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Mini review

# Flavonol triglycosides of leaves from *Maytenus robusta* with acetylcholinesterase inhibition

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ABSTRACT

Although *Maytenus robusta* aqueous infusions of leaves are used in Brazilian traditional medicine for stomach disease treatment, only a few chemical studies of this species are found in literature. The phytochemical investigation of methanol extract from *M. robusta* leaves yielded the known compound kaempferol (**3**) and two new flavonol glycosides: kaempferol-3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**) and quercetin-3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**2**). The chemical structures of **1** and **2** were elucidated by 1D/2D NMR, ESI–MS and ESI–$MS^2$ spectral data. It is the first time flavonoids have been reported from *M. robusta*. Flavonols **1** and **2** showed 66% and 80% acetylcholinesterase (AChE) inhibition, compared to 93% of the standard eserine, by the Ellman's method. These substances are one of the few active flavonols linked to a trisaccharide chain in the literature presenting this activity, and contribute to the screening for new types of natural AChE inhibitors.



**Contents**



## 1. Introduction

*Maytenus* is one of the largest genera of the Celastraceae family. About 300 *Maytenus* species are identified and found mainly in tropical and subtropical regions (McKenna et al., 2011). Several species are used in traditional medicine in the form of aqueous infusions for stomach disease treatment (Leite et al., 2001; Niero

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et al., 2011; Queiroga et al., 2000). Moreover, a variety of biological activities was found to *Maytenus* genus such as: antimicrobial, antiulcer, antiinflammatory, antinociceptive, antioxidant (Niero et al., 2011) and potential central nervous system (CNS) stimulate (Omena, 2007). Studies suggested a relationship between the biological activities and *Maytenus* secondary metabolites, such as flavonoids (Jorge et al., 2004; Leite et al., 2001; Niero et al., 2011; Souza-Formigoni et al., 1991).

*Maytenus robusta* Reissek occurs in the Brazilian Atlantic rainforest and is known as "espinheira santa" or "cancerosa". Our previous studies from hexane extracts of leaves and branches furnished a steroid together with friedelane and hopene pentacyclic triterpenes (Sousa et al., 2012a, 2014; Sousa et al. 2012b). Niero et al. (2006) studied the methanol extract of the aerial parts of this plant and only found triterpenes and a steroid. These researchers dissolved the methanol extract, previously dried, in water and partitioned it successively with hexane and ethyl acetate; however, just the latter was chromatographed on silica gel column. Therefore, to the best of our knowledge, no flavonoids have been found so far in this plant.

In this work, leaves were exhaustively macerated with hexane, chloroform, ethyl acetate and methanol at room temperature. The methanol extract was fractioned employing normal and reversed-phase chromatography furnishing kaempferol and two new flavonol glycosides with a trisaccharide chain (compound **1** and **2**). The last two were characterized by 1D/2D NMR, ESI–MS and ESI–$MS^2$ spectral data.

Flavanoids are bioactive compounds that present actions on the brain and cognitive performance, high antioxidant activity, high metal-chelating property and low toxicity (Uriarte-Pueyo and Calvo, 2011; Rendeiro et al., 2015). Uriarte-Pueyo and Calvo (2011), in their review, found only 128 flavonoids active for acetylcholinesterase (AChE) inhibition, in which only 35 were flavonols. Analyzing the group of flavonols, only 16 contain a sugar moiety but none of them contain a trisaccharide chain. In fact, no publications, to the best of our knowledge, about AChE inhibitors due to flavonols with a trisaccharide chain were found so far. The new compounds **1** and **2** showed AChE inhibition when evaluated *in vitro* by Ellman's bioassay and are one of the few examples of the flavonols linked to a trisaccharide chain presenting this activity.

## 2. Results and discussion

The methanol extract of leaves from *M. robusta* furnished the two new flavonol glycosides kaempferol-3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**) and quercetin-3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**2**), along with the known compound kaempferol (**3**) (Fig. 1).

