the first time from *sulla*. Chlorogenic acid and rutin were confirmed using authentic standards and by comparison with data from the literature.

Primary metabolites were evaluated by wet chemistry methods including the available carbohydrate (g/100g) content of 12.38 and, 14.11 and individual sugars (g/100g) were quantified; glucose (3.68 and 5.40), fructose (0.98 and 1.69), galactose and/or rhamnose (0.46 and 0.32), sucrose (1.63 and 5.50) in spring (23/09/02) and summer (21/12/01), respectively. Nutritional composition data (g/100g) by near infrared (NIR) spectroscopy has shown *sulla* to be a nutritious forage legume with high crude protein (CP; 24.4-25.1), non-structural carbohydrates (NSC; 17.1-19.7), lower neutral detergent fibre (NDF; 12.5-16.4), acid detergent fibre (ADF; 15.5-18.3) and lipid (2.6-3.3). The butanol-HCl assay showed the extractable CT content (g/100g) to be 7.6% and 5.3%, with 2.0% protein bound CT and 0.3% fibre bound CT and 1.3% protein bound CT and had no fibre bound CT, from summer (21/12/01) and spring (23/09/02) respectively.

The effects of LH-20 fractions on egg hatching (EH) and larval development (LD) assays for the nematode, *Trichostrongylus colubriformis* under *in vitro* conditions were investigated. The fractions (freeze-dried summer (21/12/01) step method 50% MeOH eluent) containing flavonoids were effective in inhibiting EH at 500 and 1000 μ g/mL, while the CT-containing fractions were not effective. All the fractions from the

fresh frozen material on the step method were not effective in inhibiting EH. However, the CT-containing fractions (LH-20 70% acetone eluent) from the freeze-dried (summer-21/12/01) and fresh frozen (spring-05/08/02) material were effective in inhibiting LD ($p < 0.001$) with certain fractions completely inhibiting the LD process. Oligomeric and polymeric CT (gradient LH-20 100% MeOH and 70% acetone eluents) fractions were effective and inhibited the larval development process at 100 $\mu\text{g/mL}$. The anti-parasitic activity of fractions in the LD and EH assay can be attributed to both the flavonoid and CT content.

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TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	v
List of Figures.....	x
List of Tables.....	xii
List of Abbreviations.....	xiv
CHAPTER ONE INTRODUCTION.....	1
1.1 Beneficial effects of CT in ruminant production.....	3
1.1.1 High performance in ruminants.....	3
1.1.2 Health Benefits.....	4
1.1.3 Environmental Benefits.....	5
1.2 Condensed tannins	5
1.3 Structure-activity relationship.....	8
1.4 Effects of CT on inhibition of protein degradation.....	9
1.5 Extraction and chromatography.....	11
1.5.1 Plant harvesting and storage.....	11
1.5.2 Extraction and purification.....	12
1.5.3 Screening with HPLC.....	12
1.6 Chemical reactions of condensed tannins.....	13
1.6.1 Acid catalysed degradation with benzyl mercaptan.....	13
1.6.2 Acid catalysed degradation with phloroglucinol.....	14
1.6.3 Butanol-HCl assay.....	16
1.7 Characterisation of phenolics with liquid chromatography-mass spectrometry (LC/MS).....	16
1.7.1 Electrospray mass spectrometry (ESI-MS) of CT oligomers.....	16
1.7.2 MALDI-TOF-MS.....	16
1.7.3 ESI-MS and APCI-MS (atmospheric pressure chemical ionisation) of flavonoids.....	17
1.8 Sulla as an anthelmintic forage for parasite control.....	18
1.8.1 Life cycle of gastrointestinal nematode <i>Trichostrongylus colubriformis</i> ...	19
1.8.2 Effects of nematodes on animal nutrition.....	20
1.8.3 Effects of CT on GI <i>in vitro</i> parasite assays.....	20

