Comprehensive characterization of naturally occurring antioxidants from the twigs of mulberry (Morus alba) using on-line high-performance liquid chromatography coupled with chemical detection and high-resolution mass spectrometry

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Abstract

Introduction: The mulberry tree (Morus alba L.) is a prolific source of biologically active compounds. There is considerable growing interest in probing M. alba twigs as a source of disruptive antioxidant lead candidates for cosmetic skin care product development.

Objective: An integrated approach using high-performance liquid chromatography (HPLC) coupled with either chemical detection (CD) or high-resolution mass spectrometry (HRMS) was applied to the hydroalcoholic extract of M. alba to detect and identify lead antioxidant compounds, respectively.

Material and methods: The twigs were weighed, powdered and homogenized using a mill and the extract was prepared using 70% aqueous ethanol. The antioxidant metabolites were detected with HPLC coupled with CD (based on the ORAC assay) and their structural identification was carried out using a Q-Exactive Orbitrap MS instrument.

Results: Using this approach, 13 peaks were detected as overall contributors to the antioxidant activity of M. alba, i.e. mulberrosides (A & E), oxyresveratrol & its derivatives, moracin & its derivatives and a dihydroxy-octadecadienoic acid, which together accounted for >90% of the antioxidant activity, highlighting the effectiveness of the integrated approach based on HPLC-CD and HPLC-HRMS. Additionally, a (3,4-dimethoxyphenyl-1-O-β-D-apiofuranosyl-(1" → 6")-O-β-D-glucopyranoside was also discovered for the first time from the twig extract and is presented here.

Conclusion: To our knowledge, this is the first report from M. alba twigs using HPLC-CD and HPLC-HRMS that identifies key compounds responsible for the antioxidant property of this native Chinese medicinal plant.
1 INTRODUCTION

Reactive oxygen species (ROS) are molecular oxygen forms that have higher reactivity than molecular oxygen. They are continually generated as by-products of normal aerobic metabolism and overproduced under environmental stress such as sunlight or pollution. Single oxygen, superoxide anion and hydroxyl radicals are the major agents causing oxidative stress, and hence form an important class of free radicals. They are generated spontaneously and continuously in any aerobic living cell, and they are both beneficial and deleterious to the skin. The ROS system is ubiquitously involved in aging, photoaging, wound healing, tumorigenesis, inflammation and other processes in the skin. The skin employs endogenous mechanisms using antioxidants, which function to protect the skin from the development of oxidative stress and in maintaining the redox equilibrium in the tissue. The nuclear-related factor 2 (Nrf2) pathway controls antioxidant enzymes, such as glutathione peroxidase or enzymes involved in glutathione synthesis. Endogenous catalase and superoxide dismutase (SOD) are enzymes that protect cells from hydroperoxide and superoxide anions, respectively. In recent years, cellular-based assays have been developed to analyze antioxidant content in fruits, vegetables and other foods. However, these assays require time and specific equipment, and therefore in vitro pre-screening assays can be very useful to get information quickly. In vitro techniques are classified based on the type of reaction, and include electron transfer (ET)-based assays and hydrogen atom transfer (HAT)-based assays. In general, ET assays are based on the reaction of an antioxidant with fluorescent or colored probes (oxidizing agent), including the Folin total phenols assay, the ferric reducing antioxidant power (FRAP) assay and the cupric reducing antioxidant capacity assay. HAT measures the capability of an antioxidant to quench free radicals by H atom donation. These assays include the β-carotene bleaching assay, the crocin bleaching assay, the total oxyradical scavenging capacity assay and the oxygen radical absorbance capacity (ORAC) assay. The latter has emerged as a method of choice in measuring peroxyl radical scavenging capacity. Several researchers have contributed immensely to the introduction of the ORAC assay. In 1993, Cao et al. developed the very first version of the ORAC assay using β-phycoerythrin (β-PE, a fluorescent protein product isolated from Porphyridium cruentum). In 2000, Naguib et al. developed a fluorescein (FL) (3′,6′-dihydroxyxyspiroisobenzofuran-1[3H], 9′[9H]-xanthen-3-one) synthetic non-protein probe which overcomes the limitations of β-PE and that follows a classic HAT reaction mechanism. The improved ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity in the presence of peroxyl radicals. It is based upon the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of azo compounds such as 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH). The ORAC assay considers both inhibition time and degree of inhibition of free radical action caused by antioxidants.

