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Polyphenols from *Allanblackia floribunda* seeds: identification, quantification and antioxidant activity

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Abstract

Oil rich seeds of *Allanblackia floribunda*, a tree from tropical Africa, have traditionally been used in food preparation. Furthermore, the therapeutic properties of various parts of this tree have long been exploited in traditional medicine. As both food and pharmaceutical
industries show growing interest in tropical tree crops, this study aimed to investigate whether *A. floribunda* seeds could also be used as a source of potentially bioactive compounds. The polyphenol profile revealed six predominant compounds which were identified by HPLC-PDA-ESI/MS² as the biflavonoids morelloflavone, Gb-2a and volkensiflavone and their respective glucosides. A range of less abundant flavones, flavonols and flavan-3-ols was also detected. All six major compounds showed antioxidant activity, with the activity of morelloflavone, its glucoside and Gb-2a-glucoside comparable with that of ascorbic acid. The main compounds accounted for approximately 10% of dry weight, making the seeds used for oil production a rich source of biflavonoids as a by-product.

**Keywords**: *Allanblackia floribunda*, phenolic compounds, biflavonoids, HPLC-PDA-ESI/MS², morelloflavone, volkensiflavone, Gb-2a, antioxidant capacity

Chemical compounds studied in this article

morelloflavone (PubChem CID: 5464454); volkensiflavone (PubChem CID: 23844069); Gb-2a (PubChem CID: 176988)

1. Introduction

*Allanblackia floribunda* Oliv. (Clusiaceae or Guttiferae) is an evergreen tree which grows in the tropical rainforests of Africa to a height of up to 30 m and is traditionally used in a variety of ways (Orwa & Munjuga, 2007; Orwa, Mutua, Kindt, Jamnadass & Anthony, 2009). Whilst the seeds consist to over 60% of oil and provide edible vegetable fat (Orwa et al., 2009; Wilfred, Adubofuor & Oldham, 2010), various parts of the tree, including roots and bark, are used in traditional medicine for the treatment of a range of ailments such as...
toothache, dysentery and coughs (Betti, 2004; Olowokudejo, Kadiri & Travih, 2008; Kayode, 2006). Furthermore, *Allanblackia floribunda* extracts have been reported to exhibit antimicrobial, anti-inflammatory and antioxidant activity (Ajibesin, Rene, Bala & Essiett, 2008; Ayoola et al., 2009; Ayoola, Ipav, Sofidiya, Adepoju-Bello, Coker & Odugbemi, 2008; Kuete et al., 2011) as well as potential antimalarial (Azebaze, Teinkela, Nguemfo, Valentin, Dongmo & Vardamides, 2015) and anticancer effects (Fadeyi, Fadeyi, Adejumo, Okoro & Myles, 2013). To date, a range of potentially bioactive compounds have been reported in a variety of extracts from *Allanblackia floribunda* and related species, including benzophenones, xanthones and biflavonoids (Locksley & Murray, 1971; Fuller, Blunt, Boswell, Cardellina II & Boyd, 1999; Nkengfack, Azebaze, Vardamides, Fomum & van Heerden, 2002). In the seed, the focus has mainly been on oil, with some information available on antioxidative properties of *A. floribunda* seed cake (Boudjeko, Ngomoyogoli, Woguia & Yanou, 2013). With growing interest in *Allanblackia* species as tree crop in particular for the food industry (IUCN, 2014), the aim of this study was to investigate the polyphenol profile of *A. floribunda* seeds in order to establish whether seed remnants after plant oil extraction could be a source of bioactive compounds with interest to the pharmaceutical sector.

2. Materials and methods

2.1 Plant material and reagents

Fruits of *Allanblackia floribunda* were collected in three different years from uncultivated farmland in Oke Igbo, Ondo State, South Western Nigeria. The fruits were authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan (voucher specimen number FHI107929). Seeds were separated from the mesocarp, and air-dried seeds were crushed,
freeze-dried and milled into a fine powder (≤1.0mm) with an automated bespoke ball mill robot (Labman Automation, Middlesborough, UK). Briefly, three stainless steel balls (3mm diameter) were added to approximately 200mg seeds in a 2mL microtube and shaken in the robot at the predetermined speed and duration to achieve the desired particle size.

