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Primitive Anthocyanin from Flowers of three Hemiparasitic African Mistletoes

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ABSTRACT

Anthocyanins were isolated using Amberlite XAD-7 column chromatography and Sephadex LH-20 gel filtration from acidified methanolic extracts of flowers of the three bird-pollinated hemiparasitic plants; Tapinanthus buvumae (Rendle) Danser, Tapinanthus constrictiflorus (Engl.) Danser and Phragmanthera usuiensis (Oliv.) M. Gilbert in the mistletoe family Loranthaceae. Their structures were shown to be cyanidin 3-O-β-glucopyranoside, elucidated by on-line diode-array detection chromatography and homo- and heteronuclear NMR techniques. In accordance with previous reports on Cynomorium coccineum (Cynomoriaceae) and Cassytha spp. (Lauraceae) simple non-acylated cyanidin 3-glycosides seem to be the principal anthocyanins in parasitic plants.

Keywords: Flowers; *Tapinanthus buvumae; Tapinanthus constrictiflorus*; *Phragmanthera usuiensis*; Cyanidin 3-*O*-β-glucopyranoside

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INTRODUCTION

The family Loranthaceae, also called mistletoe, comprises of approximately 73 genera and 900 species living on branches, twigs or roots of host plants, occurring mainly in the tropical and temperate regions [1]. They have chlorophylls, but are partial parasites by invading the host plant xylem using a special structure, the haustorium, to obtain water and minerals [2]. In some species host penetration occurs only in the phloem [3, 4]. A number have been used in traditional medicine and as such have been investigated and found to have antimicrobial [5-7], antidiabetic [8] and antiviral [9] properties. According to our knowledge, there has not been any report on the anthocyanin content of any species in the Loranthaceae family, despite their wide occurrence and the number of uses.

The objective of this paper was to determine the anthocyanin content of three bird-pollinated species, *Tapinanthus buvumae* (Rendle) Danser, *Tapinanthus constrictiflorus* (Engl.) Danser and *Phragmanthera usuiensis* (Oliv.) M. Gilbert, all from the African mistletoe family. The anthocyanin content of just a few parasitic plants has been reported previously [10-12].

MATERIALS AND METHODS

2.1. Plant Material

Flowers of three African mistletoes were collected in the same locality but from different plant hosts in Budo, Mpigi district, Uganda in November 2008. The flowers were extracted immediately after collection. The three parasitic plants were collected on the following host plants; *Ficus notalensis (Phragmanthera usuiensis)*, *Erythrina abyssinica (Tapinanthus constrictiflorus)* and *Flueggea virosa (Tapinanthus buvumae).* The plants were taxonomically identified and, voucher specimens (Voucher numbers: RB31/2008, RB32/2008, RB33/2008 for *Phragmanthera usuiensis*, *T. constrictiflorus* and *T. buvumae* respectively) have been deposited in Botany Herbarium, Makerere University.

2.2 Extraction and isolation

The flowers, *P. usuiensis* (200 g), *T. constrictiflorus* (48 g) and *T. buvumae* (200 g) were separately extracted with 1 % trifluoroacetic acid (TFA) in methanol. The filtered extract was concentrated under reduced pressure, purified by partition (several times) against ethyl acetate and applied to an Amberlite XAD-7 column. The anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 1 % TFA. The concentrated anthocyanin extract was purified by Sephadex LH-20 chromatography using 50 % aqueous methanol containing 1 % TFA as eluent.

2.3 Structure analysis

Co-chromatography included TLC and on-line HPLC using anthocyanins from blackcurrant (*Ribes nigrum*) [13] as a reference. TLC and HPLC were carried out according to earlier described procedures [14]. All the UV–Visible absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600 nm in steps of 2 nm. Based on HPLC purity, the anthocyanin pigment obtained after purification and isolation of the anthocyanins in the acidified methanolic extract of *Phragmanthera usuiensis* was chosen for NMR analysis. The NMR experiments were obtained at 600.13 and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DMX–600 instrument at 25°C. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent, CF_3COOD–CD_3OD (95:5; v/v), were used as secondary references (49.0 ppm and 3.4 ppm from tetramethylsilane for ^{1}H and ^{13}C, respectively) [15]. The 1D ^{1}H NMR and the 2D HMBC, HSQC and COSY experiments were obtained with the 5 mm TB1 probe.