Around 40% of the natural chemical scaffolds found in terrestrial plants display potentially useful biological properties. Over the past decades, natural product researchers have taken conventional approaches that involve chromatography to generate pure, single compounds of interest with the probability of discovering a disruptive hit. However, such a strategy does not offer the best approach as it limits an innovative route for the discovery of new actives. Moreover, plants are composed of complex constituents either in their active or inactive form, thereby making it challenging to identify, purify and screen them through classical biological programs. The disparity between needing to separate a compound and reducing the risk for the compound to progress to a cosmetic market is profound and therefore, the key to both is to design a disruptive tool for innovation. Thus, any alternative approaches explored needs clear and obvious reasons, if they are to change the methodology or approach to generate disruptive technologies. In recent years, hyphenated techniques together with the advancement of separation methods for complex mixtures using high-performance liquid chromatography (HPLC) and on-line (bio) chemical detection (BCD) has gained interest for the discovery of new hits. Van Elswijk et al. reported the on-line HPLC–BCD–MS method for the detection and identification of ACE inhibitors in complex mixtures. Approximately 30 ACE inhibitors were detected and identified. Phytoestrogens have attracted much attention due to their ability to prevent a range of diseases, including hormone-dependent cancers. In order to search for phytoestrogens in complex extracts, on-line HPLC–MS coupled with a β-estrogen receptor (ER) bioassay was applied to rapidly profile the estrogenic activity of the pomegranate peel extract. The flavonoids luteolin, quercetin and kaempferol were detected in the acid-hydrolyzed pomegranate peel extract by applying HPLC–BCD–MS. The on-line methods encourage innovation and exploration within the same strategy, further permitting the rapid determination of active compounds with advantages of antioxidant activity and inhibition of enzymes.

The genus Morus, which belongs to the Moraceae family, consists of over 150 species, and among them, the mulberry tree (Morus alba L) is a dominant species distributed throughout the temperate and subtropical regions of the Northern and Southern Hemispheres. It grows in a wide range of climatic, topographical and soil conditions ranging from mean sea level to altitudes as high as 4,000 m. The tree is widely distributed in Asia and is cultivated in China, Korea, Japan, Pakistan, Afghanistan, the northern part of the trans-Indus territory and the northwestern Himalayas. Various parts of this plant, including the roots, fruits, twigs, leaves and root barks, have been used as traditional Chinese medicine for centuries. The bark of the large stem is brown and rough, fissures are mostly vertical, and the bark is considered as vermifuge and purgative, while the mulberry leaves are used to feed the silkworm. Morus alba twigs have been widely used for the treatment of aches and numbness of joints in oriental folk medicine with potential health benefits against diabetes, stroke, cough...
and beriberi. Several researchers have investigated the *M. alba* twigs and reported their potent inhibitory effects on mushroom, murine and human tyrosinases as well as melanin synthesis in B-16 melanoma cells. Further, the *M. alba* twig extract, most notably oxyresveratrol, has been reported to exert remarkable anti-browning (i.e. antioxidant) effects on cloudy apple juices and fresh-cut apples slices in combination with ascorbic acid. A previous investigation of the alcoholic extract of *M. alba* twigs led to the identification of 6-geranylapigenin, 6-geranylnorarctocarpin, resveratrol, oxyresveratrol and quercetin as the main antioxidants. Additionally, Hwang et al. also reported that the ethanolic extract of *M. alba* twigs contains mulberroside A, quercetin, quercetin-3-O-β-glucoside, resveratrol and oxyresveratrol and shows high antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ORAC, FRAP and linoleic acid-based in vitro assays.