1.8.4 Effects of CT on GI using larval migration inhibition assay.....	22
1.8.5 <i>In vivo</i> parasite activity of <i>sulla</i>	22
1.9 Aims of the thesis.....	23
CHAPTER TWO MATERIALS AND METHODS.....	24
2.1 Plant material.....	24
2.1.1 Plant extraction for freeze-dried material.....	24
2.1.2. Plant extraction for fresh frozen material.....	25
2.2 Purification with Sephadex LH-20 Column.....	25
2.2.1 Packing of the LH-20 Column.....	25
2.2.2 Step fractionation method with LH-20.....	26
2.2.3 Gradient fractionation method with LH-20.....	27
2.3 Chromatographic screening assays.....	28
2.3.1 Liquid chromatography-PDA	28
2.3.2 Thin layer chromatography.....	29
2.4 Further Separation.....	29
2.4.1 Rechromatography with Sephadex LH-20 fractionation method.....	29
2.4.2 Reverse phase C18 flash chromatography.....	29
2.4.3 Solid phase extraction (SPE).....	30
2.5 Characterisation.....	30
2.5.1 LC-MS of CT oligomers.....	30
2.5.2 Selective ion monitoring-mass spectrometry (SIM-MS).....	30
2.5.3 Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) of CT oligomers.....	31
2.5.3.1 Cleaning of the target plate.....	31
2.5.3.2 Analysis of CT oligomers with MALDI-TOF-MS.....	31
2.5.4 APCI-MS and ESI-MS of flavonoids.....	32
2.5.5 ¹³ C and ¹ H NMR analysis.....	32
2.5.5.1 ¹³ C NMR analysis of CT polymers.....	32
2.5.5.2 ¹³ C NMR analysis of flavonoids.....	33
2.6 Chemical reactions.....	33
2.6.1 Thiolysis.....	33
2.6.1.1 Preparation of standards.....	34
2.6.2 Acid butanol assay for CT content	34
2.7 Near infrared spectroscopy (NIRS) for nutritional content.....	35

2.8 Analysis and identification of soluble sugars by HPLC.....	35
2.9 Parasite bioassays.....	36
2.9.1 Egg extraction.....	36
2.9.2 Egg hatch assay (EHA).....	36
2.9.3 Larval development assay (LDA).....	37
2.9.4 Calculations and statistical analysis.....	37
CHAPTER THREE CONDENSED TANNINS.....	38
3.1 Introduction.....	38
3.2 Results.....	39
3.2.1 Liquid chromatography-PDA.....	39
3.2.1.1 PDA chromatograms for step LH-20 fractionation.....	39
3.2.1.2 PDA chromatograms for gradient LH-20 fractionation.....	42
3.2.1.3 Thin layer chromatography (TLC).....	44
3.2.2 LC-ESI-MS of CT oligomers.....	45
3.2.3 Selective ion mode (SIM) mass spectroscopy for homogeneous and heterogeneous oligomers.....	48
3.2.4 MALDI-TOF mass spectrometry.....	48
3.2.5 Thiolysis.....	52
3.2.6 ¹³ C-NMR of condensed tannin polymers.....	55
3.2.7 Acid butanol assay.....	57
3.2.8 NIRS data.....	58
3.2.9 Soluble sugars and available carbohydrate.....	59
3.3 Discussion.....	60
3.3.1 LC-MS of CT oligomers and polymers.....	60
3.3.2 MALDI-TOF of CT oligomers.....	63
3.3.3 Thiolysis.....	65
3.3.4 Acid butanol assay.....	67
3.3.5 NIRS.....	69
3.3.6 Soluble sugars and available carbohydrate.....	70
3.4 Conclusions.....	71
CHAPTER FOUR CHARACTERISATION OF FLAVONOIDS.....	73
4.1 Introduction.....	73
4.2 Results and discussion.....	75

4.2.1 ESI-MS and APCI-MS of flavonoids.....	75
4.2.2 ¹³ C-NMR of flavonoids.....	81
4.3 Conclusions.....	83
CHAPTER FIVE PARASITE BIOASSAYS.....	84
5.1 Introduction.....	84
5.2 Results.....	85
5.2.1 <i>In vitro</i> egg hatch assay for LH-20 fractions.....	85
5.2.2 <i>In vitro</i> larval development assay.....	87
5.3 Discussion.....	89
5.4 Conclusion.....	92
CHAPTER SIX SUMMARY AND FUTURE WORK.....	93
6.1 Summary and Future work.....	93
REFERENCES.....	96
APPENDICES.....	110