HPLC grade solvents for extraction and analysis of phenolics were purchased from VWR (Lutterworth, Leicestershire, UK). Response factors for quantification were obtained using flavonoid standards with a minimum of 98% purity (Carbosynth Ltd, Compton, Berkshire, UK). All other analytical standards and chemicals were purchased from Sigma Aldrich (Gillingham, Dorset, UK).

2.2 Extraction of soluble phenolics

Soluble phenolics were extracted from approximately 20 mg seed powder by shaking with 5 ml 70% methanol for 15 min at room temperature. The sample was then centrifuged for 10 min at 1700g, the supernatant decanted and the pellet extracted twice more. Methanol was removed from the combined supernatants under vacuum at 60 °C before extracts were partially purified by solid phase extraction (SPE) using Sep-Pak C_{18} cartridges (Waters Ltd, Elstree, UK) as described by Hauck, Gallagher, Morris, Leemans & Winters (2014) and dried under vacuum at 60 °C. Prior to further analysis, samples were typically dissolved in 0.5 ml 70% methanol and diluted as necessary.

2.3 Liquid chromatography-tandem mass spectrometry

Secondary metabolites were analysed by reverse-phase high performance liquid chromatography with online photodiode array detection and electrospray ionisation–ion
trap tandem mass spectrometry (HPLC-PDA-ESI/MS). Structural elucidation was performed on a Thermo Finnigan LC-MS system (Thermo Electron Corp, Waltham, MA, USA) comprising a Finnigan PDA Plus detector, a Finnigan LTQ linear ion trap with ESI source and a Waters C18 Nova-Pak column (3.9 x 100 mm, particle size 4 µm), with column oven temperature maintained at 30 °C. The PDA scan range was set to 240–400 nm, and injection volume was typically 10 µl. The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B). The column was equilibrated with 95% solvent A at a flow rate of 1ml min⁻¹, with 10% going to the mass spectrometer, and the percentage of solvent B increased linearly to 65% over 60 min. MS parameters were as follows: sheath gas 30, auxiliary gas 15 and sweep gas zero (arbitrary units), spray voltage -4.0 kV in negative and 4.8 kV in positive ionisation mode, capillary temperature 320 °C, capillary voltage -1.0 V and 45 V, respectively, tube lens voltage -68 and 110 V, respectively, and normalised collision energy (CE) typically 35%.

Accurate mass measurements only were carried out on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with heated ESI source coupled to a Dionex UltiMate 3000 ultra high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Chromatographic separation was performed on a reverse-phase C18 Hypersil Gold column (20 x 2.1 mm, particle size 1.9 µm; Thermo Fisher Scientific) which was maintained at a temperature of 60 °C and with solvents A and B as described above. The column was equilibrated with 95% solvent A at a flow rate of 0.4 ml min⁻¹, and the percentage of solvent B increased linearly to 100% over 7 min, followed by isocratic elution at 100% solvent B for 3.5 min. High resolution mass spectra were acquired in both negative and positive ionisation mode at a resolution of 120,000 with an automatic gain control (AGC) target of 2e⁵ and a maximum injection time of 100 ms, with other MS parameters as
follows: vaporiser temperature 358 °C, spray voltage -2.5 kV in negative and 3.5 kV in positive ionisation mode, sheath gas 45, auxiliary gas 13 and sweep gas 1 (arbitrary units) and capillary temperature 342 °C.

### 2.4 Quantification of predominant compounds

For quantification of the predominant compounds, seed samples harvested in three different years were extracted in triplicate as described in section 2.2, followed by reverse-phase HPLC. The system comprised a Waters 996 PDA detector and a Waters C<sub>18</sub> Nova-Pak radial compression column (8 x 100 mm, particle size 4 µm), with chromatographic conditions as described by Hauck et al. (2014) and detection wavelength set to 280nm. Response factors (RF) for the flavonoid monomers naringenin, eriodictyol, apigenin and luteolin were obtained from linear standard curves over the range of 0.2 – 10 µg of standard and calculated as

$$RF = \frac{\text{amount [µg]}}{\text{peak area}_{280\text{nm}}}.$$  

All response curves had linear regression coefficients > 0.996.