The objective of our study is to re-examine the *M. alba* twigs, which contain a large reservoir of secondary metabolites, in order to identify new antioxidant compounds that could be used in cosmetic skin care product development. The analysis was conducted through an integrated approach of analytical techniques that include HPLC coupled with CD (based on the ORAC assay) for the detection of antioxidant metabolites and HPLC coupled with high-resolution mass spectrometry (HRMS) for their identification. This new methodology allowed the detection of 13 antioxidant metabolites, from which 11 were already known, namely, mulberosides (A & E), oxyresveratrol & derivatives, moracin & derivatives and a dihydroxy-octadecadienoic acid derivative that contributed for 2%, 9%, 17% and 20% of the antioxidant activity, respectively. In addition, a new antioxidant compound was identified for the first time to be responsible for 2% of the overall antioxidant activity of the *M. alba* twig extract. To the best of our knowledge, this is the first report that uses HPLC-CD and HPLC-HRMS to identify key components in the mulberry twig extract and links them to the antioxidant property of this native Chinese medicinal plant.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials and reagents

Mulberroside A, p-coumaric acid, oxyresveratrol, (+)-catechin hydrate, formic acid, AAPH and FL sodium salt with high purity were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and Merck Millipore (Darmstadt, Germany) unless otherwise specified. HPLC-grade acetonitrile and methanol, sodium phosphate dibasic dihydrate and sodium phosphate monobasic dihydrate were obtained from Sigma-Aldrich. ACS reagent-grade ammonium acetate was purchased from VWR (Radnor, PS, USA). HPLC-grade water was prepared using a Milli-Q Integral 15 system (Merck Millipore). *Morus alba* twigs were collected in 2018 from the city of Chongqing, western China and authenticated by an external consultant. A voucher specimen has been deposited at the Herbarium facility at L’Oreal (Advanced Research, Bangalore, India, under the voucher specimen number MA/L’Oreal/R&D-0014.

The twigs were crushed using an IKA® Pilotina dry milling system and sieved through a 16 μm mesh to afford a powder. The powder (10 g) was extracted using 70% aqueous ethanol (1:10, v/v) for three consecutive cycles to ensure full recovery of the extracted compounds. The pooled filtrate was reduced under pressure and then dissolved in 10–20% aqueous ethanol solution (70 mL) to obtain a transparent solution and further adsorbed over macroporous resin HP-20 by allowing the resin and sample to remain undisturbed for 4–4 hours. The resin was then packed in a glass column with a sintered glass disc and eluted with 70 mL of de-mineralized water to remove proteins. The column was further eluted using a gradient mixture containing aqueous ethanol and the eluate from 50% aqueous alcohol was collected, concentrated and dried to obtain the enriched extract containing mulberoside A, oligosaccharides and saponins. (+)-Catechin hydrate was solubilized in acetonitrile aqueous (1:1, v/v) solution to get a solution at 1 g/L. In a volumetric flask, the *M. alba* twig extract (100 mg) was dissolved in 10 mL of methanolic aqueous (1:1, v/v) solution to get a solution at 1%. The samples were filtered through a PVDF syringe filter (0.22 μm) prior to HPLC-CD and HPLC-HRMS analysis.

### 2.2 | Sample preparation

The on-line HPLC-CD (ORAC) system consisted of three parts (Figure 1): (i) an Alliance e2695 TM HPLC system equipped with a quaternary solvent manager, a degasser, a sample manager and a...
column heater coupled to a 2998 diode array detector (Waters, Milford, MA, USA) for chromatographic analysis, (ii) an Advantage™ flow splitter (Restek, Lisses, France) independent of temperature and viscosity to split the flow and orient 10% to the post-column reaction system and (iii) a post-column reaction system consisting of a Pinnacle PCX Derivatization system equipped with two pumps (Pickering laboratories, Mountain View, California, USA) coupled to a fluorescence detector 2475 (Waters) to obtain the ORAC activity profiles of the samples.