RESULTS AND DISCUSSION

All the UV–VIS spectra recorded on-line during HPLC analysis of the acidified methanolic extracts of flowers, *P. usuiensis*, *T. constrictiflorus* and *T. buvumae* showed the same anthocyanin with visible maxima around 520 nm, and $A_{440}/A_{Vis\text{-}Max}$ around 33%, indicating cyanidin 3-glycosides or peonidin 3-glycosides [16] (Table 1). The examined pigments showed similar retention times (HPLC) as cyanidin 3-glucoside found in black currant [13], and the identity was confirmed to be cyanidin 3-*O*-β-glucopyranoside by 1D and 2D NMR analysis, in agreement with data given in the literature [17] (Figure 1).

The downfield part of the 1D ^{1}H NMR spectrum showed a singlet at 9.03 ppm (H-4), a 3H AMX system at 8.36 ppm (dd, 8.7 Hz, 2.3 Hz; H-6´), 8.13 ppm (d, 2.3 Hz; H-2´) and 7.12 ppm (d, 8.7 Hz; H-5´) and an unresolved 2H AB system at 7.00 ppm (H-8) and 6.76 ppm (H-6), respectively (Table 2), in accordance with the anthocyanin, cyanidin. After the chemical shifts of the protons

were assigned, the chemical shifts of the corresponding carbons (Table 2) were assigned from the HSQC experiment.

Table 1. Chromatographic and spectral data for cyanidin 3-glucoside, 1, isolated from flowers of *Tapinanthus constrictiflorus*[a], *T. buvumae*[b], *Phragmanthera usuiensis*[c] and black currants[d] [13].

Compound	R_f (TLC), FHW	t_R (HPLC) (min)	Absorption Maxima (nm)	$A_{440}/A_{vis\text{-}max}$ (%)
a	0.22	21.07	520	36
b	0.22	21.09	520	33
c	0.22	21.05	520	33
d	0.22	21.07	520	34

The remaining quaternary C-atoms were assigned using the HMBC spectrum, which was optimized for $^2J_{CH}$ and $^3J_{CH}$ couplings. The anomeric cross peak at 5.37/102.53 ppm in the HSQC spectrum indicated one monosaccharide. Starting from the doublet at 5.37 ppm (J=7.6 Hz, H-1´´), the observed cross peak with the signal at 3.76 ppm in the DQF-COSY spectrum permitted the assignment of H-2´´. The chain of coupled protons H-2´´, H-3´´, H-4´´, H-5´´ and H-6A´´ and H-6B´´ was thereafter assigned (Table 2) from cross peaks in the same spectrum. Subsequently, the chemical shifts of the corresponding carbon atoms were assigned from the HSQC spectrum, which together with $^1H–^1H$ coupling constants were in agreement with a β-linked glucopyranose. The cross peak in the HMBC experiment at 5.37/145.22 ppm between the anomeric glucoside proton and C-3 of the aglycone, showed that the sugar moiety was linked to the aglycone 3-position. Thus, the identity was determined to be cyanidin-3-O-β-glucopyranoside (Figure 1). This is the first report on the anthocyanin content of any species in the Loranthaceae family.

Table 2: ^{1}H and ^{13}C NMR shift values (ppm) and proton-proton couplings (Hz) for cyanidin 3-O-*β*-glucopyranoside isolated from flowers of *Tapinanthus buvumae, Tapinanthus constrictiflorus* & *Phragmanthera usuiensis* dissolved in CD_3OD: CF_3COOD (95:5, v/v) at 25°C.