LIST OF FIGURES

FIGURE	Page
Figure 1.1a Monomeric units of CT polymer.....	6
Figure 1.1b Structure of hydrolysable tannin containing gallic acids ester bound to a hexose moiety.....	6
Figure 1.2 Structure of a CT polymer.....	6
Figure 1.3 A-type linkages for proanthocyanidin epicatechin-(4 β →8, 2 β →O→7)-catechin.....	7
Figure 1.4 Structure of (-) epicatechin-3-O-gallate, a major component in green tea and wine.....	8
Figure 1.5 A diagrammatic representation of protection of plant protein in the rumen.....	10
Figure 1.6 HPLC-PDA chromatograms for a 50% MeOH and a 70% aqueous acetone eluted from an LH-20 column from sulla leaves.....	13
Figure 1.7 Thiolytic degradation of CT polymer using benzyl mercaptan yielding free flavan-3-ols (terminal units) and thioylether derivatives (extender units).....	14
Figure 1.8 The basic life cycle of <i>Trichostrongylus colubriformis</i> nematode in sheep.....	20
Figure 1.9 Inhibition of development of <i>Trichostrongylus colubriformis</i> eggs to L3 in the presence of CT.....	21
Figure 2.1 Flow chart for separation of aqueous crude extract through the Sephadex step LH-20 column chromatography.....	27
Figure 3.1 PDA chromatograms recorded at 280 nm for step LH-20 fractions OTA02801-04 (at 240-360 nm) and OTA02805-07 (at 280 nm) from the freeze-dried sulla harvested summer (21/12/01).....	41
Figure 3.2 PDA chromatograms for the gradient LH-20 fractions OTA04301-05 (at 240-360 nm) from fresh frozen plant material collected in spring (23/09/02).....	44
Figure 3.3 TLC chromatogram for the aqueous crude extract, ethyl acetate extract 50% MeOH fractions (OTA02801-4, OTA03101-4), 70% acetone fractions (OTA02805-7, OTA03105-7) from sulla collected in summer (21/12/01).....	45

Figure 3.4	Mass spectra obtained for CT oligomers using LC-ESI-MS in a negative ion mode for the gradient LH-20 fractionation a) OTA02806 (70% acetone), b-d) OTA04307-OTA04309 (100% MeOH) e) OTA04310 (70% acetone).....	48
Figure 3.5	MALDI-TOF spectra acquired in a positive reflectron mode for CT oligomers fractions a) OTA04309 b) OTA09806 c) OTA09807 d) OTA09808 and e) OTA09809.....	52
Figure 3.6	HPLC-UV chromatograms (at 280 nm) for elution of free flavan-3-ols and thioylether derivatives obtained through thiolysis reaction for 70% acetone fractions a) OTA02805 b) OTA02806 c) OTA03706 and d) OTA07008.....	53
Figure 3.7	a) Chemical structure of CT polymer b) ^{13}C NMR data for CT polymer (OTA02806, 70% acetone, Fr-6).....	56
Figure 3.8	Calibration curve for the standards in determination of the concentration of the CT from plant material.....	58
Figure 4.1	Flavonoid basic core structure.....	73
Figure 4.2	The main classes of flavonoids found in plants.....	74
Figure 4.3	Mass spectral data for OTB08801 SPE and OTB08408 RP-C18 fractions characterised using APCI $[\text{M}+\text{H}]^+$ and ESI $[\text{M}-\text{H}]^-$	77
Figure 4.4	Structures of flavonoids and iso flavonoids derivatives isolated from <i>sulla</i> and characterised with LC-ESI-MS and APCI-MS.....	79
Figure 4.5	UV spectrum for formononetin characterised from fraction OTB08612 C18F.....	80
Figure 5.1	The effect of <i>sulla</i> step LH-20 fractions with increasing concentrations (100 to 1000 $\mu\text{g}/\text{mL}$) on hatching of <i>Trichostrongylus colubriformis</i> eggs incubated for 24 hrs at 26 $^\circ\text{C}$	86
Figure 5.2	The effect of <i>sulla</i> gradient LH-20 fractions with increasing concentrations (100 to 1000 $\mu\text{g}/\text{mL}$) on hatching of <i>Trichostrongylus colubriformis</i> eggs incubated for 24 hrs at 26 $^\circ\text{C}$	87
Figure 5.3	The effect of <i>sulla</i> step LH-20 fractions with increasing concentrations (25 to 250 $\mu\text{g}/\text{mL}$) on larval development (LD) of <i>Trichostrongylus colubriformis</i> eggs incubated for seven days at 24 $^\circ\text{C}$	88
Figure 5.4	The effect of <i>sulla</i> gradient LH-20 fractions with increasing concentrations (25 to 200 $\mu\text{g}/\text{mL}$) on LD of <i>Trichostrongylus colubriformis</i> eggs incubated for seven days at 24 $^\circ\text{C}$	88

