Metabolomic fingerprint classification of *Brachychiton acerifolius* organs via UPLC-qTOF-PDA-MS analysis and chemometrics

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*Brachychiton acerifolius*, or *Sterculia acerifolia* as formerly known, is a member of a genus reported for a myriad of bioactive compounds. Metabolome analysis of *B. acerifolius* – leaves, flowers and seeds – and quantification of its major compounds are demonstrated in this study. Metabolites were analysed via UPLC-PDA-qTOF-(±) ESI-MS and UPLC/ITMS, with a total of 56 metabolites characterised including 30 flavonoids, 2 anthocyanins, 6 phenolic acids (i.e. citric and hydroxycitric acid conjugates) and 8 fatty acids (FAs). Multivariate data analyses (i.e. principle component analysis and orthogonal partial least square-discriminate analysis) were applied to identify metabolite markers for each organ. Pelargonidin-**O**-glucoside and naringenin-**O**-glucuronide were found exclusively in flowers versus flavone enrichment in leaves (i.e. luteolin-**O**-glucuronide and apigenin-**O**-rhamnosyl glucuronide). Gas chromatography/mass spectrometry analysis revealed the presence of toxic cyclopropene FAs in seeds which may restrict its use. Antioxidant activity assessment for the three organs was performed in comparison with vitamin C as positive control. Leaves showed the highest activity (IC50 0.015 mg/mL).

**Keywords:** *Brachychiton acerifolius*; UPLC/MS analysis; chemometrics; antioxidant activity

1. Introduction

*Brachychiton acerifolius* (F. Malvaceae), formerly known as *Sterculia acerifolia* (F. Sterculiaceae), is an Australian tree and a member of a well-known genus reported as anti-diabetic (Desoky & Youssef 1997), anti-hypercholesterolaemic, anticancer (Vital et al. 2010), antimicrobial (Vital et al. 2010) and in the treatment of constipation (Langmead & Rampton 2001). Previous phytochemical studies on that genus have revealed a plethora of chemical classes including alkaloids (Ali et al. 1983; Wang RF et al. 2003; Kassem 2007), flavonoids (Ali et al. 1983; Dubey & Tiwari 1991; Kassem 2007), terpenes/sterols (Anjaneylu & Raju 1987) and coumarins (Ali et al. 1983).

With regard to *B. acerifolius*, very little information is reported on its bioactive chemicals except for one report on the presence of waxes, esters of terephthalic acids, phytosterols, triterpenes and flavonoids in its leaves (De Laurentis et al. 2003). Flavonoids of common occurrence in that species include luteolin, kaempferol, isorhamnetin, quercetin, rutin and hyperoside. In this study, we detail the first metabolome analysis of *B. acerifolius* major metabolite classes, namely flavonoids, phenolic acids, alkaloids and fatty acids (FAs) in one
chromatographic run (Figure S1) and also demonstrate the utility of chemometric methods [principle component analysis (PCA) and orthogonal partial least square-discriminate analysis (OPLS-DA)] for the classification of its organs and identification of metabolites unique for each organ. UPLC-qTOF-PDA-MS is a powerful technique for metabolite analysis in biological samples based on their mass and UV spectra and is increasingly used in plant metabolomics projects (Farag & Wessjohann 2012; Farag et al. 2013). This study provides not only the first detailed map for secondary metabolites composition in B. acerifolius but rather for the whole genus and draws more insight into its possible medicinal uses.

