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

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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 ^{13}C-nuclear magnetic resonance (NMR) spectroscopy.

Acid catalysed degradation of the CT polymer with benzyl mercaptan afforded catechin, epicatechin, gallocatechin and epigallocatechin in both the terminal and extender units. Epigallocatechin was the major extender unit (69%) while gallocatechin 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. ^{13}C NMR provided information on the stereochemistry of the heterocyclic C-ring and the existence of procyanidin and prodelphinidin units in the B-ring. The ^{13}C 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 ad 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 μg/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 μg/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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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 penduculatus
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
Mw	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 (Mw, 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