Response factors of biflavonoid aglycones were calculated as the mean of the relevant flavonoid monomer RFs, and for the quantification of biflavonoid glucosides these values were multiplied by the following correction factor to allow for the difference in molecular weight ($M_r$):

$$\frac{(M_r \text{ aglycone} + 162)}{M_r \text{ aglycone}}.$$  

### 2.5 Acid hydrolysis and analysis of sugar moieties and flavonoid aglycones

To identify the aglycone part of the flavonoid glycosides, acid hydrolysis of total extract was carried out by combining equal volumes of aqueous extract and 2 mol L<sup>-1</sup> HCl. After
heating the solution to 90 °C for 1 h, the pH was adjusted to 3 – 4 with NaOH prior to partial
purification by SPE as described in section 2.2. Flavonoid aglycones were then analysed by
HPLC-PDA-ESI/MS as described in section 2.3 and identified by direct comparison with
relevant flavonoid standards.

For identification of the sugar moieties of the main glycosylated metabolites, compounds
were purified by collecting the HPLC eluent of individual peaks from the Waters system
described in section 2.4. After reducing the volume under vacuum at 60 °C acid hydrolysis
was performed by combining equal volumes of aqueous extract and 2 mol L⁻¹ HCl. The
solution was heated to 90 °C for 1 h and subsequently the pH was adjusted to 6 - 7 with
NaOH. The samples were then loaded onto Strata-X-A strong anion exchange columns
(Phenomenex, Macclesfield, UK) which had been conditioned with 100% methanol and
equilibrated with water. The sugar containing non-binding fraction of the samples was
collected for further analysis.

Monosaccharides were analysed by high-performance anion-exchange chromatography
with pulsed amperometric detection (HPAEC-PAD). Separation of monosaccharides was
achieved on a Dionex ICS-5000 system (Thermo Fisher Scientific, Waltham, MA, USA) fitted
with an Eluent Generator and a Dionex CarboPac SA10 column (250 x 4 mm; Thermo Fisher
Scientific) with an SA-10 guard column (50 x 4 mm). The operating temperature of the
column was 45 °C with an isocratic mobile phase of 1 mmol L⁻¹ KOH pumped at a rate of 1.5
ml min⁻¹. Monosaccharides were identified by comparison of retention times with glucose
and galactose standards.

2.6 Antioxidant activity of selected metabolites
The predominant metabolites were purified by collecting the HPLC eluent of individual peaks from the Waters system described in section 2.4 and then dried down and resuspended in 50% methanol at a concentration of 100 µg mL$^{-1}$. The antioxidant activity of individual compounds was measured in triplicate by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams, Cuvelier & Berset, 1995) and by ferric ion reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996).

For the DPPH assay, 0.5 mL of DPPH solution (140 µmol L$^{-1}$ in 50% methanol) was combined with an equal volume of sample at a concentration of 5-100 µg mL$^{-1}$ and left to stand at room temperature for 30 min. After this time absorbance (A) was measured on a Pharmacia Biotech Ultraspec 4000 UV/Visible spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, UK) at 517 nm, including 50% methanol as control, and % inhibition (I) was determined as

$$ I = \frac{(A - A_{\text{blank}})_{\text{control}} - (A - A_{\text{blank}})_{\text{sample}}}{(A - A_{\text{blank}})_{\text{control}}} \times 100 $$

with IC$_{50}$ defined as the concentration which resulted in 50% inhibition.

The FRAP assay measures reduction of the Fe$^{3+}$ / tripyridyl-s-triazine complex (TPTZ) to the blue ferrous form and was performed as follows: acetate buffer (300 mmol L$^{-1}$, pH 3.6), TPTZ (10 mmol L$^{-1}$ in 40 mmol L$^{-1}$ HCl) and ferric chloride (20 mmol L$^{-1}$) were mixed in the ratio of 10 : 1 : 1 to obtain the FRAP reagent. Sample volumes of 125 µL at a concentration of 100 µg mL$^{-1}$ were added to 1 ml FRAP reagent and after 10 min absorbance was measured at 593 nm. A ferric chloride calibration curve ranging from 100 to 1000 µmol L$^{-1}$ was prepared to estimate Fe$^{2+}$ concentration.

2.7 Statistical analysis
Quantitative analyses were carried out in triplicate and results are presented as mean ± standard error of the mean. Where appropriate results were statistically analysed by one-way ANOVA and significant differences were determined by the Bonferroni post hoc test, GenStat 14th edition.