2. Results and discussion

Chemical constituents of B. acerifolius organs were analysed via reversed-phase UPLC/PDA/ESI-qTOF-MS, using a gradient mobile phase consisting of acetonitrile and aqueous formic acid that allowed for a comprehensive elution of plant analytes within 15 min (ca. 900 s). Metabolites were identified by comparing retention time, UV/Vis spectra and MS data (accurate mass, isotopic distribution and fragmentation patterns) of the compounds detected with Brachychiton compounds reported in the literature and phytochemical dictionary of natural product database. Identifications were confirmed with standard compounds whenever available in-house. All extracts were analysed in both the positive- and negative-ion electrospray ionisation (ESI) modes, as changes in ESI polarity can alter competitive ionisation and suppression effects revealing new metabolites. The positive-ion ESI mass spectra were characterised by cations corresponding to pseudo-molecular species (i.e. (M + H)⁺) and several lower mz fragments ions attributed to the sequential loss of sugar moieties that typically allowed for the determination of molecular weight and aglycone molecular weight. From these data, the majority of the structures could be tentatively identified and then confirmed through the co-analysis of authentic standards. The identification of metabolites observed in the positive-ion ESI mode was also validated using negative-ion ESI. In general and compared with positive ESI, negative-ion had better sensitivity and revealed more observable peaks. Phenolic acids and FAs were better detected in negative ionisation mode due to the presence of freely ionised carboxylate group, whereas the identification of flavonoids and anthocyanins was observed and presented mostly in positive-ion ESI mode. In an attempt to detect the presence of commonly reported purine alkaloids in this genus (Ali et al. 1983; Kassem 2007) (Figure 1(D)), methanol extract of seeds was analysed in both ESI modes, but their presence could only be detected upon acid hydrolysis identified as caffeine (data not shown), which suggests its natural presence as a conjugate or being polymerised with other forms, i.e. tannic acid that could have evaded elution and/or detection.

A total of 56 chromatographic peaks belonging to various metabolite classes were derived from the three organs’ samples including flavonoids, phenolic acids and FAs, as listed in Table S1. A representative UPLC–MS base peak chromatogram of extracts of leaves, flowers and seeds is shown in Figure S1, and the structures of metabolites commonly found in B. acerifolius and discussed throughout the manuscript are shown in Figure 1.

2.1. Flavonoids and anthocyanins

In MS/MS analysis, the nature of sugars was revealed through the natural loss, that is 162 amu (hexose; glucose or galactose), 146 amu (rhamnose), 176 amu (glucuronic acid) or 132 amu (pentose). Photodiode array (PDA) detection provided an overview of the main flavonoid subclasses in B. acerifolius. UV spectra (200–500 nm) were recorded and provided the basis for the differentiation of various flavonoid subclasses including 12 flavonols, 10 flavones, 6 flavanones, 2 anthocyanins in addition to flavanonol and a flavanol (Figure 1(A),(B)) as each
subclass exhibits UV absorbance at certain ranges (Mabry et al. 1970). The identification of flavonoids was based on assigning molecular ion, its chemical formula and MS fragments. For example, kaempferol-\(O\)-dihexosyl rhamnoside (peak 12) in flower sample showed molecular ion \((M + H)^+\) at \(/m/z/ 757.2219, C_{33}H_{41}O_{20}\) and yielding fragments \(/m/z/ 611, 595, 449, 433 \text{ and } 287\) suggesting the presence of 2 hexose and rhamnosyl moieties to kaempferol aglycone, \(/m/z/ 287, C_{15}H_{11}O_6\). This explains the reduction in UV maximum absorbance (283, 340 nm) compared with the free aglycone as multiple hydroxyl groups are occupied (Mabry et al. 1970). Similarly, MS spectra interpretation allowed for the detection of kaempferol (\(/m/z/ 287, C_{15}H_{11}O_6^+\); in peaks 25 and 28), quercetin (\(/m/z/ 303, 16 \text{ amu higher than kaempferol, } C_{15}H_{11}O_7^+\); in peaks 20, 21, 22 and 40) and isorhamnetin (\(/m/z/ 317, 14 \text{ amu higher than quercetin, } C_{16}H_{13}O_7^+\); in peaks 26, 33 and 47). Quercetin-\(O\)-[hydroxy-methylglutaryl] glucopyranoside (21) found only in leaves was assigned based on its \((M + H)^+\) at \(/m/z/ 609.1509, C_{27}H_{29}O_{16}^+\) with main fragments at \(/m/z/ 465, C_{21}H_{21}O_9^+\) for 3-hydroxy-methylglutaryl moiety and \(/m/z/ 303, C_{12}H_{11}O_7^+\) for quercetin aglycone \((−162 \text{ amu, hexose})\), in agreement with a standard compound. This metabolite is of relatively rare distribution and was suggested for phylogenetic-based analyses in other families (Porter et al. 2012). The readily cleaved sugar moieties from aglycone infers \(-\text{O-}\) type glycosides and found in most identified flavonol peaks, contrary to \(C\)-glycosides that give rise to non-homogenous fragments as found in (peak 13) apigenin-6,8-\(C\)-diglucoside. Fragmentation pathways most characteristic of \(C\)-glucosyl flavonoids include dehydration and cross-ring cleavages of the glucose moiety, i.e. 0.2 cross-ring cleavage \((−120 \text{ u})\) for 6-\(C\)-glycosides and 0.3 cross-ring cleavage \((−90 \text{ u})\) for 8-\(C\)-glycosides (Davis & Brodbelt 2004), consequently peak 13 [(\(M – H\))\(^+\), \(/m/z/ 593.1540\)] was assigned as apigenin-6,8-\(C\)-diglucoside.
(vicine II) from its product ions: m/z 575 (−18 amu, H2O), 503 (−90 amu), 473 (−120 amu) and 353 (M−2 × 120 amu).