Separation was carried out on an XBridge™ RP18 column (5 μm, 150 mm × 4.6 mm, Waters, Milford, MA, USA) maintained at 25°C. Gradient elution using a mobile phase composed of A (ammonium acetate, 0.02 M, pH 6.8) and B (acetonitrile) was used as follows: 0–12.5 min, 2% B → 10% B; 12.5–16 min, 10% B; 16–25 min, 10% B → 20% B; 25–28 min, 20% B; 28–29 min, 20% B → 25% B; 29–35 min, 25% B; 35–37 min, 25% B → 50% B; 37–40 min, 50% B; 40–40.1 min, 50% B → 2% B; 40.1–45 min, 2% B. The flow rate was 1 mL/min. The on-line UV–Visible spectra were recorded in the range of 240–600 nm; the chromatograms were extracted in MaxPlot. The flow (flow rate, 1 mL/min) was split in order to obtain a flow rate of 0.1 mL/min at the entrance of the post-column reaction system.

The eluent from the HPLC gradient after the split (flow rate, 0.1 mL/min) entered the Pinnacle derivatization system to be mixed with AAPH solution (8.75 g/L in 30 mM phosphate buffer, pH 7.4, 0.2 mL/min) and FL solution (500 ng/L in 30 mM phosphate buffer, pH 7.4, 0.2 mL/min). The mixture was immediately introduced into a reaction coil (15 m, I.D. 0.01100, 1 mL) maintained at 60°C. The fluorescence of the reaction mixture was then recorded by the fluorescence detector (λex = 488 nm, λem = 515 nm, data rate: 5 pts/s, gain: 10) to display the ORAC activity profile. Different parts of the instrument were connected by polyether ether ketone tubes (I.D. 0.25 mm). Of each sample, 10 mL was injected for the on-line analysis. Prior to the experiment, a catechin solution was used to control the performance of the system.

### 2.4 | High-performance liquid chromatography coupled high-resolution mass spectrometry (HPLC-HRMS)

The HPLC separation was performed as described above using an Ultimate 3000 chromatography system (Thermo Fisher Scientific) equipped with a vacuum degasser, an autosampler, a binary pump, a temperature-controlled column compartment and a photodiode array (PDA) detector. The LC system was coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) fitted with an electrospray source operated in the positive and negative ion modes. The mass spectrometer was calibrated before analyses in both polarities following the manufacturer’s recommendations (external calibration).

The Orbitrap Fusion mass spectrometer was operated with a capillary voltage of ~3 kV in negative ionization mode and 3.5 kV in positive ionization mode. Capillary temperature was set at 325°C. The sheath gas pressure and the auxiliary gas pressure were set at 30 and 5 arbitrary units with nitrogen gas, respectively. The mass resolution power of the analyzer was 120,000 m/Δm, with full width at half maximum (FWHM) at m/z 200, for singly charged ions. The detection in full scan mode was achieved from m/z 100 to 1,000 in both negative and positive ionization modes. All data were manually inspected using the Qualbrowser module of Xcalibur software version 4.1. Inclusion lists were set for MS/MS experiments. Non-resonant induced dissociation experiments using higher collision-induced dissociation (HCD) with nitrogen gas and multistage resonant-induced dissociation with helium experiments were performed at normalized collision

