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Novel flavonol-3-*O*-methylethers from *Zanthoxylum pistaciifolium* Griseb. (Rutaceae)

Zanthoxylum pistaciifolium Griseb. is a tree endemic to Cuba, occasionally used in herbal medicine. Previously, the antitrypanosomal activity of a *n*-hexane–2-butanone extract of *Z. pistaciifolium* leaves and of its constituent skimmianine were published. In the current study a more thorough examination of the respective extract is performed, which led to the isolation and identification of three flavonoids, more specifically, the flavonol-3-*O*-methylethers kaempferol-3-*O*-methylether (**1**) and novel compounds kaempferol-3-*O*-methylether-5-*O*-*β*-D-glucoside (**2**) and kaempferol-8-hydroxy-3,7-*O*-dimethylether-5-*O*-*β*-D-glucoside (**3**). All compounds were screened for their antimicrobial and antiprotozoal activity and cytotoxicity towards MRC-5 SV2 cells. Compound **1** showed a moderate to weak activity against *Trypanosoma cruzi* (IC_{50} 30.8 μM), *T. brucei* (IC_{50} 15.4 μM) and *Plasmodium falciparum* (IC_{50} 53.8 μM), but also showed cytotoxicity (CC_{50} 19.0 μM). Compounds **2** and **3** did not display activity in any of the assays (IC_{50} and CC_{50} > 64 μM).

Keywords: *Zanthoxylum pistaciifolium*; flavonoids; antitrypanosomal; antifungal; cytotoxicity

1. Introduction

The genus *Zanthoxylum* (family Rutaceae) comprises about 549 plant species distributed worldwide, but mainly in tropical and temperate regions, and in Cuba around 25 different species of the genus *Zanthoxylum* can be found of which 15 are endemic (Diéguez et al. 2004; Javier-Patiño et al. 2012). This plant genus has great importance due to its alimentary, industrial and medicinal applications, and in herbal medicine, *Zanthoxylum* species are applied for the treatment of diarrhea, intermittent fever and ear aches (Diéguez-Hurtado et al. 2003; Javier-Patiño et al. 2012), which may occur as a consequence of microbial infections. A wide range of biological activities has already been reported for plants belonging to the *Zanthoxylum* genus, including antimicrobial, antiparasitic, antiviral, anthelmintic, insecticidal, anti-inflammatory, antioxidant, antitumor and antinociceptive activity, while also effects on the central nervous system were found (Javier-Patiño et al. 2012). Phytochemical studies revealed the presence of various classes of secondary metabolites in the *Zanthoxylum* genus, including alkaloids, amides, coumarins, flavonoids, terpenes, sterols and lignans (Javier-Patiño et al. 2012).

Zanthoxylum pistaciifolium Griseb. (also called "palo vencedor") is one of the *Zanthoxylum* species endemic to Cuba and a decoction of its bark and leaves is used to treat ear pain, pulmonary infections and the common cold (Roig 2012). Two synonyms are described for *Z. pistaciifolium*, namely *Fagara pistacifolia* (Griseb.) Krug & Urb. and *Zanthoxylum flavum* subsp. *pistaciifolium* (Griseb.) Reynel of which the latter is the official name. However, in view of consistency with previous publications, the name *Z. pistaciifolium* is used throughout this article.

Only a few reports have been published about this species, which mentioned the presence of the alkaloid skimmianine and the coumarins marmesin and byakangelicin in the bark (Fajardo et al. 1987; Heredia-díaz et al. 2020), while also the essential oils of the leaves were studied and mainly led to the identification of terpenoids (monoterpenes and sesquiterpenes) (Heredia-díaz et al. 2016). Previously, activity of a *n*-hexane–2-butanone extract of *Z. pistaciifolium* was

reported against *Trypanosoma cruzi* and *Candida* spp., together with the activity of one of its major constituents: skimmianine against *T. cruzi* (Heredia-díaz et al. 2020). However, no further examination of the extract was carried out. Therefore, the present study aims to further characterize the *n*-hexane–2-butanone extract of *Z. pistaciifolium* leaves, and to identify and assess the antimicrobial and antiprotozoal activity and cytotoxicity of some of its constituents.

2. Results and Discussion

Three compounds were purified from the *n*-hexane–2-butanone extract of *Z. pistaciifolium* leaves by means of column chromatography and semi-preparative HPLC-DAD-MS. One compound was obtained from the dichloromethane – ethyl acetate (50:50 V/V) fraction, while two compounds were derived from the ethyl acetate fraction. The structures of the three purified compounds are shown in Figure 1. All three compounds belong to the class of flavonol-3-*O*-methylethers, and compounds **1**-**3** are the first flavonoids to be reported in *Z. pistaciifolium*.