Hydroxyluteolin-O-dirhamnoside (peak 24) was characterised by an (M + H)+ at m/z 595.1711 with UV λmax (266, 345 nm) and product ions corresponding to the sequential loss of two rhamnosyl moieties appearing at m/z 449 and 303 (aglycone, C15H11O7). It was previously reported in Sterculia genus as 6-hydroxy luteolin (Nair et al. 1976) and 8-hydroxy luteolin (Xia et al. 2009), whereas tandem MS spectrum of m/z 303 in peak 24 revealed the presence of RDA fragments corresponding to ring A1,3 m/z 151, C7H3O4 and ring B1,3 m/z 153, C8H9O3 suggesting the presence of the extra hydroxyl group on the B ring. It should be noted that leaf sample was enriched in several methylated flavones of apigenin (peaks 39 and 42), and luteolin (i.e. diosmetin, peaks 30 and 34), whereas flowers contained methylated flavanones such as methyl naringenin (peak 43) and hesperetin (peaks 35, 36 and 41) as evident from the characteristic loss of methyl from its methoxy group (−15 Da). It is believed that methylated flavonoids play a role in protecting young developing flower and leaf buds from mechanical stress, damaging UV-radiation and/or serving as antimicrobials (Andersen & Markham 2005). In terms of its health effect, O-methylated flavonoids exhibit a superior anticancer activity than the corresponding hydroxylated derivatives (Bernini et al. 2011).

As observed in Figure S1, seeds showed the least amount of flavonoids, except for an abundant flavanol; catechin (291.0885, (M + H)+, peak 4) identified only in seeds in which it is commonly distributed to serve as building block for seed coat formation (Duenas et al. 2003). Another flavanonol found exclusively in flowers includes dihydroquercetin-O-glucuronide (peak 14, m/z 481.1022, C21H21O13) with an aglycone mass fragment at m/z 305, C15H11O7 likely to serve as precursor for anthocyanin biosynthesis in flowers (Schram et al. 1981). Anthocyanins are water-soluble pigments that impart red, purple or blue colouration to fruits and flowers. In case of B. acerifolius, or the flame tree as commonly regarded, flower colour was confirmed to be attributed due to the presence of pelargonidin-3-O-glucoside (peak 8, m/z 433.1176, C21H21O10) with characteristic λmax at 520 nm, and product ion for pelargonidin at m/z 271, C15H11O5. Another minor anthocyanin found in flowers includes cyanidin-O-rutinoside (peak 7, m/z 595.1508 (M + H)+), see Table S3.