![FIGURE 2](A) HPLC-photodiode array (PDA) detection of M. alba twig extract. (B) On-line measurement of fluorescein fluorescence loss through peroxyl-radical formation by the breakdown of 2,2'-azobis-2-methylpropanimidamide dihydrochloride (AAPH) of M. alba twig extract. The green signal corresponds to the blank signal (AAPH replaced by water) to detect false positive signals (due to native fluorescence of extract compounds) (λex = 488 nm, λem = 515 nm). The X signal corresponds to a false positive
**TABLE 1** Antioxidant compounds detected with HPLC-CD-HRMS

| Peak no. | Tentative assignment | t<sub>HPLC-MS</sub> (min) | t<sub>HPLC-CD</sub> (min) | λ<sub>max</sub> (nm) | [M-H]⁻ | Molecular formula | MS/MS fragmentation | References |
|----------|----------------------|---------------------------|---------------------------|---------------------|--------|-------------------|---------------------|-----------|
| 1        | p-coumaric acid      | 5.2                       | 7.5                       | 284.0               | 163.0395 | C<sub>9</sub>H<sub>8</sub>O<sub>3</sub> | @CID25: [M-H]⁻ 163.0395 (25%); [M-H-CO₂]⁻ 119.0490 (100%) | Chan et al. (Ref. 28) |
| 2        | 3,4-Dimethoxyphenol-β-D-apiofuranosyl-({1",6")-β-D-glucopyranoside | 12.9                      | 15.2                      | 336.0               | 447.1503 | C<sub>19</sub>H<sub>28</sub>O<sub>12</sub> | @CID25: [M-C<sub>8</sub>H<sub>10</sub>O<sub>3</sub>]⁻ 293.08699 (100%); [M-C<sub>10</sub>H<sub>14</sub>O<sub>5</sub>]⁻ 233.06562 (5.3%); [M-C<sub>12</sub>H<sub>16</sub>O<sub>6</sub>]⁻ 191.05495 (1.6%); [M-αglucosyl-glucoside]⁻ 149.0441 (1.1%); [M-αglucosyl-glucoside-H<sub>2</sub>O]⁻ 131.03401 (0.6%) | Ferrari et al. (Ref. 29) |
| 3        | 5,7-dihydroxycoumarin 7-O-β-D-apiofuranosyl-(1- > 6)-O-β-D-glucopyranoside | 14.4                      | 16.7                      | 318.1               | 471.1139 | C<sub>20</sub>H<sub>24</sub>O<sub>13</sub> | @CID25: [M-C<sub>11</sub>H<sub>18</sub>O<sub>9</sub>]⁻ 177.019 (100%) | Chan et al. (Ref. 28) |
| 4        | trans-Mulberroside A | 20.0                      | 22.0                      | 324.0 (sh)          | 567.1714 | C<sub>26</sub>H<sub>32</sub>O<sub>14</sub> | @HCD25: [M-H]⁻ 567.1721 (2.4%); [M-glucoside]⁻ 405.1190 (16.8%); [M-2glucoside]⁻ 243.0660 (100%) | Chan et al. (Ref. 28) |
| 5        | Mulberroside E       | 21.0                      | 23.2                      | 303.7               | 551.1765 | C<sub>26</sub>H<sub>32</sub>O<sub>13</sub> | @CID25: [M-glucoside]⁻ 389.1241 (100%); [M-2 glucoside]⁻ 227.0708 (53%) | Hano et al. (Ref. 30) |