3. Results and discussion

3.1 Phenolic profile

Figure 1 shows the profile of soluble polyphenols extracted from Allanblackia floribunda seeds which showed little variation between years. Analysis by HPLC-PDA-ESI/MS\textsuperscript{n} revealed six abundant and a range of minor compounds which were tentatively identified based on their UV/vis absorbance and mass spectral characteristics summarised in tables 1 and 2 and figure 2. The compounds which were identified all belonged to the flavonoids, with biflavonoids as the main class. The main compounds were collected individually from peaks 1 to 6 (figure 1) and purified as pale yellow to orange crystalline powders. Molecular weights ($M_r$) of the main compounds are based on accurate mass measurements in both negative and positive ionisation mode, whilst $M_r$ of less abundant compounds are nominal mass based on general MS scans in both ionisation modes. MS\textsuperscript{n} analyses were carried out in negative ionisation mode unless stated otherwise.

3.1.1 Identification of biflavonoids

Based on accurate mass data (table 2) compound 5 was assigned the elemental composition $C_{30}H_{20}O_{11}$ with $M_r$ 556.1005 Da. The corresponding UV/vis spectrum showed absorbance maxima at 275(sh), 290 and 348 nm, a combination of the absorbance spectrum typical of a flavone (with two major absorption bands in the region of 300-380 nm for band I...
and 240-280 nm for band II; Mabry, Markham & Thomas, 1970) with that of a flavanone (with predominant absorption band II and a much reduced band I) and in close agreement with the absorbance characteristics reported for the biflavonoid morelloflavone, a naringenin-luteolin conjugate (Herbin, Jackson, Locksley, Scheinmann & Wolstenholme, 1970). MS$^2$ fragmentation of the parent ion at m/z 555 in negative mode resulted in loss of 126 Da, yielding one main product ion with m/z 429. This can be attributed to cleavage of ring C (figure 3a), typical of the fragmentation of flavonoids (Cuyckens & Claeys, 2004; Fabre, Rustan, de Hoffmann & Quetin-Leclercq, 2001), here breaking bonds 1 and 4 and producing fragment ion [M-H-$^{1,4}$A]$^-$ with the remainder of the upper moiety of the dimer still attached to the lower moiety. A low intensity fragment with m/z 403 was also observed. This corresponds to loss of 152 Da and stems from cleavage of bonds 1 and 3 of ring C. Fragmentation of naringenin standard in negative mode showed cleavage of corresponding bonds, yielding ion $^{1,3}$A$^- \text{m/z} 151$ as base peak and ion $^{1,3}$B$^- \text{m/z} 119$ among the less intense fragment ions. In the dimer, ring B is still attached to the lower moiety, thus resulting in fragment [M-H-$^{1,3}$A]$^-$. The minor fragments also included ions at m/z 449 (loss of 106 Da) and m/z 461 (loss of 94 Da, [M-H-B]$^-$), and corresponding losses were also seen when fragmenting naringenin standard.

With normalised collision energy (CE) set to default (35%), MS$^3$ fragmentation of the predominant MS$^2$ ion at m/z 429 occurred only to a small extent, an observation also made with luteolin standard. Raising CE to 70% resulted in the neutral loss of a number of small molecules such as CO, CO$_2$ and H$_2$O, also typical of flavonoid fragmentation (Cuyckens & Claeys, 2004; Fabre et al., 2001). A further MS$^3$ product ion with m/z 295 (loss of 134 Da) can be accounted for by cleavage of bonds 1 and 3 of the heterocycle of the luteolin moiety, producing [M-H-$^{1,4}$A-$^{1,3}$E]$^-$. Cleavage of corresponding bonds was also observed in luteolin.
standard, resulting in a fragment with m/z 151. Overall, fragmentation of the flavonoid dimer mirrored a combination of the fragmentation patterns observed for naringenin and luteolin standards. The data presented here is in close agreement with mass spectral data reported by Carrillo-Hormaza et al. (2016) and supports the tentative identification of compound 5 as morelloflavone (naringenin-1,3-ll,8-luteolin).