Compound **1**, obtained from fraction D was identified as kaempferol-3-*O*-methylether (isokaempferide, 5,7,4′-trihydroxy-3-methoxyflavone) by comparison of its NMR data (Table S1) to literature and confirmed by HRMS (Nakatani et al. 1991; Csapi et al. 2010). Kaempferol-3-*O*-methylether is a commonly occurring flavonoid and has been reported in many plant species of various families (Wollenweber 1994).

The NMR spectra of compound **2** showed some similarities to compound **1**, but several additional signals, indicating the presence of a glycoside moiety, were observed. With the aid of 2D-NMR, the aglycon moiety could be identified as kaempferol-3-*O*-methylether. Moreover, the glycoside moiety was identified as *β*-D-glucopyranoside, based on comparison of the ^{13}C chemical shift values and comparison with published data (Markham and Chari 1982).The *β*-configuration was deduced from the *J*-coupling of H-1, which was 7.6 Hz, while

a smaller *J*-value would be expected in case of the *α*-configuration (Ravindra et al. 2007; Roslund et al. 2008; Zhang et al. 2014). From the HMBC-spectrum, it could be deduced that this glucoside moiety was connected to the A-ring of the flavonoid. More specifically, a HMBC correlation with a quaternary carbon at δ_C 160.1 was observed, indicating that this carbon is *O*-glucosylated (Figure S1). Nevertheless, additional evidence to determine the exact position of the glucose moiety (position 5 or position 7) could not be deduced from the HMBC spectrum, but was obtained by chemical shift considerations.

The assignment of the ^{1}H and ^{13}C-signals of positions 6 and 8 of compound **2** was done by comparison to the NMR data of compound **1** and to literature data, which showed that the C-6 signal is generally more downfield to the C-8 signal, in the case of substitution of C-5 and C-7 with a hydroxyl or *O*-glycosidic constituent (δ_C-6 104.4; δ_C-8 98.9; δ_H-6 6.79; δ_H-8 6.65) (Markham and Chari 1982). Since both H-6 and H-8 showed HMBC correlations with the same carbon signals, namely quaternary carbons at δ_C 109.6, 160.1 and 164.9, in addition to showing correlations with C8 or C6, respectively, the position of the hydroxyl and glucose moiety could not be determined based on these data. However, by comparison to literature data, it was also found that C-5 is usually more upfield than C-7, in case of an OH- or *O*-glycosidic substituent in these two positions (Markham and Chari 1982; Hase et al. 1995; Aquino et al. 2002; Ho and Chen 2002; Khan et al. 2006). This implies that the signal at δ_C 160.1 should be assigned to C-5 and the signal at δ_C 164.9 to C-7 and thus, the *O*-glucose moiety is present in position 5. In addition, the C-1 signal of glucose typically has a chemical shift value of 104.3 ppm when bound to the flavonoid 5-position, while a glucoside in position 7 typically shows a δ_C-1 at 100.4 (Markham and Chari 1982). This is in correspondence to our data (Glc δ_C-1 104.9) and confirms that in compound **2**, the glucose moiety is present in position 5. Moreover, the chemical shift of C-4 (δ_C 176.4) is in agreement with a 5-*O*-glucoside moiety; in case of a 5-hydroxy substitution, the C-4 chemical shift would be shifted more downfield (≈δ_C

180)(Markham and Chari 1982; Hase et al. 1995; Ho and Chen 2002; Khan et al. 2006). Thus, this compound was identified as kaempferol-3-*O*-methylether-5-*O*-*β*-D-glucoside (7,4′-dihydroxy-3-methoxyflavone-5-*O*-*β*-D-glucoside), which, to the best of our knowledge, was not reported before.

Accurate mass measurements revealed a monoisotopic mass of 463.1245 for the $[M+H]^+$ ion, corresponding to a molecular formula of $C_{22}H_{23}O_{11}$ (Δ 1.08 ppm). In addition, an ion appearing at 301.0710 was observed, corresponding to $[M\text{-glucose}+H]^+$, and formed by spontaneous hydrolysis of the glycosidic bond during mass analysis.