LIST OF TABLES

TABLE	Page
Table 1.1 Composition of terminal and extender units of CT fractions from <i>Lotus corniculatus</i> and <i>Lotus pendunculatus</i>	15
Table 1.2 Parasite checklist of nematodes of sheep in New Zealand.....	19
Table 2.1 Batches of sulla plant extraction yielding ethyl acetate and aqueous layer.....	25
Table 2.2 Step LH-20 fractionation for crude extracts from freeze-dried and fresh frozen sulla material.....	26
Table 2.3 Sulla gradient LH-20 fractions from freeze-dried and fresh frozen plant material.....	28
Table 2.4 Ions for SIM-MS for the determination of CT oligomers.....	31
Table 2.5 Commercially available standards used for calibration in analysis of thiolysis adducts and free flavan-3-ols.....	34
Table 2.6 Working standards prepared from the stock solution using butanol-HCl method in determination of sulla CT concentration.....	35
Table 3.1 Observed and expected $[M+Na]^+$ and $[M+K]^+$ ions from MALDI-TOF mass spectra employed in a positive reflectron mode from the 100% MeOH fractions.....	49
Table 3.2 Comparison of % extender and terminal units of proanthocyanidin, mean degree of polymerization (mDP), <i>cis:trans</i> ratio from sulla LH-20 fractions by thiolysis.....	54
Table 3.3 Concentration of free CT, protein bound and fibre bound CT (g/100g) from sulla plants measured by the butanol-HCl method (Terrill et al., 1992) with literature comparisons.....	57
Table 3.4 Nutritional composition (g/100g) of sulla plants collected on different dates and analysed using NIRS with literature comparisons.....	58
Table 3.5 Soluble sugars content and available carbohydrate (g/100g) from sulla leaves.....	59
Table 3.6 Comparison of mDP by acid catalysis and the mass range estimates by MALDI-TOF-MS and ESI-MS.....	62
Table 4.1 Identification of flavonoids from C18 SPE fractions and reverse phase C18 flash chromatography fractions using APCI-MS and ESI-MS.....	81

Table 4.2	^{13}C -NMR spectral data (δ) of quercetin moiety from fraction OTA10305 (75% MeOH).....	82
Table 5.1	Effect of CT fractions from sulla step LH-20 fractionation on the hatching rate and development to L3 of <i>Trichostrongylus colubriformis</i> eggs <i>in vitro</i> compared with the chemical composition as determined by thiolysis.....	85
Table 5.2	Effect of CT fractions from sulla gradient LH-20 fractionation on the hatching rate and development to L3 of <i>Trichostrongylus colubriformis</i> eggs <i>in vitro</i> compared with chemical composition as determined by thiolysis.....	86

LIST OF ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
AQ	aqueous
BSA	bovine serum albumin
Bu-HCl	butanol hydrochloric acid
C	catechin
CP	crude protein
CT	condensed tannins
COSY	correlated spectroscopy
DHB	2,5-dihydroxy benzoic acid
DCM	dichloromethane
DHQ	dihydroquercetin
DM	dry matter
DP	dietary protein
EC	epicatechin
EGC	epigallocatechin
EHA	egg hatch assay
ESI	electrospray ionization
FAB	fast atom bombardment
GC	gallocatechin
HMBC	heteronuclear multiple quantum coherence
HMQC	heteronuclear multiple bond connectivity
HMW	high molecular weight
HT	hydrolysable tannin
IS	internal standard
LC	liquid chromatography
LCS	lotus corniculatus
LDA	larval development assay
LMI	larval migration inhibition
LMW	low molecular weight
LP	lotus pendunculatus
LSI	liquid secondary ionisation
LSU	large subunit
MALDI-TOF	matrix aided laser desorption ionization, time of flight

mDP	mean degree of polymerisation
MMW	medium molecular weight
MS	mass spectrometry
M_w	molecular weight
NDF	neutral detergent fibre
NIRS	near infrared reflectance spectroscopy
NMR	nuclear magnetic resonance
NP-HPLC	normal phase high performance liquid chromatography
NSC	non-structural carbohydrates
ODS	octadecyl silica
OMD	organic matter digestibility
PA	proanthocyanidins
PC	procyanidins
PD	prodelphinidins
PDA	photodiode array
PEG	polyethylene glycol (M _w , 3500)
RP-HPLC	reverse phase high-performance liquid chromatography
RRF	relative response factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIM	selective ion mode
SSU	small subunit
UV-VIS	ultra violet-visible
VFI	voluntary feed intake