The UV/vis spectrum of peak 6 showed absorbance maxima at 274, 290 and 328 nm (figure 2), similar to those of peak 5 but with absorbance band I at a lower wavelength, consistent with the absorbance characteristics reported for volkensiflavone, a naringenin-apigenin conjugate (Herbin et al., 1970). Accurate mass measurements (table 2) suggest the elemental composition $\text{C}_{30}\text{H}_{20}\text{O}_{10}$ with $M_r$ 540.1056 Da for this compound. The fragmentation pattern in negative mode was also similar to that of morelloflavone (table 1, figure 3a) and yielded product ions consistent with a naringenin moiety, namely $[\text{M-H-}1,4\text{A}]^{-}$ with m/z 413, $[\text{M-H-B}]^{-}$ with m/z 445, $[\text{M-H-106}]^{-}$ with m/z 433 and $[\text{M-H-}1,3\text{A}]^{-}$ with m/z 387, whereas fragmentation of the $\text{MS}^2$ base peak at m/z 413 with raised CE resulted in losses similar to those observed with apigenin standard, yielding product ions at m/z 295 (loss of 118 Da, $[\text{M-H-}1,4\text{A-}1,3\text{E}]^{-}$) and 293 (loss of 120 Da). The findings for compound 6 are in close agreement with the fragmentation pattern reported for volkensiflavone (naringenin-1,3-ll,8-apigenin) by Carrillo-Hormaza et al. (2016).

The UV/vis spectrum of peak 3 (figure 2) showed a band II absorbance maximum at 293 nm with band I reduced to a small shoulder, a characteristic of flavanones (Mabry, Markham & Thomas, 1970). Based on accurate mass measurements (table 2) the molecular formula $\text{C}_{30}\text{H}_{22}\text{O}_{11}$ with $M_r$ 558.1162 Da was assigned to this compound. Fragmentation in negative ionisation mode followed a pattern very similar to that described above (table 1, figure 3b). $\text{MS}^2$ analysis of the parent ion at m/z 557 yielded a predominant product with m/z 431 (loss
of 126 Da, [M-H-1,4A-] as well as low abundance ions consistent with the loss of 152 Da (at m/z 405), 106 Da (at m/z 451) and 94 Da (at m/z 463) from a naringenin moiety. MS³ analysis of the fragment at m/z 431 produced a base peak with m/z 295 (loss of 136 Da, [M-H-1,4A-1,3E-]; Fabre et al., 2001) and less intense ions at m/z 269 (loss of 162 Da, [M-H-1,4A-1,4E-]), in agreement with the fragmentation pattern observed for eriodictyol standard. Based on the data presented here compound 3 was tentatively identified as Gb-2a (naringenin-I,3-II,8-eriodictyol), a conclusion supported by the mass spectral data of Carrillo-Hormaza et al. (2016).

Peaks 1, 2 and 4 had UV/vis spectra which were almost identical to those of peaks 3, 5 and 6, respectively (figure 2) but with $M_r$ 162 Da bigger than the corresponding biflavonoids (tables 1 and 2), and they were also more polar as shown by the earlier retention times. The MS² spectra of the compound 1, 2 and 4 parent ions included products [M-H-162-], indicating the loss of an O-linked hexose (Vukics & Guttmann, 2010), while other product ions resulted from fragmentation of the biflavonoid core with or without simultaneous loss of the sugar moiety. In addition, MS⁶ fragmentation of the presumed aglycone fragments at m/z 557, 555 and 539, respectively, followed the common pattern seen for compounds 3, 5 and 6, thus confirming biflavonoids as core molecules of the glycosides, with neutral loss of 126 Da producing the diagnostic fragments [M-H-1,4A-], while subsequent MS⁶ analysis included product ions [M-H-1,4A-1,3E-] with m/z 295.

HPAEC analysis of the purified, hydrolysed compounds revealed glucose as the sugar moiety of all three glycosides. Although it is not possible to determine the precise position of the glucose part with the methods employed here, fragmentation data for compounds 1 and 2, which have different substitution patterns on rings B and E, suggests that the glucose may be attached to one of the hydroxyl groups of the lower flavonoid unit. In the MS²
spectra of both compounds there were product ions characteristic for the fragmentation of
the naringenin moiety with the sugar unit still attached, namely [M-H-1,4A]⁻ (loss of 126 Da),
[M-H-1,3A]⁻ (loss of 152 Da) and low intensity ions indicating the loss of ring B ([M-H-B]⁻, loss
of 94 Da).