The ^{1}H-NMR spectrum of compound **3** showed two doublets, each integrating for two protons (δ_H 8.12 and δ_H 6.94, *J*= 8.9 Hz), typically found for a flavonoid B-ring with para-substitution. The HMBC-spectrum revealed that, like in compounds **1** and **2**, this compound bears a 4′-hydroxy substituent. Also, one methoxy-signal (δC 60.3/ δH 3.78) was observed, typical for a methoxy-group in the 3-position, and similar to compounds **1** and **2**. However, opposed to the former two compounds, the ^{1}H-NMR spectrum of compound **3** showed only one more signal in the aromatic region (δ_H 7.19, singlet), one signal at δ_H 4.00 with an integration of three, corresponding to a second methoxy-signal, and several signals indicating the presence of a glycoside moiety. As was the case for compound **2**, also for this compound the exact positions of the different substitutions in the A-ring could not be determined solely based on the HMBC spectrum, but a comparison to literature data of similar compounds aided in the assignment.

Firstly, the position of the aromatic proton was defined with the aid of NOESY. Since substitution with hydroxy, methoxy, or *O*-glycoside moieties preferentially occurs in the 5- or 7-position, the proton is most likely positioned at C-6 or C-8. The NOESY spectrum revealed correlations of the proton at δ_H 7.19 with the anomeric proton of the *O*-glycoside (δ_H 4.78), and

also with the methoxy-signal at δ_H 4.00 ppm (Figure S1). Thus, the aromatic proton was expected to be located in proximity of these two substituents, which is not possible in case of a H-8, but which is possible in case of a H-6. In addition, the ^{13}C chemical shift of the protonated carbon was δ_C 102.3, which is more typical for the position 6 than 8, since a more upfield shift would be expected in case of H-8 substitution. Thus, the NMR signal at δ_C 102.3 and the NMR signal at δ_H 7.19 were assigned to C-6 and H-6, respectively. The HMBC spectrum revealed correlations of H-6 with quaternary C-signals at δ_C 111.1, 132.4, 151.1 and 152.7, but not with δ_C 146.5 and thus, the latter could be assigned to C-9, in para-position of C-6 in ring A. Also, the signal at δ_C 111.1 could be assigned to C-10, since this carbon signal is typically the most upfield quaternary carbon signal in the flavonoid A-ring. Thus, three quaternary carbon signals are remaining: one at δ_C 151.1, which is glycosylated, as deduced from the HMBC correlation of the anomeric proton with this carbon signal; one at δ_C 152.7, which is methoxylated, as deduced from the HMBC correlation with the proton signal at δ_H 4.00, and one at δ_C 132.4, which must bear an OH-substitution, since other types of substitution would result in additional NMR signals. As already mentioned above, the H-6 was in close proximity to the glycosylated and methoxylated carbons, indicating that those are present in positions 5 and 7. This infers that the signal at δ_C 132.4 can be assigned to C-8, which is thus hydroxylated. Moreover, in case of a hydroxyl-group in positions 5 or 7, the C-5 or C-7 signal would be more downfield (> 150 ppm) (Markham and Chari 1982; Hase et al. 1995; Ho and Chen 2002). In addition, C-4 is found at δ_C 176.9 and would correspond to a more downfield signal in case of a 5-OH substitution, vide supra.

Thus, only the position of the methoxy-group and the *O*-glycoside moiety have to be determined still. As mentioned before, typically the C-5 has a chemical shift value more upfield compared to C-7 (Markham and Chari 1982; Hase et al. 1995; Aquino et al. 2002; Ho and Chen 2002; Khan et al. 2006). This implies that the glycosidic moiety is present at position 5 (δ_C-5

151.1) and the methoxy-signal is present at position 7 (δ_C-7 152.7). The glycosidic moiety was identified as *β*-D-glucopyranoside by comparison to literature data (Markham & Chari 1982). The rather downfield shift of C-1'' (δ_C 106.4) complies with a 5-*O*-glucoside, rather than a 7-*O*-glucoside. The *β*-configuration was deduced from the *J*-coupling of H-1, which was 7.5 Hz (Ravindra et al. 2007; Roslund et al. 2008; Zhang et al. 2014).

HRMS analysis of this compound revealed a molecular ion at 493.1346, corresponding to the $[M+H]^+$ ion ($C_{23}H_{24}O_{13}$, Δ 0.00 ppm). As was the case for compound **2**, spontaneous hydrolysis of the glycosidic bond occurred during the MS analysis, resulting in the detection of a signal with *m/z* 331.0809, corresponding to the $[M\text{-glucose}+H]^+$ ion.