Compound **1** was obtained as a light-yellow solid and its molecular formula, $C_{33}H_{40}O_{20}$, was established by HR-ESI–MS (*m*/*z*: 757.2185 $[M+H]^+$, calc. 757.2183). The IR spectrum presented absorption bands correspondent to hydroxyl (3416 and 1074 $cm^{-1}$), carbonyl (1656 $cm^{-1}$), and phenyl (1610 and 1178 $cm^{-1}$) groups. Its $^1H$ NMR spectrum showed four doublet signals of aromatic protons: two *meta*-coupled, at $\delta_H$ 6.19 (1H, d, $J$ = 2.0 Hz, H-6) and 6.38 (1H, d, $J$ = 2.0 Hz, H-8), and two *ortho*-coupled, at $\delta_H$ 6.89 (2H, d, $J$ = 8.8 Hz, H-3'/H-5') and 8.04 (2H, d, $J$ = 8.8 Hz, H-2'/H-6'). This information identifies compound **1** as a kaempferol derivative. The signals of three anomeric protons and carbon atoms in the $^1H$ and $^{13}C$ NMR spectra suggested compound **1** as a trisaccharide. Based in the aglycone carbon chemical shifts, we concluded the trisaccharide is linked to C-3. The anomeric proton signals correlated at $\delta_H$ 5.70 (1H, d, $J$ = 7.6 Hz, H-1") and $\delta_C$ 100.4; at $\delta_H$ 5.25 (1H, d, $J$ = 1.5 Hz, H-1"') and $\delta_C$ 102.4; and at $\delta_H$ 4.55 (1H, d, $J$ = 7.8 Hz, H-1"") and $\delta_C$ 105.8 in the HSQC spectrum. HSQC-TOCSY spectrum secured the assignments for one rhamnose and two glucose molecules. The large $^3J_{H1\text{-}H2}$ coupling constants ($J_{H1"\text{-}H2"}$ = 7.6 and $J_{H1""\text{-}H2""}$ = 7.8 Hz) established the β-configurations for the glucose units. The small $^3J_{H1\text{-}H2}$ coupling constant ($J_{H1"'\text{-}H2"'}$ = 1.5 Hz) established the α-configuration for the rhamnose unit (Sannomiya et al., 1998). The saccharide chain (glucose-rhamnose-glucose) was established considering the correlations observed between H-1""/C-3"' and H-1"'/C-2" in the HMBC