Analysis of the smaller peaks revealed further compounds of this type. In particular, a
compound with \( M_r = 542 \) Da eluting at 41.6 min followed a similar fragmentation pattern as
described above (figure 3b) and was tentatively identified as dinaringenin, and a hexoside of
this with \( M_r = 704 \) Da was found at \( t_R = 36.4 \) min. A further biflavonoid with \( M_r = 574 \) Da eluted at
\( t_R = 30.9 \) min and had a fragmentation pattern consistent with naringenin as upper and an
unidentified flavonoid with \( M_r = 304 \) Da as lower unit, and a hexoside of this with \( M_r = 736 \) was
seen at 21.8 min. In addition to morelloflavone and morelloflavone-glucoside, a compound
with \( M_r = 880 \) Da eluting at 27.5 min showed a fragmentation pattern consistent with
morelloflavone-dihexoside. Some larger related molecules were also detected amongst the
low abundance compounds. For example, fragmentation of a compound with \( M_r = 1006 \) Da at
\( t_R = 28.4 \) min produced a fragment ion with \( m/z = 717 \). Further MS³ analysis of this fragment
was consistent with morelloflavone-hexoside, and the compound was tentatively identified
as morelloflavone conjugate. Similarly, MS² analysis of two compounds with \( M_r = 1008 \),
eluting at 25.6 and 30.2 min, produced fragments with \( m/z = 719 \), and MS³ analysis of this ion
product was consistent with the fragmentation pattern of Gb-2a-hexoside.

Both morelloflavone and volkensiflavone have previously been reported in \textit{A. floribunda}
heartwood (Locksley & Murray, 1971) and stem bark (Brusotti et al., 2016) as well as in
various tissues of related genera such as fruits, leaves and heartwood of \textit{Garcinia} (Herbin et
al., 1970; Yang et al., 2010; Stark, Lösch, Wakamatsu, Balemba, Frank & Hofmann, 2015;
Carrillo-Hormaza et al., 2016). Whilst Gb-2a is also well documented in \textit{Clusiaceae} (Herbin et
al., 1970; Stark et al., 2015; Carrillo-Hormaza et al., 2016), this is to our knowledge the first report of Gb-2a in *Allanblackia*.

3.1.2 Identification of other flavonoids

In addition to the biflavonoids discussed in section 3.1.1, a range of low abundance compounds from different flavonoid classes were also detected (table 1). Epicatechin and luteolin were identified by direct comparison with analytical standards. Due to the absence of catechin in the extract, the flavan-3-ol oligomers found at several retention times were assumed to consist of epicatechin units. There was also a range of flavone and flavonol glycosides which were tentatively identified by their fragmentation patterns following the principles outlined by Vuciks & Guttman (2010) and Ferreres, Gil-Izquierdo, Andrade, Valentão & Tomás-Barberán (2007), whilst their flavonoid cores were confirmed by MS$^n$ analyses and acid hydrolysis. Interestingly, *A. floribunda* seed extract did not contain detectable amounts of xanthones or benzophenones which were previously reported in other tissues of *A. floribunda* and other species of the *Clusiaceae* family (Locksley & Murray, 1971; Fuller et al., 1999; Nkengfack et al., 2002; Azebaze et al., 2009; Yang et al., 2010).

3.2 Characterisation of biflavonoids

3.2.1 Quantification

The six main biflavonoids were quantitated in µg mg$^{-1}$ seed powder using response factors based on HPLC standard curves obtained with analytical standards as described in section 2.4. The total biflavonoid content was high, constituting approximately 10% of dried seed powder in comparison to seeds of the related species *Garcinia madruno* where Carrillo-Hormaza et al. (2016) reported a total biflavonoid content of less than 2%.
Morelloflavone and its glucoside were the main biflavonoids present in *A. floribunda* seeds (table 2). Similarly, Locksley & Murray (1971) reported morelloflavone as the predominant metabolite in *A. floribunda* heartwood.

### 3.2.2 Antioxidant activity

The antioxidant activities of the six main biflavonoids were analysed by the DPPH-radical scavenging and the ferric reducing antioxidant power (FRAP) assays. All six compounds demonstrated antioxidant activity which differed significantly (*P*≤0.05) and showed a similar ranking of activity with both assays apart from Gb-2a aglycone and volkensiflavone-glucoside whose order was reversed with the DPPH compared with the FRAP assay (table 3).