2.2. Phenolic acids

Phenolic acids readily ionise in negative ESI mode and are mainly found as conjugates with sugars, organic acids or bound to cell wall structures (Clifford 2000). UPLC–MS analysis revealed the presence of three hydroxycinnamic acids (i.e. caffeic, ferulic and p-coumaric acids; Figure 1(C)) conjugated with either iso/citric acid, hydroxyxycitric acid or hexose as evident from characteristic UV maximum absorbance at 280–290 and 315–330 nm (Pink et al. 1994). Flowers showed the least content of phenolic acids with one hydroxycinnamic acid derivative, in common with leaves, and identified as caffeoyl iso/citrate (peak 11, m/z 353.0536, C15H13O10) yielding fragments at m/z 191, C6H3O7− 179, C9H7O24 for iso/citric acid and caffeic acid, respectively. The low phenolic acid content observed in flowers may be attributed to their degree of maturation (Schmitzer et al. 2009). In contrast, seeds and leaves showed relatively higher amounts of phenolic acids. The seeds UPLC–MS trace showed deprotonated molecular ion at m/z 353.0515 for p-coumaroyl hydroxy iso/citrate (peak 15, C15H13O10) and yielding fragments at m/z 207, 189 and 163 for hydroxyxycitric acid, dehydrated hydroxyxycitric acid and p-coumaric acid moieties, respectively. Similarly, ferulic acid was identified in peaks 9 and 17 at m/z 193, C10H9O4 as hexoside and hydroxy iso/citrate conjugate, respectively. An increasing interest in hydroxyxycitric acid conjugates effect as antihyperlipidaemic and anti-obesity agent is given as in Hibiscus sabdariffa (F. Malvaceae) (Rao & Sakariah 1988; Carvajal-Zarrabal et al. 2009).
2.3. Fatty acids

In the second half of the chromatographic run (400–900 s), negative ionisation MS revealed the presence of several FAs as major peaks, most abundant in seed extract (Figure S1). Genus Sterculia is reported for the presence of toxic FA containing cyclopropene ring (Pasha & Ahmed 1992). These FAs were not detected in seeds extract analysed via UPLC–MS analysis. The possibility exists that these FAs evaded detection, considering that extraction and or elution gradient method was not optimised for this group of metabolites. Gas chromatography/mass spectrometry (GC/MS) analysis was adopted for the comprehensive profiling of FA in seed extract and revealing for the presence of 47 FA analysed as their methyl esters (Table S2). The most notable cyclopropane containing FAs were those of methyl 8-(2-hexylcyclopropyl) octanoate, methyl-2-octylcyclopropene-1-heptanoate and methyl sterculate, later forms containing unsaturated cyclopropene ring, see Table S2. These FAs constitute 0.4% of the total identified FAs, albeit might still present a health hazard that restrict the use of Sterculia seeds for nutritional and/or medicinal use. Whether production of lines containing low or no cyclopropane FAs is feasible as in oil rape seeds containing low levels of toxic erucic acid, has yet to be achieved in that genus (El-Beltagi & Mohamed 2010; Richter et al. 2010). A study comparing the chemical profiles of other Brachychiton species could also help provide a better understanding of FA biosynthesis and accumulation patterns within that genus.

2.4. Quantitative determination of major compounds

Metabolites showing differential accumulation among organs were subjected to absolute quantification using standards. A total of 10 phenolics were quantified including luteolin and kaempferol conjugates, naringenin glucuronide and the anthocyanin ‘pelargonidin-3-O-glucoside’. Flavones were detected mostly in leaves whereas flavanones and anthocyanins were major peaks in flowers. Details on the quantification of flavonoids and anthocyanins are provided in Table S3.