Cyanidin	^{1}H δ (ppm)	^{13}C δ (ppm)
2		163.98
3		145.22
4	9.03 *d* 0.9	136.55
5		158.67
6	6.76 *d* 1.9	103.29
7		169.88
8	7.00 *dd* 1.9, 0.9	95.18
9		157.39
10		112.77
1'		120.86
2'	8.13 *d* 2.3	118.05
3'		147.12
4'		155.52
5'	7.12 *d* 8.7	117.48
6'	8.36 *dd* 8.7, 2.3	128.04
3-*O*-β-glucopyranoside		
1"	5.37 *d* 7.6	102.53
2"	3.76 *dd* 7.6, 9.0	73.88
3"	3.65 *m*	78.08
4"	3.53 *dd* 9.3, 9.0	69.79
5"	3.82 *m*	77.42
6A"	4.13 *dd* 1.9, 11.3	62.21
6B"	3.73 *m*	

The same simple anthocyanin as found in the present study (cyanidin 3-glucoside) has previously been identified as the major pigment (92%), together with another simple anthocyanin, cyanidin 3-rutinoside, in the parasitic plant *Cynomorium coccineum* from the Cynomoriaceae family [10, 11]. These two cyanidin-derivatives together with cyanidin 3-arabinoside were altogether reported to occur in six *Cassytha* taxa (Lauraceae) [12]. All previous papers including this work, on the anthocyanin content of parasitic plants seem to reveal simple non-acylated cyanidin 3-glycosides.

Harborne (1993) has provided evidence that natural selection for particular colors in different environments depends upon the most active pollinators present [18].

Selection has worked in two directions: from the aglycone cyanidin as the most basic or most primitive type of plant pigment, to flowers with scarlet orange colors containing the aglycone pelargonidin arising by loss mutations. In the second direction, gain mutations have occurred in temperate climates producing the delphinidin colors common in bee-pollinated families. Scogin (1988) has reported that bird-pollinated flowers are much more likely to contain pelargonidin derivatives and much less likely to contain delphinidin-derivatives than other flowers generally [19]. However, a difference in pigment composition has been reported in comparisons between perching-bird and hummingbird-visited flowers with a greater prevalence of cyanidin-derivatives, as opposed to pelargonidin-derivatives, in perching-bird flowers. In his study Scogin reported that six out of eight perching-bird pollinated species of the Old World (Africa) contained cyanidin-derivatives. The African taxa in the present study (*Tapinanthus* and *Phragmanthera*) are reported to be pollinated mainly by sunbirds (perching-birds) [20]. The finding of the most "primitive" anthocyanin, cyanidin 3-glucoside, in the three examined species is consistent with the results of Scogin (1988) [19].

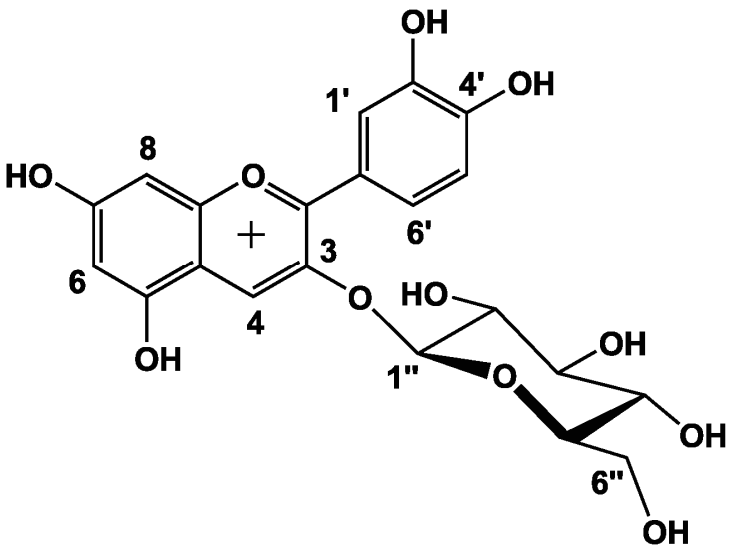

Figure 1: The structure of cyanidin 3-*O*-β-glucopyranoside isolated from flowers of *Tapinanthus constrictiflorus, T. buvumae,* and *Phragmanthera usuiensis.*

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