The highest activities were observed with Gb-2a and morelloflavone-glucosides and the lowest activities with volkensiflavone aglycone, volkensiflavone-glucoside and Gb-2a aglycone. Morelloflavone-glucoside and aglycone and GB-2a-glucoside showed radical scavenging activities comparable with ascorbic acid (IC$_{50}$ values 16.87, 21.26, 18.38 and 19.36 µg ml$^{-1}$, respectively). Radical scavenging and FRAP activities observed with morelloflavone aglycone and glucoside also reflect results reported by Kuete et al. (2011) and Carillo-Hormaza et al. (2016) where the aglycone showed lower activity than the glucoside. This pattern was observed with all biflavonoids tested here, indicating that glycosylation enhances antioxidant activity. The higher IC$_{50}$ values reported by Kuete and co-workers may be due to the higher DPPH concentration used in their assays.

Burda & Oleszek (2001) observed a relationship between certain structural features of flavonoids and antioxidant behaviour. Their studies demonstrated that a hydroxyl group in the *para* position on the B-ring is essential for activity and that this activity is enhanced by a second hydroxyl on the B-ring in the *ortho* position and a double bond between C2 and C3.
on the C ring. These findings are consistent with aglycone activities observed in the current study. Overall, the data presented here demonstrates that Allanblackia seeds are an abundant source of highly active antioxidant phenolic components in common with seeds of the fruits of other tropical species including jaboticaba (Myrciaria cauliflora; Hacke et al., 2016) and guaraná (Paullinia cupana; Majhenič, Škerget & Knez, 2007; Marques et al. 2016).

4. Conclusion

The comprehensive profile of soluble phenolics presented here confirms biflavonoids as the main phenolic compound class present and as major constituents of A. floribunda seeds. Due to their antioxidant activity and reported therapeutic properties, these compounds are of increasing interest to the pharmaceutical industry. With oil from A. floribunda seeds attracting attention from the food sector as a potential ingredient of margarine and other products (Cernansky, 2015), they could provide an excellent source of biflavonoids as a by-product.

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The authors declare that they have no conflict of interest.

References


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Figure 1. HPLC chromatogram of soluble secondary metabolites from *Allanblackia floribunda* seeds detected by photodiode array at 280nm. Peak numbering refers to the six predominant compounds.

Figure 2. UV/vis spectra of biflavonoids from *Allanblackia floribunda* seeds.

Figure 3. Structures and fragmentation patterns of (a) flavanone-flavone dimers (volkensiflavone, morelloflavone) and (b) flavanone-flavanone dimers (Gb-2a, dinaringenin) extracted from *Allanblackia floribunda* seeds.
RT: 0.00 - 60.00

NL: 4.05E5 nm=279.5-280.5 PDA seed1_041

Relative absorbance (%)

Time (min)

509

510
peak 5  
peak 6  
peak 3  
peak 2  
peak 4  
peak 1
upper moiety: naringenin
lower moiety: apigenin (R=H) or luteolin (R=OH)

upper moiety: naringenin
lower moiety: naringenin (R=H) or eriodictyol (R=OH)
Figure 1. HPLC chromatogram of soluble secondary metabolites from *Allanblackia floribunda* seeds detected by photodiode array at 280nm. Peak numbering refers to the six predominant compounds.

Figure 2. UV/vis spectra of biflavonoids from *Allanblackia floribunda* seeds.

Figure 3. Structures and fragmentation patterns of (a) flavanone-flavone dimers (volkensiflavone, morelloflavone) and (b) flavanone-flavanone dimers (Gb-2a, dinaringenin) extracted from *Allanblackia floribunda* seeds.
<table>
<thead>
<tr>
<th>$t_\text{s}$ (min)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$M_\text{r}$</th>
<th>MS$^+$ fragments in -ve mode unless stated otherwise (base peak in bold)</th>
<th>MS$^+$ fragments of MS$^-$ base peak (base peak in bold)</th>
<th>additional diagnostic MS$^+$ fragments (parent ion in bracket)</th>
<th>tentative ID (figure 1 peak no. in brackets)</th>
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<td>10.9</td>
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<td>287, 289, 407, <strong>425</strong>, 451, 559</td>
<td>179, 205, 245 (289)</td>
<td>epicatechin dimer</td>
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<tr>
<td>13.7</td>
<td>279</td>
<td>290</td>
<td>179, 205, <strong>245</strong></td>
<td>287, 289 (577)</td>
<td>epicatechin trimer</td>
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<td>293, 295, 369, 385</td>
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</table>

nd: not detected, either due to low absorbance or coeluting compounds
1: identified by direct comparison with reference compounds
Table 2
Accurate mass and content of predominant biflavonoids in seeds of *Allanblackia floribunda*