| 6        | cis-Mulberroside A   | 22.7                      | 25.0                      | 281.1 (sh)          | 567.1714 | C<sub>26</sub>H<sub>32</sub>O<sub>14</sub> | @HCD25: [M-glucoside]⁻ 405.1190 (27.6%); [M-2 glucoside]⁻ 243.0660 (100%) | Chan et al. (Ref. 28) |
| 7        | trans-Oxyresveratrol-3-O-β-D-glucopyranoside | 26.4                      | 28.7                      | 324.0 (sh)          | 405.1186 | C<sub>20</sub>H<sub>22</sub>O<sub>6</sub> | @HCD25: [M-H]⁻ 405.1190 (27.6%); [M-glucoside]⁻ 243.0660 (100%) | Choi et al. (Ref. 23) |
| 8        | cis-Oxyresveratrol-3-O-β-D-glucopyranoside | 27.0                      | 29.3                      | 328.8               | 405.1186 | C<sub>20</sub>H<sub>22</sub>O<sub>6</sub> | @HCD25: [M-H]⁻ 405.1190 (27.6%); [M-glucoside]⁻ 243.0660 (100%) | Choi et al. (Ref. 23) |
| 9        | Moracin-6-O-glucoside | 31.8                      | 34.0                      | 315.7 (sh)          | 403.1029 | C<sub>20</sub>H<sub>20</sub>O<sub>9</sub> | @HCD45: [M-glucoside]⁻ 410.504 (100%); [M-C<sub>2</sub>H<sub>2</sub>O]⁻ 199.0391 (21.8%); [M-C<sub>2</sub>O]⁻ 197.060 (53%) | Piao et al. (Ref. 31) |
| 10       | Oxyresveratrol       | 34.6                      | 36.9                      | 327.6 (sh)          | 243.0657 | C<sub>14</sub>H<sub>12</sub>O<sub>4</sub> | @HCD25: [M-H]⁻ 243.066 (39%); [M-H<sub>2</sub>O]⁻ 225.0552 (32%); [M-C<sub>2</sub>H<sub>6</sub>O]⁻ 199.076 (61%); [M-C<sub>3</sub>O<sub>2</sub>]⁻ 175.076 (100%) | Choi et al. (Ref. 23) |
| 11       | Unknown              | 36.3                      | 38.5                      | 271.6               | 545.334 | C<sub>20</sub>H<sub>49</sub>O<sub>10</sub> | 404.2165, 371.1145, 364.2599, 320.2708, 293.1497, 231.2499, 180.0658 | - |
| 12       | Dihydroxy-octadecadienoic acid derivative | 38.5                      | 41.2                      | 279.9               | 311.2219 | C<sub>32</sub>H<sub>34</sub>O<sub>4</sub> | @HCD25: [M-H]⁻ 231.2219 (64.5%); [M-H<sub>2</sub>O]⁻ 293.2110 (11.7%); [M-2 (H<sub>2</sub>O)]⁻ 275.2016 (7.1%); 235.1691 (10.6%); [M-H<sub>2</sub>O-C<sub>5</sub>H<sub>12</sub>]⁻ 223.1695 (100%) | Bastos et al. and Leyva-Jimenez et al. (Refs. 32 and 33) |
| 13       | Moracin/moracin M   | 39.5                      | 41.8                      | 315.7               | 241.0501 | C<sub>14</sub>H<sub>10</sub>O<sub>4</sub> | @HCD35: [M-H]⁻ 241.050 (65%); [M-C<sub>2</sub>H<sub>6</sub>O]⁻ 199.0391 (100%); [M-C<sub>3</sub>O<sub>2</sub>]⁻ 197.060 (53%); 157.028 (60%) | Choi et al. (Ref. 23) |