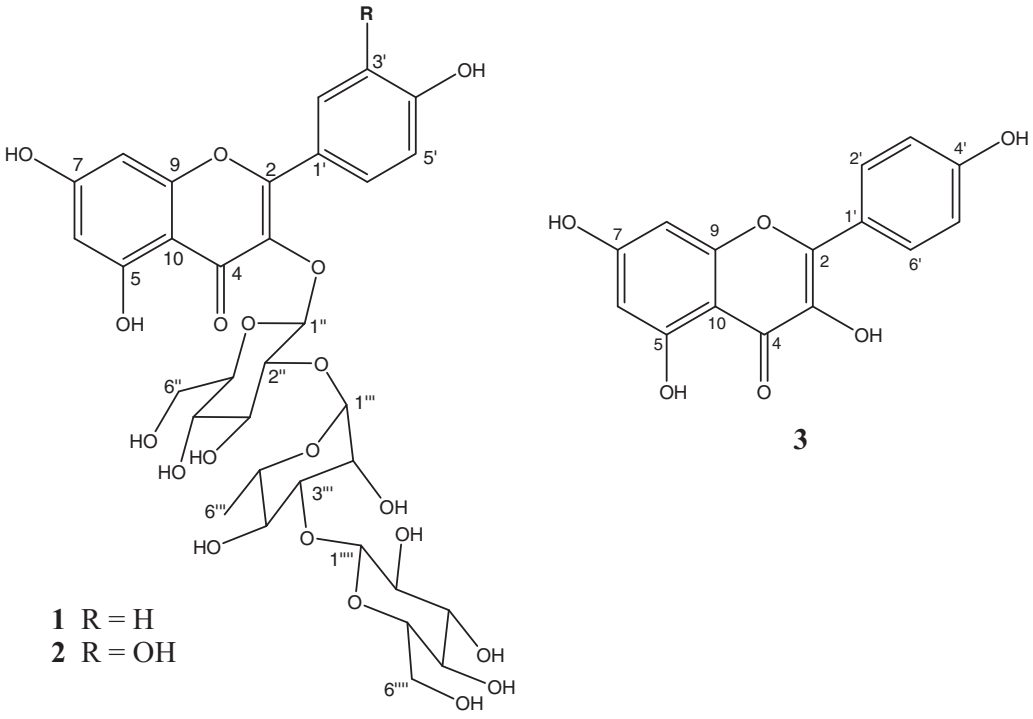


**Fig. 1.** Flavonoids isolated of leaves from *Maytenus robusta*.

**Table 1**
NMR spectral data of compound **1.**

| Atom | δC | Type | δH | HMBC | HSQC-TOCSY |
|---|---|---|---|---|---|
| 2 | 158.6 | C | | | |
| 3 | 134.5 | C | | | |
| 4 | 179.5 | C | | | |
| 5 | 163.3 | C | | | |
| 6 | 99.8 | CH | 6.19, d, *J* = 2.0 | 5, 7, 8, 10 | 8 |
| 7 | 165.8 | C | | | |
| 8 | 94.6 | CH | 6.38, d, *J* = 2.0 | 6, 7, 9,10 | 6 |
| 9 | 158.5 | C | | | |
| 10 | 106.0 | C | | | |
| 1′ | 123.1 | C | | | |
| 2′ | 132.2 | CH | 8.04, d, *J* = 8.8 | 2, 4′, 6′ | 3′ |
| 3′ | 116.2 | CH | 6.89, d, *J* = 8.8 | 1′, 4′, 5′ | 2′ |
| 4′ | 161.4 | C | | | |
| 5′ | 116.2 | CH | 6,89; d; *J* = 8.8 | 1′, 3′, 4′ | 6′ |
| 6′ | 132.2 | CH | 8.04, d, *J* = 8,8 | 2, 2′, 4′ | 5′ |
| 1′′ | 100.4 | CH | 5.70, d, *J* = 7.6 | | 2”, 3”, 4”, 5” |
| 2′′ | 80.0 | CH | 3.61 | 1”, 3” | |
| 3′′ | 78.9[a] | CH | 3.57[b] | | |
| 4′′ | 71.6 | CH | 3.28 | 5” | |
| 5′′ | 78.5[a] | CH | 3.22[b] | | 4”, 6” |
| 6′′ | 62.7 | $CH_2$ | 3.50 | | |
| | | | 3.72 | | |
| 1′′′ | 102.4 | CH | 5.25, d, *J* = 1.5 | 2”, 3”’, 2”’, 5”’ | 2”’ |
| 2′′′ | 71.6 | CH | 4,27–429, m | | 1”’ |
| 3′′′ | 83.3 | CH | 3.89 | | |
| 4′′′ | 72.8 | CH | 3.51 | 5”’ | |
| 5′′′ | 69.7 | CH | 4.08–4.13, m | | |
| 6′′′ | 17.7 | $CH_3$ | 0.98, d, *J* = 6.3 | 4”’, 5”’ | 3”’, 4”’, 5”’ |
| 1′′′′ | 105.8 | CH | 4.55, d, *J* = 7.8 | 3”’ | 2””, 3””, 4””, 5”” |
| 2′′′′ | 75.5 | CH | 3.28 | 3”” | |
| 3′′′′ | 77.8 | CH | 3.36 | | 1””, 4”” |
| 4′′′′ | 71.2 | CH | 3.36 | | |
| 5′′′′ | 77.8 | CH | 3.36 | | 1””, 4”” |
| 6′′′′ | 62.4 | $CH_2$ | 3.59 | | |
| | | | 3.87 | | |

$CD_3OD$, 100 or 400 MHz, δ ppm, *J* = Hz.
[a,b] The signals could be exchanged.