To the best of our knowledge, compound **3** was not reported before, and in line with the other two purified compounds, it was named kaempferol-8-hydroxy-3,7-*O*-dimethylether-5-*O*-*β*-D-glucoside.

For compounds **1**-**3** the antimicrobial activity and antiprotozoal activity, as well as their cytotoxicity towards MRC-5 SV2 cells (human lung fibroblasts) were determined. Compound **1** was weakly active against *T. cruzi* (IC_{50} 30.8 µM) and *T. brucei* (IC_{50} 15.4 µM). Against *P. falciparum* an IC_{50}-value of *53.8* µM was found. With regard to the cytotoxicity, a CC_{50} of 19.0 µM was determined. These results are in line with previous reports on the antitrypanosomal, antiplasmodial and cytotoxic activity of kaempferol-3-*O*-methylether (Gadelha Militão et al. 2005; Omosa et al. 2016; Boniface & Ferreira 2019). No activity was found for compound **1** against *S. aureus* and *C. albicans* (IC_{50} >64 µM) and compounds **2** and **3** did not show activity in any of the assays (IC_{50} >64 µM). The moderate antitrypanosomal activity of the *n*-hexane–2-butanone extract previously reported (Heredia-díaz et al. 2020), may therefore in part be attributed to the presence of compound **1**. However, further research is required in order to determine which constituents are responsible for the activity against Candida spp., which had

previously been reported for the same extract as well (Heredia-díaz et al. 2020).

3. Experimental

3.1: General Experimental Procedures

A semi-preparative HPLC system with DAD and ESIMS detectors was used for isolation of pure compounds and was comprised of a sample manager, injector and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, an HPLC pump (515), a photodiode array detector (2998), and a Micromass Quattro mass spectrometer with TQD, all from Waters (Milford, MA, USA). MassLynx version 4.1 was used to process the data. Compounds were separated on a Luna C18 column (250 mm x 10.0 mm, particle size 5 µm) from Phenomenex (Utrecht, the Netherlands) and a C18 guard column (10 mm × 10 mm, particle size 5 µm) from Grace (Hesperia, CA, USA).

NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), equipped with either a 3 mm broadband inverse (BBI) probe or a 5 mm dual $^{1}H/^{13}C$ probe, using standard Bruker pulse sequences and operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Apart from ^{1}H- and ^{13}C-spectra, also Distortionless Enhancement by Polarization Transfer spectra (DEPT-135 and DEPT-90) were recorded. As for the 2D NMR experiments, COSY, HSQC, HMBC, and if required, NOESY NMR experiments were performed, revealing ^{1}H-^{1}H, direct ^{1}H-^{13}C, indirect ^{1}H-^{13}C connections, and ^{1}H-^{1}H proximity in space, respectively. Methanol-d_4 (99.8% D) was used as solvent and was purchased from Sigma-Aldrich. The spectra were acquired with Topspin version 1.3.

HRMS data were obtained on a UPLC-QTOF-MS system with an Acquity UPLC and Xevo G2-XS QTOF (Quadrupole Time-Of-Flight) mass spectrometer (Waters). Samples were injected on a UPLC HSS T3 column (2.1 x 100 mm, 1.8 µm) kept at 40 °C and a general gradient starting at 97% H_2O + 0.1% formic acid and 3% acetonitrile + 0.1% formic acid, and

linearly changing up till 100% acetonitrile + 0.1% formic acid was applied. The mass spectrometer was operated in ESI+ mode and set under the following conditions: capillary voltage: 2.0 kV, sampling cone voltage: 40 V, source temperature: 120 °C, desolvation temperature: 550 °C, cone gas flow: 50 L/h, desolvation gas flow: 1000 L/h. The MS system was operated at a resolution of approximately 20,000.

Optical rotations were determined using a Jasco P-2000 polarimeter (de Meern, the Netherlands).

3.2: Plant Material

Z. pistaciifolium leaves were collected in January 2018 at "El Palenque", Siboney community (Lat 19.961442, Lon -75.714852), Santiago de Cuba, Cuba. The plant material was identified and authenticated by taxonomist Felix Acosta Cantillo. A voucher specimen with number 21660 was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity (BIOECO, Spanish acronym), Santiago de Cuba. Fresh leaves were dried in the shade until residual humidity values lower than 12%, milled, sieved and conserved for further steps.