Note: Error value ±/− 5 ppm.
energies of 15, 25, 35 and 45. Note that the PDA detector was used in both systems (HPLC-CD and HPLC-HRMS) allowing the use of the UV profile of the peaks in order to link them in the chromatograms.

3 | RESULTS AND DISCUSSION

As mentioned, the objective was to identify disruptive antioxidant hits from the hydroalcoholic extract of M. alba twigs using an integrated approach including HPLC-CD (Figure 2) based on the ORAC assay for the detection of antioxidant metabolites and HPLC-HRMS for their identification. This approach allowed us to detect 13 peaks as contributors to the antioxidant activity (Figures 2 and Table 1) of M. alba. Eleven peaks (1, 3, 4, 5, 6, 7, 8, 9, 10 and 13) were assigned to known metabolites of M. alba based on retention time, UV spectra and MS data (Figure 3 and Table 1), further supported by literature.23-33

4 | ANTIOXIDANT ACTIVITY

The M. alba hydroalcoholic twig extract was selected based on its antioxidant activity. HPLC-CD based on the ORAC assay revealed the distribution of 13 antioxidant compounds (Figure 2) that exhibited protective effects against peroxyl radicals (ROO•). The chemical detection signal of the compounds was used to determine their contribution to the antioxidant activity of the twig extract by the following equation34:

\[ \text{Participation rate } X(\%) = 100 \times \frac{\text{BCD area } (X)}{\sum (\text{BCD area})} \]  

The M. alba antioxidant activity is shown in Figure 4. Our results are in agreement with several previous literature findings. For instance, Chang et al. reported the superoxide radical scavenging activity of the ethanolic extract of M. alba twigs, and showed a 20–58% inhibition in superoxide production at concentrations of 10–50 μg/mL in comparison to the positive control (catechin).35 trans-Mulberroside A 4 and compound 12 contributed to 30% and 21% of the antioxidant potential of the twig extract, respectively. On the other hand, the mulberrosides 4, 5 and 6 were responsible for 40%, while oxyresveratrol and its glucosides (i.e. 7, 8 and 10) were responsible for 9% of the antioxidant potential of the twig extract, respectively. These results are consistent with earlier findings, supporting the notion that mulberroside A (4) is a major component of the polar fraction and plays a significant role in the antioxidant activity of M. alba, together with oxyresveratrol (10).36 It is noteworthy to mention here that Hwang et al. also reported the ethanolic extract of M. alba twigs containing mulberroside A, quercetin, quercetin-3-O-
β-glucoside, resveratrol and oxyresveratrol exhibited in vitro DPPH activity (10.70 g TE/100 g), ABTS (92.15 g TE/100 g) and ORAC (150.23 g TE/100 g) results in the assays.\(^2\) However, we neither detected quercetin nor its derivatives in our hydroalcoholic extract, which could be explained by differences in the nature of our extracts. Additional studies with mulberroside A (4) are currently underway to identify the affected antioxidant enzyme pathways (glutathione-degrading enzyme inhibitors, ChaC1, ChaC2), its singlet oxygen quenching properties and the impact of peroxidation of skin lipids on DNA, protein and cellular damage, to better define its antioxidant properties and these results will be reported elsewhere. Further, moracin and its glucosides were also detected in our twig extract, as previously encountered by other researchers and were responsible for 17% of the antioxidant potential of the M. alba extract. These results indicate that not a single metabolite of this complex mixture is directly responsible for the antioxidant property of Morus twigs, but rather suggests a synergism between the secondary metabolites that could explain for the overall antioxidant property of M. alba.

5 | PHYTOCHEMICAL PROFILE AND STRUCTURAL CHARACTERIZATION

A new peak corresponding to compound 2 was assigned by comparing the molecular formula with that of the published known components. It is reported here for the first time from M. alba twigs.