**Table 2**
NMR spectral data of compound (**2**).

| Atom | δC | Type | δH | HMBC | HSQC-TOCSY |
|---|---|---|---|---|---|
| 2 | 158.2 | C | | | |
| 3 | 134.6 | C | | | |
| 4 | 179.3 | C | | | |
| 5 | 163.2 | C | | | |
| 6 | 99.9 | CH | 6.25, sl | 5, 7, 8, 10 | 8 |
| 7 | 165.8 | C | | | |
| 8 | 94.7 | CH | 6.45, sl | 6, 7, 9, 10 | 6 |
| 9 | 158.4 | C | | | |
| 10 | 105.9 | C | | | |
| 1′ | 123.2 | C | | | |
| 2′ | 117.3 | CH | 7.66, d, *J* = 1.6 | 1′, 3′, 4′, 6′ | |
| 3′ | 146.2 | C | | | |
| 4′ | 149.7 | C | | | |
| 5′ | 116.3 | CH | 6.93, d, *J* = 8.4 | 1′, 3′, 4′, 6′ | 6′ |
| 6′ | 123.2 | CH | 7.63, dd *J* = 1.6 e 8.4 | 2, 1′, 4′, 2′ | 5′ |
| 1” | 100.3 | CH | 5.70; d, *J* = 7.6 | | 2”, 5” |
| 2” | 79.7 | CH | 3.65 | 1”, 3”, 1”’ | 1” |
| 3” | 78.9[a] | CH | 3.58[b] | | 1” |
| 4” | 71.8 | CH | 3.36 | | |
| 5” | 78.6[a] | CH | 3.24[b] | | |
| 6” | 62.6 | $CH_2$ | 3.56[c] | | |
| | | | 3.74[c] | | |
| 1”’ | 102.2 | CH | 5.23, sl | 2”, 3”’, 2”’, 5”’ | 2”’ |
| 2”’ | 71.4 | CH | 4.29, sl | | |
| 3”’ | 83.4 | CH | 3.88 | | 6”’ |
| 4”’ | 72.7 | CH | 3.51 | | 6”’ |
| 5”’ | 69.6 | CH | 4.07–4.11, m | | 6”’ |
| 6”’ | 17.9 | $CH_3$ | 0.98, d, *J* = 6.1 | 4”’, 5”’ | |
| 1”” | 105.9 | CH | 4.58; d, *J* = 7.9 | 3”’ | 2””, 3””, 4””, 5”” |
| 2”” | 75.6 | CH | 3.29 | 1””, 3”” | 1”” |
| 3”” | 77.9 | CH | 3.37 | 4”” | 1”” |
| 4”” | 71.2 | CH | 3.32 | | 1”” |
| 5”” | 77.8 | CH | 3.42 | | |
| 6”” | 62.4 | $CH_2$ | 3.74[c] | | |
| | | | 3.88[c] | | |

$CD_3OD$ + DMSO-$d_6$, 100 or 400 MHz, δ ppm, *J* = Hz.
[a,b,c] The signals could be exchanged.

spectrum. Finally, compound **1** was identified as kaempferol-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (Fig. 1). Table 1 shows the complete NMR data for this flavonol. The ESI–$MS^2$ (*m*/*z*: 757.2183 $[M+H]^+$) spectrum of **1** exhibited the fragment ions at *m*/*z* 449.1080 [M – glucosyl – rhamnosyl + H]$^+$ and at *m*/*z* 287.0551 [M – glucosyl – rhamnosyl – glucosyl + H]$^+$ corresponding to the loss of sugars.

Compound **2** was isolated as a dark-yellow solid and its molecular formula, $C_{33}H_{40}O_{21}$, was determined by HR-ESI–MS (*m*/*z*: 773.2134 $[M+H]^+$, calc. 773.2140). The IR spectrum of **2** showed absorptions bands at 3418 $cm^{-1}$ (OH), 1658 $cm^{-1}$ (C=O), 1610 $cm^{-1}$ (C=C), 1204 $cm^{-1}$ (phenol C-O) and 1074 $cm^{-1}$ (alcohol C-O). Different from compound **1**, the $^1H$ NMR spectrum of **2** exhibited two broad signals ($\delta_H$ 6.25, 1H; and $\delta_H$ 6.45, 1H), two doublet signals ($\delta_H$ 6.93, 1H, d, *J* = 8.2 Hz; and $\delta_H$ 7.66,1H, d, *J* = 1.6 Hz), and a doublet of doublet ($\delta_H$ 7.63,1H, d, *J* = 1.6 e 8.8 Hz), attributed to aromatic protons of a quercetin derivative. The $^1H$ and $^{13}C$ NMR spectra of **2** showed three signals of anomeric protons and carbon. The HSQC spectrum exhibited correlations between the anomeric atoms: $\delta_H$ 5.70 (1H, d, *J* = 7,6 Hz) and $\delta_C$ 100.3; $\delta_H$ 5.23 (1H, broad signal) and $\delta_C$ 102.2; and $\delta_H$ 4.56 (1H, d, *J* = 7,9 Hz) and $\delta_C$ 105.9. We concluded that compound **2** presents the same sugar molecules of compound **1** after analysing its HSQC-TOCSY spectrum. In the HMBC spectrum, correlations were observed between H-1””/C-3”’ and H-1”’/C-2” (Table 2). Therefore, compound **2** displays the same trisaccharide sequence than **1** with a different aglycon, and was identified as quercetin-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (Fig. 1). Its ESI–$MS^2$ (*m*/*z*: 773.2134 $[M+H]^+$) spectrum showed fragment ions at *m*/*z* 465.1038 [M – glucosyl – rhamnosyl + H]$^+$ and at *m*/*z* 303.0499 [M – glucosyl – rhamnosyl – glucosyl + H]$^+$ corresponding to the loss of sugars.