<table>
<thead>
<tr>
<th>Figure 1 peak no. (compound ID)</th>
<th>t&lt;sub&gt;r&lt;/sub&gt; (min)</th>
<th>measured mass [M-H]&lt;sup&gt;−&lt;/sup&gt;</th>
<th>calculated mass [M-H]&lt;sup&gt;−&lt;/sup&gt;</th>
<th>mass difference (ppm)</th>
<th>measured mass [M-H]&lt;sup&gt;−&lt;/sup&gt;</th>
<th>calculated mass [M-H]&lt;sup&gt;−&lt;/sup&gt;</th>
<th>mass difference (ppm)</th>
<th>content (µg mg&lt;sup&gt;−1&lt;/sup&gt; seed powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Gb-2a-glucoside)</td>
<td>30.1</td>
<td>719.1615</td>
<td>719.1618</td>
<td>0.42</td>
<td>721.1774</td>
<td>721.1763</td>
<td>1.53</td>
<td>16.73 ± 0.906</td>
</tr>
<tr>
<td>2 (morelloflavone-glucoside)</td>
<td>33.7</td>
<td>719.1459</td>
<td>719.1461</td>
<td>0.28</td>
<td>719.1616</td>
<td>719.1607</td>
<td>1.25</td>
<td>58.26 ± 3.872</td>
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<tr>
<td>3 (Gb-2a)</td>
<td>35.4</td>
<td>557.1087</td>
<td>557.1089</td>
<td>0.36</td>
<td>559.1238</td>
<td>559.1235</td>
<td>0.54</td>
<td>2.02 ± 0.136</td>
</tr>
<tr>
<td>4 (volkensiflavone-glucoside)</td>
<td>39.2</td>
<td>701.1514</td>
<td>701.1512</td>
<td>0.29</td>
<td>703.1666</td>
<td>703.1657</td>
<td>1.28</td>
<td>4.03 ± 0.297</td>
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<tr>
<td>5 (morelloflavone)</td>
<td>40.3</td>
<td>555.0929</td>
<td>555.0933</td>
<td>0.72</td>
<td>557.1079</td>
<td>557.1078</td>
<td>0.18</td>
<td>16.54 ± 0.935</td>
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<tr>
<td>6 (volkensiflavone)</td>
<td>44.5</td>
<td>539.0984</td>
<td>539.0984</td>
<td>0.00</td>
<td>541.1134</td>
<td>541.1129</td>
<td>0.92</td>
<td>2.02 ± 0.136</td>
</tr>
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</table>

*The content of individual compounds is expressed as mean ± standard error of the mean (n=9).*
Table 3
Antioxidant capacity of biflavonoids from *A. floribunda* seeds

<table>
<thead>
<tr>
<th>Figure 1 peak no. (compound ID)</th>
<th>FRAP (µmol Fe²⁺ mg⁻¹)</th>
<th>DPPH IC₅₀ (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Gb-2a-glucoside)</td>
<td>13.67 ± 0.281</td>
<td>18.38 ± 0.112</td>
</tr>
<tr>
<td>2 (morelloflavone-glucoside)</td>
<td>15.35 ± 0.082</td>
<td>16.87 ± 0.337</td>
</tr>
<tr>
<td>3 (Gb-2a)</td>
<td>3.82 ± 0.350</td>
<td>27.20 ± 0.292</td>
</tr>
<tr>
<td>4 (volkensiflavone-glucoside)</td>
<td>2.61 ± 0.012</td>
<td>26.42 ± 0.006</td>
</tr>
<tr>
<td>5 (morelloflavone)</td>
<td>6.33 ± 0.016</td>
<td>21.26 ± 0.059</td>
</tr>
<tr>
<td>6 (volkensiflavone)</td>
<td>2.12 ± 0.272</td>
<td>33.92 ± 0.382</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>17.91 ± 0.243</td>
<td>19.36 ± 0.036</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard error of the mean (n=3).
Highlights

- The phenolic profile of *Allanblackia floribunda* seeds was studied by LC-PDA-MS².
- *A. floribunda* seeds contain approximately 10% biflavonoids on a dry weight basis.
- Morelloflavone, volkensiflavone and Gb-2a were the predominant biflavonoids.
- Antioxidant activity of the main biflavonoids was comparable with ascorbic acid.