3.3: Extraction, Isolation and Structure Elucidation

An ethanol extract was prepared from the air-dried and powdered *Z. pistaciifolium* leaves (1 kg) by percolation. The obtained extract was filtered and dried under reduced pressure at 40 °C. Next, this extract (120 g) was suspended in 50% ethanol and partitioned with *n*-hexane. The remaining 50% ethanol phase was alkalinized with NaOH until pH 8.5 and successively partitioned with *n*-hexane–2-butanone (1:1) , dichloromethane, *n*-butanol and methanol. The *n*-hexane–2-butanone phase was further fractionated by column chromatography using silica gel as stationary phase and *n*-hexane (A), *n*-hexane-dichloromethane (50:50 V/V) (B), dichloromethane (C), dichloromethane – ethyl acetate (50:50 V/V) (D) and ethyl acetate (E) as eluting solvents, thus resulting in 5 fractions.

Fractions D and E were submitted to semi-preparative HPLC-DAD-MS. As mobile phase, H_2O + 0.1% formic acid (A) and acetonitrile (B) were used. Linear gradients were applied and the flow rate was set at 4.75 mL/min. For fraction D the gradient conditions were: 0-5 min 5% B, 40 min 55% B, 45-50 min 100% B, 55-60 min 5% B and for fraction E: 0-5 min 15% B, 25 min 30% B, 30-35 min 100% B, 40-45 min 15% B. The DAD spectrum was recorded from 200 nm to 400 nm and mass spectra were taken in (+)-ESI MS mode, with MS scan range: *m/z* 100 to 800 and 100 to 1100 for fractions D and E, respectively. Capillary voltage 3.00 kV, cone voltage 50 V, extractor voltage 3 V, V_{RF}-lens 0.2 V, T_{source} 135 °C, TDesolvation 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h. Interesting peaks were selected based on the UV-spectrum and the *m/z*-value of each peak. For fraction D, the peak with *m/z* 286 was collected and for fraction E, the peaks with *m/z* 331 and 301. The collection of the eluate was triggered as long as the intensity of selected m/z values exceeded the set threshold. Collected fractions of the same *m/z* and retention time were combined and dried under reduced pressure. In this way, compound **1** (5 mg) was obtained from fraction D, while two compounds were derived from fraction E (compound **2**: 14 mg and compound **3**: 6 mg). Both 1D- and 2D-NMR spectroscopy and MS data were used for structure elucidation.

*3.3.1 kaempferol-3-O-methylether (**1**)*

Yellow powder (5 mg), UV λmax 265 nm, 346 nm. HRMS: *m/z* 301.0710, corresponding to molecular formula $C_{16}H_{13}O_6$ ($[M+H]^+$, Δ -0.66 ppm). ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data can be consulted in the supplementary data, table S1.

*3.3.2 Kaempferol-3-O-methylether-5-O-β-D-glucoside (**2**)*

Yellow powder (14 mg), $[\alpha]_D$ −101.3 (c 0.5, CH_3OH), UV λmax 259 nm, 340 nm. HRMS: *m/z* 463.1245, corresponding to molecular formula $C_{22}H_{23}O_{11}$ ($[M+H]^+$, Δ 1.08 ppm). ^1H NMR

(CD_3OD, 400 MHz) δ_H 3.49 (2H, overlapping, H-4′′ and H-5′′), 3.53 (1H, overlapping, H-3′′), 3.65 (1H, t, 8.3 Hz, H-2′′), 3.75 (3H, s, 3-OCH_3), 3.78 (1H, overlapping, H-6′′), 3.96 (1H, d, 11.8 Hz, H-6′′), 4.87 (1H, d, 7.6 Hz, H-1′′), 6.65 (1H, br s, H-8), 6.79 (1H, br s, H-6), 6.92 (2H, d, 8.7 Hz, H-3′ and H-5′), 7.96 (2H, d, 8.7 Hz, H-2′ and H-6′); ^{13}C NMR (CD_3OD, 100 MHz) δ_C 176.4 (C-4), 164.9 (C-7), 161.5 (C-4′), 160.1 (C-5), 159.6 (C-9), 156.3 (C-2), 141.3 (C-3), 131.3 (C-2′ and C-6′), 122.5 (C-1′), 116.6 (C-3′ and C-5′), 109.6 (C-10), 104.9 (C-1′′), 104.4 (C-6), 98.9 (C-8), 78.6 (C-5′′), 77.3 (C-3′′), 74.7 (C-2′′), 71.2 (C-4′′), 62.5 (C-6′′), 60.3 (3-OCH_3).