2.5. Multivariate PCA and OPLS-DA analyses

Although difference in metabolite patterns was observed by visual inspection of the different organs UPLC–MS traces (Figure S1), we attempted to analyse data in a more holistic way using PCA. The PCA score plot (Figure S2(A)) shows clear classification of samples with PC1 and PC2 accounting for more than 90% of the variance. Replicate measurements from the same organ were found to be highly reproducible, as the scores were more or less superimposed. Flower sample is positioned on the left side of the vertical line representing negative PC1 value whereas leaf and seed samples are positioned on the right side (positive PC1 value), indicating that the three samples are chemically distinct. The separation observed among samples can be explained in terms of identified compounds using PCA loading plot (Figure S2(B)). Four major chemical classes appear to have the most discriminating effect among samples according to MS/RT signals appearing in loading plot. In detail, anthocyanins and flavanones (i.e. pelargonidin glucoside (8) and naringenin glucuronide (31)), in addition to kaempferol-O-rutinoside (25) were found almost exclusively in flower. The co-accumulation of naringenin conjugates (flavanone) along with anthocyanins in flowers prove its role as its biosynthetic precursor via the action of an FHT, DFR and ANS enzymes along the anthocyanins biosynthetic pathway (Yan et al. 2005). Functional characterisation of these enzymes has yet to be reported in B. acerifolius. In contrast, flavones were found most enriched in leaves (i.e. luteolin glucuronide (23), apigenin rhamnosyl glucuronide (27) and apigenin glucuronide (32)) consistent with their possible role as UV protectants in aerial plant parts. PCA loading plot revealed that seed sample contains a
discriminating unknown metabolite (peak 52) within the FA region at \( R_t \) 700 s with \( m/z \) 351.2553 (\( M + H \))^+, molecular formula \( C_{21}H_{35}O^+ \) which has yet to be identified.

OPLS-DA technique is a particularly another useful tool for producing a model of clearer interpretation and to help reduce complexity of data-set, as it removes structured noise from the given data-set by collecting information in predictive latent variables (Consonni et al. 2009). The S-plot obtained from OPLS-DA model is used in identifying the variables responsible for sample differentiation and thus are sometimes considered metabolite markers. OPLS-DA score plot for flower and leaf samples, each modelled separately versus other organs (Figure S3(A), (B)), confirmed that they are chemically distinct and as revealed from the derived S-plot for flowers and leaves data-set showing anthocyanin peaks distinct in flowers (Figure S3(C)) versus flavones peaks (Figure S3(D)) in leaves, and in agreement with PCA loading plot results.

2.6. Antioxidant effect of B. acerifolius organ extracts

Antioxidant effects of plant extracts are known to mediate several of their biological effects, and thus serve as a good prediction of extracts potential biological activities (Thill et al. 2012). To address the potential variation in bioactivity, the ability of ethanol extracts prepared from different organs to scavenge DPPH free radicals was determined and expressed as IC \(_{50}\) values. A low IC \(_{50}\) value indicates strong antioxidant activity. Inhibitions of DPPH after incubation with different extracts and with vitamin C as positive control at concentrations 10, 50 and 100 mg/mL were recorded (Figure S4). Leaf sample showed the highest antioxidant activity against DPPH \(^-\) (IC \(_{50}\), 0.015 mg/mL) followed by flowers (IC \(_{50}\), 6.5 mg/mL), a finding that could be attributed to its high flavonoid content, whereas seeds extract showed the lowest antioxidant effect (IC \(_{50}\), 56.46 mg/mL). Vitamin C (ascorbic acid) used as positive drug control exhibited an IC \(_{50}\) of 12.2 mg/mL, suggesting for the potential antioxidant effect in leaf sample with an IC \(_{50}\) of 0.015 mg/mL.

3. Experimental

3.1. Plant material

B. acerifolius leaves, flowers and seeds were collected during April–May 2011 from Agriculture Museum, Giza Governorate, Egypt. The plant material was kindly identified by Agricultural Engineer Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and ex-director of Orman botanical garden, Giza, Egypt. Plant specimens have been deposited at the Faculty of Pharmacy, Cairo University (voucher No.: 16-6-2014).