The $^{13}C$ NMR spectral data of **3** were similar to the one related by Agrawal et al. (1989) and it was identified as kaempferol (Fig. 1).

Compounds **1** and **2** were evaluated *in vitro* for acetylcholinesterase (AChE) inhibition using the colorimetric method established by Ellman but adapted for 96-well microplate (Rhee et al., 2001). The assay using Ellman's method is relatively simple, fast and straightforward (Ingkaninan et al., 2000). Flavonoids **1** and **2** demonstrated 66 ± 3% and 80 ± 2% of AChE inhibition, respectively. The inhibition of AChE has been one of the most used strategies for Alzheimer's disease (AD). Alkaloids such as galantamine or alkaloid-related synthetic compounds, such as rivastigmine, are considered useful drugs to alleviate cognitive symptoms in patients with AD. However, this class of substances provides a

series of side-effects, such as hepatotoxicity and gastrointestinal disorders (Xie et al., 2014). Compared to alkaloids, only a few flavonoids are documented as AChE inhibitors (Williams et al., 2011). In a study developed by Orhan et al. (2007), the flavonols quercetin and kaempferol-3-*O*-galactoside were screened for their *in vitro* AChE inhibitory activity employing Ellman's method adapted for 96-well microplate. Only quercetin was found to inhibit AChE 76.2% (1 mg mL$^{-1}$), while kaempferol-3-*O*-galactoside showed no inhibition. Compound **2**, a quercetin derivative, demonstrated an inhibition of 80% of AChE and compound **1**, a kaempferol trisaccharide, an inhibition of 66% of this enzyme at the same concentration. These substances are one of the few active flavonols linked to a trisaccharide chain in the literature presenting this activity, and contribute to the screening for new types of natural AChE inhibitors.

## 3. Experimental

### 3.1. General experimental procedures

Optical rotations were obtained using an ADP220 Bellinghan + Stanley Ltd polarimeter. UV spectra were measured on a Thermo Scientific™ Multiskan GO spectrometer. IR spectra were recorded on a Shimadzu IR-408 spectrometer, in KBr discs. NMR spectra were recorded on Bruker DRX400 *Avance* spectrometer, using TMS as an internal standard and $CD_3OD$ or $CD_3OD$ and DMSO-$d_6$ as solvent. LC–MS analyses were performed on a Nexera UHPLC-system (Shimadzu) hyphenated to a maXis ETD hight-resolution ESI-QTOF mass spectrometer (Bruker) operating in a positive ion mode from *m*/*z* 40–1000. Samples were injected on a Shimadzu Supelco Titan column (C18, 1.9 μm, 2.1 × 100 mm) under a flow rate of 200 μL/min with phases of 0.1% (v/v) formic acid in both water and acetonitrile. UV-chromatograms were recorded at λ 214 and λ 254 nm. Column chromatography (CC) processes were carried out using silica gel 60 (70–230 mesh) or Sephadex LH-20 (Pharmacia). Reversed-phase medium pressure chromatography was performed on Isolera One Biotage® apparatus equipped with a UV/VIS detector (set at λ 270 and 350 nm) and a KP-C18-HS SNAP column. Thin layer chromatography (TLC) was carried out using precoated silica gel plates.

### 3.2. Plant material

Leaves from *M. robusta* were collected in June 2010 at the Parque Estadual do Itacolomi, Ouro Preto City, Minas Gerais, Brazil. The species was identified by Dra. Maria Cristina Teixeira Braga Messias (Universidade Federal de Ouro Preto). A voucher specimen (OUPR: 25559) was deposited in the Herbário Professor José Badini, Universidade Federal de Ouro Preto.