The UV absorption and HRMS data (Table 1) of 2 were consistent with those published previously.\(^3\) The characterization of 2 began with side-by-side interpretation of MS/MS data, with the spectral characterization of 2a also serving as an obvious point of reference (Figure 5). Thus, the molecular formula of 2 was established as C\(_{19}\)H\(_{28}\)O\(_{12}\), with a precursor ion at m/z 447.1487 [M-H], and it differed from that of 2a by one methoxyl substituent.\(^4\) The presence of an apiofuranosyl glucoside moiety was supported by a prominent mass fragment at m/z 293.08699, suggesting the cleavage of the glycoside linkage between the phenol and the hexose moiety, followed by dehydration to generate a chromene apiofuranosyl moiety (Figure 5-2b). Additionally, MS/MS experiments (Figure 5-2c) showed a fragment at m/z 233.06562 which could be attributed to an apiose-1-oxyetheno-ethen-one fragment generated through a remote hydrogen rearrangement via a ring opening pathway of the hexose, while a fragment at m/z 191.05495 was as a result of a
hydroxyethyleneoxy-apiofuranosyl fragment (Figure 5-2d). Similarly, the placement of the apiofuranosyl moiety as the terminal sugar of the phenolic glycoside was supported by the fragment ion for an apiose at m/z 149.0444 (Figure 5-2e). The remaining MS/MS evidence was linked to the presence of an apiose sugar in compound 2 as observed for a fragment peak at m/z 131.03401 (Figure 5-2f) through the loss of a C2” hydroxyl substituent.39 The molecular formula of 2 differed from that of kelampayoside A (2a) by just a methoxylated substituent. Collectively, these data indicate a molecular formula of C_{16}H_{25}O_{12} for 2 (compare to that for 2a, C_{20}H_{30}O_{13}). Finally, compound 2 was conclusively identified as 3,4-dimethoxyphenyl-1-O-β-D-apiofuranosyl-(1’’→6’’)→β-D-glucopyranoside (Table 1 and Figure 5), which was previously reported from Nauclea officinalis (commonly known as Danmu), which belongs to the madder family growing in southern China.34,35 Based on the stereochemistry already published for kelampayoside A (2a), the relative stereochemical configuration is illustrated for 2 in Figure 5 and a proposed fragmentation pathway is described in Figure 6. There is a striking biosynthetic link between the apiofuranosyl glucosides from the bark of M. alba, which include 5,7-dihydroxycoumarin 7-(6-O-β-D-apiofuranosyl-β-D-glucopyranoside),40 isoscopoletin 6-(6-O-β-apiofuranosyl-β-glucopyranoside)41 and umbelliferone-6-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside.42 The only source of 2 from the Moraceae family has been the stem bark of Soroea ilicifolia however, this is the first report of compound 2 from M. alba twigs.

Compound 12 displayed a molecular weight of 312.2219, corresponding to C_{18}H_{33}O_{4} with characteristic ions at m/z 311.2219,

![Figure 6](image_url)
corresponding to [M–H]– by a loss of hydrogen (Table 1), suggesting it to be a member of the linoleic acid family with two hydroxy substituents.32 Even if these fragments gave intense signals, they did not provide unique structural information highlighting the position of the hydroxyl substituent.

Finally, our attempts to characterize compound 11 were unsuccessful, as we were unable to distinguish the peak signal from the signal-to-noise (S/N) ratio, despite MS optimization through improvements in sensitivity, sample pre-treatment strategies, mobile phase composition, LC column characteristics and manipulation of the S/N ratio. It is interesting to note that in addition to the identification of the antioxidant metabolites in M. alba twig extract, kelampayoside A and morusimic acid A hexoside were also detected by HPLC-HRMS, however, these two compounds did not contribute to the antioxidant properties of M. alba twigs.

The genus Morus continues to be a prolific source of new biologically active compounds. As an example, M. alba produces a large variety of secondary metabolites, including the mulberrosides based on stilbenes, flavonoids, benzofuran derivatives and coumarins.32 The major biological properties discovered for M. alba metabolites include (i) inhibition of ultraviolet B irradiation-induced melanogenesis by mulberroside A,43 (ii) anti-inflammatory properties of oxyresveratrol44 and (iii) the ability to prevent diseases characterized by chronic inflammation through inhibition of NF-κB and antioxidant effects of the polyphenols.45 The identification of the new 3,4-dimethoxyphenyl-1-O-β-D-apiofuranosyl-(1′→6′)-O-β-D-glucopyranoside as well as all three mulberrosides, which accounted for 42% of the antioxidant activity, highlights the effectiveness of the integrated approach based on HPLC-CD and HPLC-HRMS methods as an initial discovery tool to rapidly identify the key antioxidant compounds in complex mixtures. Most importantly, these results provide encouragement that the study of Morus members will lead to the discovery of additional natural antioxidant products.

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