3.3.3 Kaempferol-8-hydroxy-3,7-O-dimethylether-5-O-β-D-glucoside ***(3)***

Yellow powder (6 mg), $[\alpha]_D$ -44.5 (c 0.6, CH_3OH), UV λmax 268 nm, 356 nm. HRMS: *m/z* 493.1346, corresponding to molecular formula $C_{23}H_{24}O_{13}$ ($[M+H]^+$, Δ 0.00 ppm). ^1H NMR (CD_3OD, 400 MHz) δ_H 3.40 (1H, d, 9.0 Hz, H-4′′), 3.50 (1H, overlapping, H-5′′), 3.52 (1H, overlapping, H-3′′), 3.63 (1H, dd, 9.3; 7.8 Hz, H-2′′), 3.75 (1H, overlapping, H-6′′), 3.78 (3H, s, 3-OCH_3), 3.98 (1H, overlapping, H-6′′), 4.00 (3H, s, 7-OCH_3), 4.78 (1H, d, 7.8 Hz, H-1′′), 6.94 (2H, d, 8.9 Hz, H-3′ and H-5′), 7.19 (1H, s, H-6), 8.12 (2H, d, 8.9 Hz, H-2′ and H-6′); ^{13}C NMR (CD_3OD, 100 MHz) δ_C 176.9 (C-4), 161.7 (C-4′), 156.7 (C-2), 152.7 (C-7), 151.1 (C-5), 146.5 (C-9), 141.2 (C-3), 132.4 (C-8), 131.6 (C-2′ and C-6′), 122.8 (C-1′), 116.5 (C-3′ and C-5′), 111.1 (C-10), 106.4 (C-1′′), 102.3 (C-6), 78.7 (C-5′′), 77.3 (C-3′′), 75.0 (C-2′′), 71.7 (C-4′′), 62.9 (C-6′′), 60.3 (3-OCH_3), 57.1 (7-OCH_3).

3.4: Antimicrobial and Antiprotozoal Activity and Cytotoxicity

The inhibitory activity of the three purified compounds was assessed against *Staphylococcus aureus* ATCC 6538, *Candida albicans* B59630, *Trypanosoma cruzi* Tulahuen CL2 (B-Gal)m *Trypanosoma brucei* Squib 427 and *Plasmodium falciparum* K1, while cytotoxicity was

determined on MRC-5 SV2 cells (human lung fibroblasts), up to a concentration of 64 μM, as previously reported (Cos et al. 2006; Baldé et al. 2010; Heredia-díaz et al. 2020). Doxycycline (IC_{50} 0.2 μM), flucytosine (IC_{50} 0.4 μM), benznidazol (IC_{50} 1.8 μM), suramine (IC_{50} 0.04 μM), chloroquine (IC_{50} 0.1 μM) and tamoxifen (CC_{50} 9.9 μM) were used as positive controls, respectively. A detailed description of the experimental methodology can be found in the supplementary data.

4. Conclusions

In view of the previously reported activity of a *n*-hexane–2-butanone extract of leaves of *Zanthoxylum pistaciifolium* against *T. cruzi* and *Candida* spp, the current research intended to further characterize this extract. Three flavonol-3-*O*-methylethers were isolated from the *n*-hexane–2-butanone extract: kaempferol-3-*O*-methylether (**1**) and two novel flavonol-3-*O*-methylethers, kaempferol-3-*O*-methylether-5-*O*-*β*-D-glucoside (**2**) and kaempferol-8-hydroxy-3,7-*O*-dimethylether-5-*O*-*β*-D-glucoside, which were identified by extensive NMR analysis and HRMS. All compounds were screened for their antimicrobial and antiprotozoal activity and cytotoxicity. While compounds **2** and **3** did not display activity in these assays, compound **1** showed moderate to weak activity against *Trypanosoma cruzi, T. brucei* and *Plasmodium falciparum*, as well as cytotoxicity. Therefore, compound **1** may contribute to the antitrypanosomal activity previously found for the *n*-hexane–2-butanone extract. This is the first report about the identification of flavonoids in *Z. pistaciifolium*.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Supplementary data

A table with NMR data of compounds **1-3** is provided in Table S1. Figure S1 shows the key

NOESY and HMBC correlations of compounds **2** and **3**. All recorded NMR spectra can be consulted in figures S2-S17.

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Figure 1. Structure of compounds **1**-**3**