### 3.3. Extraction and isolation

*M. robusta* leaves were dried at room temperature and then powdered. The powder material (888.8 g) was subjected to exhaustive subsequent extraction with hexane, chloroform, ethyl acetate and methanol (3 L of each solvent, 5 days, room temperature, 2 times). After the solvent removal, the MeOH extract (130 g) was obtained. A portion of the extract (25 g) was chromatographed on silica gel CC eluted with pure chloroform and in gradient mode with methanol, adding the last 10% by 10% until the use of pure methanol. Fractions were combined in groups according to their TLC analysis. Group 1 (fractions 15–22; chloroform-methanol 8:2; 355.6 mg) was chromatographed on a Sephadex LH-20 column eluted with methanol, providing a yellow solid (3.0 mg) which was identified as kaempferol (**3**). Group 2 (fractions 36–58; chloroform-methanol 1:1; 6.3 g) was chromatographed on a Sephadex LH-20 column eluted with methanol and the fractions were combined into 2 groups, 2a and 2b. Group 2a (fractions 14–18; 2.7 g) and 2b (fractions 19–26; 2.1 g) were purified by medium pressure chromatography on a reversed-phase KP-C18-HS SNAP column. The mobile phase consisted of two solvents: water containing 0.1% (v/v) formic acid (A), and methanol (B). Group 2a was eluted in gradient mode (10–100% of B) to obtain kaempferol-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**1**) (fractions 159–169; 51–56% of B; 128.3 mg). Group 2b was eluted in gradient mode (20–100% of B) to obtain quercetin-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**2**) (fractions 22–27; 35–40% of B; 48.9 mg).

### 3.4. Compound characterization

#### 3.4.1. Kaempferol-3-O-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D- glucopyranoside (**1**)

Light-yellow solid; mp 186 °C dec.; $[a]_D^{23}$ −76.47 (c 0.68, MeOH); UV (MeOH): $\lambda_{max}$ 289, 350 nm; IR (KBr) ν/cm$^{-1}$ 3416, 1656, 1610, 1178, 1074; NMR ($CD_3OD$) data of $^1$H (400 MHz) and $^{13}$C (100 MHz), see Table 1; HR-ESI–MS (positive-ion mode): 757.2185 [M + H]$^+$, calc. 757.2191. MS$^2$ of *m*/*z* 757.2183: 449.1080 (*m*/*z* calc. 449.1084) [M − glucosyl − rhamnosyl + H]$^+$, 287.0551 (*m*/*z* calc. 287.0556) [M − glucosyl − rhamnosyl − glucosyl + H]$^+$.

#### 3.4.2. Quercetin-3-O-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**2**)

Dark-yellow solid; mp 187 °C dec.; $[a]_D^{23}$ −65.96 (c 0.94, MeOH); UV (MeOH): $\lambda_{max}$ 282, 354 nm; IR (KBr) ν/cm$^{-1}$ 3418, 1658, 1610, 1204, 1074; NMR ($CD_3OD$ + DMSO-$d_6$) data of $^1$H (400 MHz) and $^{13}$C (100 MHz), see Table 2; HR-ESI–MS (positive-ion mode): 773.2134 [M + H]$^+$, calc. 773.2140. MS$^2$ of *m*/*z* 757.2134: 465.1038 (*m*/*z* calc. 465.1033) [M − glucosyl − rhamnosyl + H]$^+$, 303.0499 (*m*/*z* calc. 303.0505) [M − glucosyl − rhamnosyl − glucosyl + H]$^+$.

### 3.5. In vitro acetylcholinesterase inhibition

The AChE inhibition of the new compounds **1** and **2** (25 μL, 10 mg mL$^{-1}$ in DMSO, resulting in a concentration of 0.25 mg per well or 1 mg mL$^{-1}$) was measured using a 96-well microplates reader based on an adapted Ellman's method (Rhee et al., 2001). The assay was performed in quintuplicate. Physostigmine (eserine; 0.25 mg per well) was used as positive control and DMSO as blank. Using an Elisa thermoplate microplate reader, the absorbance was measured at λ 405 nm before and after addition of acetylcholinesterase, every 60 s for eight and ten times, respectively. The absorbance increase relative to substrate hydrolysis was corrected by reaction rate variation before and after addition of the enzyme. Inhibition percentage was calculated comparing the rates of the sample with the blank.

## 4. Conclusions

We isolated and elucidated two new flavonol glycosides and a known flavonoid from *M. robusta* methanol leaf extract: kaempferol-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**1**), quercetin-3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**2**) and kaempferol (**3**). This is the first work that reports flavonoids from this plant. *In vitro* assays demonstrated significant AChE inhibition for flavonols **1** and **2**. This data highlights these compounds as one of the few, if not the unique, flavonols linked to a trisaccharide chain presenting this activity.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.10.024.

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