3.2. Chemicals and reagents

Acetonitrile and formic acid (LC/MS grade) were obtained from J. T. Baker (Deventer, The Netherlands), milliQ water was used for UPLC analysis. Rutin, isorhamnetin, kaempferol-3-O-\(\beta\)-D-rutinoside and kaempferol were obtained from Chromadex (Wesel, Germany). All other chemicals and standards were provided from Sigma-Aldrich (St Louis, MO, USA).

3.3. Extraction procedure and sample preparation for UPLC-qTOF-PDA-MS analysis

Dried leaves, flowers and seeds were ground with a pestle and mortar using liquid nitrogen. Leaf and seed powders (18 mg) were then homogenised with 1.8 mL 70% MeOH containing 10 \( \mu \)g/mL umbelliferone (as internal standard for relative quantification using UPLC/MS) using ultrasonic bath for 30 min. Extracts were then vortexed vigorously and centrifuged at 12,000 g for 5 min to remove plant debris and filtered through 22 \( \mu \)m pore size filter. Three microlitres
were used for the analysis. Samples were analysed in positive and negative ionisation mode. In case of flowers, 30 mg was extracted in 1.8 mL 70% MeOH containing 2% HCl and processed as mentioned earlier.

### 3.4. High-resolution UPLC-qTOF-PDA-MS analysis

Chromatographic separations were performed on an Acquity UPLC system (Waters, Milford, MA, USA) equipped with an HSS T3 column (100 mm × 1.0 mm, particle size 1.8 µm; Waters) following the exact procedure described by Farag et al. (2013).

### 3.5. Quantification of flavonoids via UPLC-PDA-MS

Flavonoids and anthocyanins were quantified using calibration curves derived from PDA detection of kaempferol and cyanidin standards, prepared at concentrations spanning 2, 20 and 200 mg/mL. Assays were carried out in three independent biological replicas.

### 3.6. MS data processing and multivariate analysis

Relative quantification of *B. acerifolius* metabolite profiles derived from UPLC–MS was performed using XCMS data analysis software, which can be downloaded without charge as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) (Smith et al. 2006). This software approach employs peak alignment, matching and comparison following the exact procedure described by Farag and Wessjohann (2012). PCA was performed on the MS-scaled data using custom script under the R 2.9.2 environment. OPLS-DA was performed with the program SIMCA-P Version 13.0 (Umetrics, Umeå, Sweden).

### 3.7. GC/MS analysis

Chromatographic separations were obtained via GC/MS analysis adopting the following conditions: fused silica capillary column, 30 m length, 0.32 mm ID and 0.25 µm packed with TR-5MS (5% phenyl polysil phenylene siloxane) and injecting helium gas at 1 mL/min, 13 psi. Gradient temperature was programmed at 60°C isothermal for 3 min then 60–280°C at a rate of 5°C/min and finally at 280°C isothermal for 10 min.

### 3.8. Antioxidant activity

Serial extract concentrations spanning from 10 to 100 mg/mL were assessed for its antioxidant effect by the method of Chen et al. 2007, where DPPH free radicals react with plant antioxidants, and the decrease in absorbance (A) of DPPH was calculated in relation to the absorbance of the control (blank) as follows:

$$\text{Percentage inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100.$$  

Vitamin C was used as positive control at same concentrations.

### 4. Conclusion

To our knowledge, this study provides the most complete map for secondary metabolites composition particularly flavonoids in *B. acerifolius* on an organ basis using untargeted large-scale metabolite analysis. The approach utilised herein allowed for the assignment of 56 metabolites, most of which belong to the flavonoid subclass with quantification of the major
peaks. Differences among organs mainly emanate from the flavonoid and FA content, relevant classes of *Brachychiton* chemical composition. More *in vivo* and *in vitro* studies along with detailed phytochemical investigation are needed to underpin the perspective use of *B. acerifolius* leaf for the prevention or therapy of diseases involving free radicals.

**Supplementary material**

Experimental details relating to this paper are available online, alongside Tables S1–S3 and Figures S1–S4.

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